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RESEARCH ARTICLE

Exploration of the Medicinal Origin and Clinical Anti-Cancer Value of Wumei (*Mume fructus*)

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ABSTRACT: Wumei is the nearly mature fruit of *Prunus mume (Sieb.) Sieb. Et Zucc.*, a deciduous tree of the Rosaceae family. It possesses unique advantages and characteristics in anti-cancer properties. Thus, the study aims to examine the historical origins and evolution of traditional Chinese medicine Wumei (*Mume fructus*) and to investigate its potential anti-cancer effects using network pharmacology research techniques. This study uses network pharmacology techniques to examine the historical origins and evolution of Wumei (*Mume fructus*) and its potential anti-cancer effects. Initially, a comprehensive review of the plant's distribution, medicinal properties, and processing techniques was conducted. Active compounds of Wumei were identified using the TCMSP database, and the SwissTargetPrediction database was used to predict their potential targets. A protein-protein interaction network was established to investigate the primary targets and pathways involved in Wumei's anti-tumor mechanism. Eight effective components of Wumei were identified, predicting 282 potential targets. The PPI network includes key proteins such as VEGFA, SRC, AKT1, ESR1, and BCL2L1. Biological enrichment analysis indicates potential interactions with cancer-related pathways, including the PI3K Akt, Rap1, Ras, and MAPK signaling pathways. Wumei targets various tumors, including medulloblastomas, astrocytomas, lymphomas, mesothelioma, prostate cancer, breast cancer, and head and neck cancers. Wumei is a traditional Chinese medicine with both medicinal and edible properties, offering diverse clinical health benefits.

KEYWORDS: Robotics and Intelligent machines, C, Anti-Cancer, TCM, Wumei.

Introduction

Wumei is a dry, nearly mature fruit of *Prunus mume (Sieb.) Sieb.et Zucc*, a deciduous tree of Rosaceae. It is native to China and is commonly found in Yunnan, Fujian, Sichuan, Anhui, and Guizhou.[1] Statistics show that there are 50-60 different artificially cultivated varieties in China, and all of them can be used for Wumei processing.[2] Wumei is a traditional Chinese medicine with the effects of astringing lung qi, astringent intestines, relieving diarrhea, promoting fluid production, quenching thirst, relieving vexation, and relieving ascariasis, it has a long history of medication, which is flat in nature, sour and astringent in taste, and belongs to liver, spleen, lung and large intestine meridians.[3] Modern pharmaceutical studies have confirmed that the effective components of Wumei include citric acid, ursolic acid, stickers, sterols, flavonoids, alkaloids, and so on.[4] Wumei is a product with homology of medicine and food. As early as 2002, the National Health and Health Commission of China listed Wumei in the catalog of homology of medicine and food, which not only has good edible value but also has good medicinal value. Especially, it has unique advantages and characteristics in anti-cancer. In recent years, the emerging network pharmacology research method has found widespread application in traditional Chinese medicine research, facilitating in-depth exploration of drug-biological system interactions through computer technology and informatics methods.[5] Network pharmacology holds promise for systematically elucidating the potential anti-cancer mechanism of Wumei. Thus, this study utilizes a combination of literature review and network pharmacology research to investigate the anti-cancer properties of Wumei preliminarily. Based on the study of ancient and modern literature, this paper systematically summarizes the medicinal value of Wumei from the resource distribution, historical origin, and traditional Chinese medicine understanding, combined with modern network pharmacology research, and puts forward the clinical value of Wumei against cancer.

1. Medicinal origin of Wumei:

1.1 Distribution of plant resources of Wumei:

The cultivation history of Wumei, also known as smoked plum, black plum, plum seed, and orange plum meat, dates back to ancient times as documented in the Notes on Materia Medica Classic. The plum originated in China and is cultivated and found in both wild and cultivated forms across a wide geographic area encompassing the Qinling Mountains, south of the Huaihe River, and between latitudes 23-33 degrees north.[6] The provinces of Taiwan, Guangdong, Jiangsu, and Zhejiang are primarily engaged in the introduction and cultivation of plum fruit. The cultivation of fruit plums predominantly consists of original variety plums, while wild plums are primarily found in the western and southwestern regions of Sichuan, Yunnan, and Guizhou provinces. According to the "Southern Cooperative Group for Variety Arrangement and Quality Research of Commonly Used Chinese Herbal Medicines" and "Chinese Medicinal Materials Science," Wumei is primarily cultivated in Fujian, Sichuan, Zhejiang, Hunan, and Guangdong provinces.[7,8] Among the different regions where Wumei is produced, the quality of Wumei from Zhejiang is notable, while the largest output comes from Sichuan. There

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are three prevalent processing methods for Wumei: drying, smoking, and baking, with the primary method being the cultivation of variant plums. The drying method includes hot drying and steaming drying, while the smoking method can be categorized into pine smoking and miscellaneous wood smoking, each utilizing distinct fuels. The predominant method of consumption for this clinical medicinal product is smoking, and due to the superior quality of the product from Zhejiang Changxing, it is colloquially referred to as "black plum smoked by green fruit," highlighting the company's dominant position in the market.

1.2 Tracing back to the medicinal value of Wumei:

Shennong's classic of materia medica records the medicinal use of Wumei for the first time, which is listed as a medium product, flat in nature, sour and astringent in taste, and belongs to the lung, liver, spleen, kidney, stomach, and large intestine meridians.[9] Its functions mainly include astringent lung qi, astringent intestine to stop diarrhea, promoting fluid production to quench thirst, relieving vexation, etc. It is used for chronic cough due to lung deficiency, asthenia, heat to quench thirst, chronic diarrhea, and constipation. The Materia Medica of past dynasties has described the efficacy of Wumei in detail, and *Shennong's classic of materia medica* puts forward "governing qi, removing heat and annoyance, reassuring, limb pain, being dry and heartless, killing muscles, removing green moles and evil meat." It is pointed out that Wumei can remove green moles and evil meat, which should be the earliest anti-tumor record of Wumei. *LiuJuanZi Ghost* left a prescription that also records the efficacy of Wumei in removing evil meat. In the book, Wumei meat is used to burn and preserve, and it can be eliminated overnight. Meng Shen's *Dietetic Materia Medica* records that Wumei can relieve constipation for external use, and its description of symptoms is similar to intestinal obstruction. It says, "The stool is impassable, and the qi is running to death. Ten Wumeis are placed in soup, and the nucleus must be removed. The pestle is as big as a jujube, and the lower part is accepted, which can be passed when it is young." *Compendium of Materia Medica* points out that Wumei can be used to treat gastric cancer and esophageal cancer, saying: "Converging lungs and astringing intestines, treating chronic cough, diarrhea, nausea and choking diaphragm, vomiting, detumescence, phlegm, killing insects, relieving fish poison, horse sweat poison and sulfur poison." Symptoms such as joint pain, skin paralysis, boredom, and diarrhea are also common clinical problems of cancer, and Wumei also has very good effects. As pointed out in the List of Famous Doctors, Wumei has the functions of stopping dysentery, quenching thirst, and dredging tendons. *Rihuazi Materia Medica* also said: "Eliminate labor, treat bone steaming, relieve boredom, astringent intestines, stop dysentery, eliminate alcohol toxicity, treat paralysis of dry skin, remove black spots, and make people sleep." When *Materia Medica Jingshu* talks about the treatment of limb pain and hemiplegia with Wumei, it thinks that its mechanism lies in "because moisture is immersed in meridians, the tendons are relaxed or painful; Liver is the main tendons, acid enters the liver and nourishes the tendons, and the liver is nourished, so the bones are soft and the organs are beneficial." In addition, *Materia Medica Qiuyuan* talks about how Wumei can be used for blood syndrome, which has good curative effect on hematuria, hematochezia, and various blood diseases. The use method of Wumei emphasizes that it should be dipped in salt when it is used raw, which can warm gallbladder and promote fluid production, and has better effect. At the same time, it also points out that pregnant women can eat more, especially the Wumei, before Xiaoman has the best effect. Wumei, as a traditional Chinese medicine with the homology of medicine and food, has been widely concerned at home and abroad. It contains a large number of organic acids, sugars, and vitamins, and has the effects of helping to lower blood sugar, clearing the throat and moistening the throat, improving skin moisture, stimulating appetite, regulating mood, relieving headaches after drinking, and resisting tumors. Because it has the effects of relieving cough (chronic cough, dry cough), relieving deficiency sweating (spontaneous sweating due to qi deficiency, night sweating due to yin deficiency), stopping metrorrhagia (Wumei charcoal), stopping diarrhea (both deficiency and excess, cold and heat), stopping leukorrhagia (yellow leukorrhagia, thin white matter, red and white), etc., Chinese medicine calls it a good medicine for "five stops."[10]

1.3 Processing and application of Wumei:

As shown in Figure 2, Wumei has different processed products with different effects, such as clean Wumei, Wumei meat, vinegar Wumei, Wumei charcoal, and so on. The records of Wumei, Wumei meat, Wumei charcoal, steamed Wumei, and vinegar Wumei are included in the 1963 edition of I*ntegration of Traditional Chinese Medicine Processing Experience*.[11] The Pharmacopoeia of the Chinese pharmacopeia in different periods has different records on processed products of Wumei, and the 1963 edition contains Wumei, Wumei meat, and Wumei charcoal.[12] The 1977 edition did not contain Wumei meat, and the 1985 edition added Wumei meat: "Take clean Wumei, moisten it to make it soft or steam it to remove the core."[13,14] At present, the processing methods (Code for Processing Chinese Herbal Medicine), Chinese Pharmacopoeia (2000 edition), and Integration of Traditional Chinese Medicine Processing Experience are vinegar, charcoal frying, and steaming.[11,15,16] The four common methods for preparing black plums are shown in Figure 1. According to comprehensive data analysis, the processing methods of Wumei include frying, baking, steaming, boiling, roasting, and charcoal making without auxiliary materials, and vinegar making, wine making, salt making, and honey making with auxiliary materials. In the Han Dynasty, *Jin Kui Yu Han Jing* was recorded as the earliest processing method of Wumei. "Soak Wumei in bitter wine for one night, remove the core, steam it under five buckets of rice, and pound it into mud when the rice is cooked."[17]

In various regions of China, two types of Wumei decoction pieces are utilized: pure Wumei meat and nuclear combination. Currently, they are commonly employed without nuclear removal for several reasons. Firstly, the clinical efficacy of Wumei remains unaffected by the removal of the nucleus, and the process of removal is deemed laborious and time-consuming. Additionally, the inclusion of the nucleus in Wumei facilitates its storage, transportation, preservation, and processing, there

by extending its shelf life and reducing the likelihood of decay. Furthermore, the presence of the kernel in Chinese medicine Wumei aids in distinguishing authentic from counterfeit Wumei, thus justifying the retention of the kernel.[18]

Various processed products of Wumei exhibit distinct clinical applications, including the preservation of acidity in their raw form and the promotion of salivation, alleviation of vexation, and enhancement of astringency and hemostasis following charcoal preparation. Wumei is abundant in organic acids, notably malic acid and citric acid, which are considered the most significant. It has been observed that the organic acids present in Wumei products predominantly exist in diverse salt forms. Following vinegar treatment, these acids are converted into salt organic acids, subsequently reverting to a free state, thereby enabling them to exert a distinct pharmacological effect.[18]

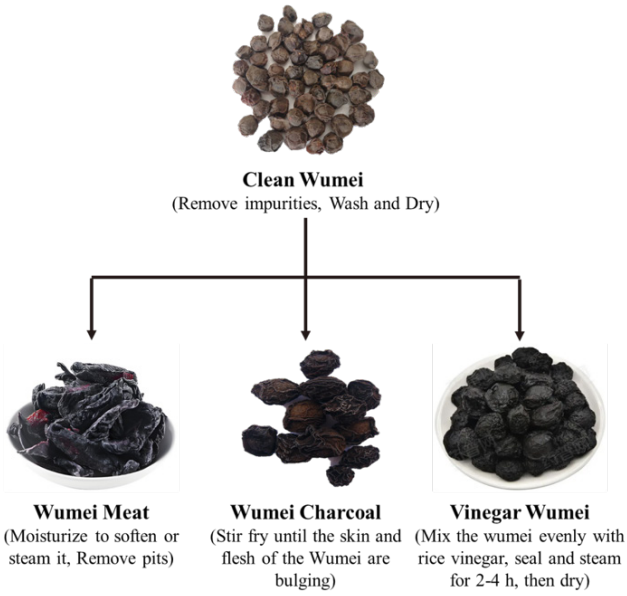

Figure 1: Four common Wumei processed products. This picture mainly shows four common processed products of Wumei: Clean Wumei, Wumei Meat, Wumei Charcoal, and Vinegar Wumei.

1.4 Clinical value of Wumei against cancer:

The prevention and treatment of cancer through the lens of both Chinese and Western medicine is a topic of significant interest. Extensive clinical observations, in conjunction with traditional Chinese medicine's perspective on malignant tumors, suggest that cancer can be viewed as a manifestation of a yin-yang imbalance. Central to the pathogenesis of cancer transformation is the interplay between healthy qi and cancer toxins. One aspect of disease development involves the healthy dissipation of qi and positive deficiency loss in solid absorption. In contrast, another aspect involves the consumption of healthy qi by cancer toxins, which promote cancer diffusion and metastasis. In light of this characteristic, a fixation-based treatment method has been proposed for clinical cancer treatment. This approach aims to strengthen body resistance and fixation to prevent the outward dispersion of healthy qi, correct deficiencies, and prevent or reduce cancer spread and metastasis.

Contemporary research has validated that Wumei contains various chemical compounds such as organic acids, flavonoids, terpenoids, and polysaccharides. Specifically, the organic acids and volatile components exhibit notable antioxidant properties, while flavonoids, terpenoids, and sterols demonstrate significant anti-tumor effects. The terpenoids ursolic acid and oleanolic acid, found in the pulp of Wumei, are identified as natural anticancer agents. Oleanolic acid demonstrates potent inhibitory properties against the proliferation and progression of malignant tumors, leading to the restoration of serum protein levels in tumor-bearing mice and suppression of DS-PAGE protein component expression.[19] Oleanolic acid has been shown to upregulate the expression of the P21 protein and downregulate the expression of the Ki-67 protein in lymphoma-bearing mice, thereby exerting control over the proliferation of tumor cells in mice.[20] Ursolic acid, a naturally occurring compound, exhibits anti-tumor properties by effectively inhibiting the growth of A172 glioma cells, SGC7901 gastric cancer cells, and A-549 lung cancer cells.[21] Research conducted by Xu Chao has demonstrated the efficacy of Wumei extract in inhibiting colon cancer and cervical cancer. Additionally, Chao's research indicates that Wumei extract can effectively impede the migration of colon cancer HT29 cells.[22] Furthermore, the extract has been found to alleviate hematochezia and physical deterioration in patients with colon cancer, potentially preventing the progression from colitis to cancer. The extract of Wumei pulp demonstrates promising preventive properties against various types of cancer, including liver cancer, gastric cancer, renal cancer, melanoma, leukemia, endometrial adenocarcinoma, and ovarian cancer. Several traditional Chinese medicine formulations incorporating Wumei exhibit notable anti-cancer efficacy, such as Wumei pills, Wumei-Siwu decoction, and allergic decoction. The traditional Chinese medicine prescription known as the Wumei Pill consists of various herbs such as Wumei, asarum, dried ginger, Coptis chinensis, Angelica sinensis, Heishun tablets, Chuanjiao, cassia twig, ginseng, and Phellodendron amurense exhibit therapeutic properties including wind-calming and astringent effects, yang-supporting and yin-strengthening actions, as well as warming and tonifying properties. This prescription is commonly used in clinical practice for the treatment of pancreatic cancer, lung cancer, cervical cancer, and liver cancer, among others, and has demonstrated positive clinical outcomes. In a study conducted by Huang Jinchang *et al.*, [21] patients diagnosed with advanced pancreatic cancer were administered the Wumei Pill.[23] Following a 3-month treatment period, the findings indicated an appetite improvement rate of 80.00%, an abdominal pain relief rate of 52.63%, and a clinical effective rate of 71.43%. Chu Shirong clinically selected 45 patients with gastric cancer and treated them with the Wumei Pill.[24] The results showed that it had the best curative effect on stage II patients, which could improve the quality of life, hemogram, and T cell subsets of patients. Contemporary pharmacological research has demonstrated that Wumei and its associated compounds exhibit anti-tumor properties through various mechanisms, including the inhibition of precancerous lesions, modulation of gene expression, alteration of protein activity

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and signal pathway expression, suppression of tumor cell proliferation and metastasis, and facilitation of tumor cell apoptosis.

Methods

Table 1: Research on anti-cancer effects of Wumei in the PubMed database.

By searching the PubMed database for relevant experimental research literature, the anti-cancer mechanism of Wumei formula preparation was summarized.

References	Single herb/Formula	Study type	Cancer type	Mechanism
[25]	Formula (Wumei pill)	*in vivo and in vitro*	Colon Cancer	AHCY-mediated hedgehog signaling pathway
[26]	Formula (Wumei pill)	*in vivo*	Colon Cancer	PI3K/Akt signaling pathway
[27]	Formula (Compound Wumei Powder)	*in vivo*	Gastric Cancer	Cox-2/PGE2-PI3K/AKT/GSK3 β/ β-Catenin Signaling Pathway

1. Network pharmacological analysis of Wumei against cancer:

Traditional Chinese medicine emphasizes the importance of a holistic approach to disease treatment. Modern pharmacological research has demonstrated that traditional Chinese medicine can effectively address diseases through the manipulation of multiple targets and pathways. The development of a network action mode based on multiple targets and pathways further exemplifies the holistic perspective inherent in traditional Chinese medicine. In recent years, the network pharmacology research approach has been commonly employed to investigate the mechanism of action of traditional Chinese medicine. Following the principles of network pharmacology research, this study conducts an initial investigation into the principal bioactive compounds of Wumei and their potential anticancer mechanisms.

1.1 Data and Methodology:

1.1.1 Determination of Active Components in Fructus Mume:

In this study, the TCMSP platform (http://tcmspw.com/tcmsp.php) was utilized to identify the constituents of Wumei. Subsequently, the drug components were filtered based on their bioavailability (OB) ≥ 30% and drug-likeness (DL) ≥ 0.18.[28]

1.1.2 Target analysis of bioactive components in Wumei:

According to the screening results, the active components of Wumei were imported into the PubChem database (https://pubchem.ncbi.nlm.nih.gov/) to obtain the SMILES structure of each active component. The SMILES structure was used to detect the targets on the SwissTargetPrediction platform (http://www.swisstargetprediction.ch/), and the predicted targets were screened according to Probability > 0.

1.1.3 Construction of the target protein interaction (PPI) network of efficacious substances in Wumei:

The target of active components of Wumei was imported into the STRING database (https://string-db.org/), and the mapping relationship of the protein interaction network (PPI) was obtained, which was also visualized by the Cytoscape software. In addition, through the Cytoscape plug-in MCODE topology analysis of the PPI network, we can get the key sub-modules in the network, and the targets in the key modules are the key targets of Wumei.

1.1.4 Biological enrichment analysis:

In this study, biological enrichment analysis was carried out on the targets of efficacious substances in Wumei, including KEGG pathway enrichment analysis, DisGENET disease enrichment analysis, and PaGenBase cell and tissue enrichment analysis. Biologic enrichment analysis was performed by the online tool Metascape (http://metascape.org/), and the screening threshold of enrichment results was $P < 0.05$.

Results and Discussion

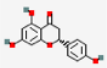
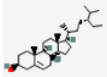
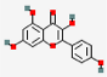
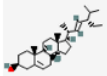
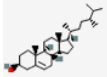
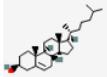
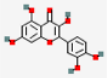
1. Result

1.1 Screening of effective substances in Wumei:

Forty compounds of Wumei were retrieved by the TCMSP data platform. According to oral bioavailability (OB) ≥ 30% and drug-like (DL) ≥ 0.18, eight effective substances were screened. See Table 1 for specific molecular structures and parameters.

1.2 Prediction of the action target of effective substances in Wumei:

Table 2: Efficacious Substances of Fructus Mume. The data is sourced from the TCMSP database and PubChem database, summarizing the main active ingredients of Wumei, including molecule name, 2D structure, oral bioavailability, etc.

Mol ID	Molecule Name	2D Structure	OB (%)	DL
MOL001040	(2R)-5, 7-dihydroxy-2-(4-hydroxyphenyl) chroman-4-one		42.36	0.21
MOL000358	Beta-sitosterol		36.91	0.75
MOL000422	Kaempferol		41.88	0.24
MOL000449	Stigmasterol		43.83	0.76
MOL005043	campest-5-en-3beta-ol		37.58	0.71
MOL008601	Methyl arachidonate		46.9	0.23
MOL000953	CLR		37.87	0.68
MOL000098	Quercetin		46.43	0.28

The SwissTarget platform was used to predict the action targets of 8 pharmacodynamic substances in Wumei. After screening out repeated targets, a total of 282 were obtained. Further, a network of "Wumei-pharmacodynamic substances

-action targets" with 291 nodes and 568 edges was constructed by Cytoscape (Figure 3).

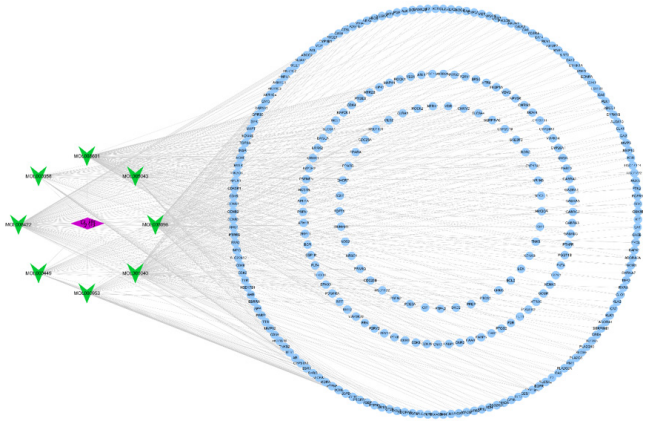


Figure 2: Wumei-pharmacodynamic substances-target network. The red diamond-shaped nodes represent Wumei, the green arrow nodes represent medicinal substances, and the blue circular nodes represent targets.

1.3 PPI network of action targets of Wumei pharmacodynamic substances:

The potential targets of Wumei obtained above are introduced into the STRING platform to build the PPI network, which contains 277 nodes and 2548 edges (Figure 4). The network topology analysis is carried out by the CytoHubba plug-in. The 20 most critical targets in the network (from high to low according to MCC value) are screened out: VEGFA, SRC, AKT1, ESR1, BCL2L1, IGF1R, MAPK1, PTGS2, EGFR, MDM2, MAP2K1, MCL1, CCNB1, MMP9, PIK3CA, AR, GSK3B, MAPK14, MMP2 and KDR. (Figure 5, Table 2)

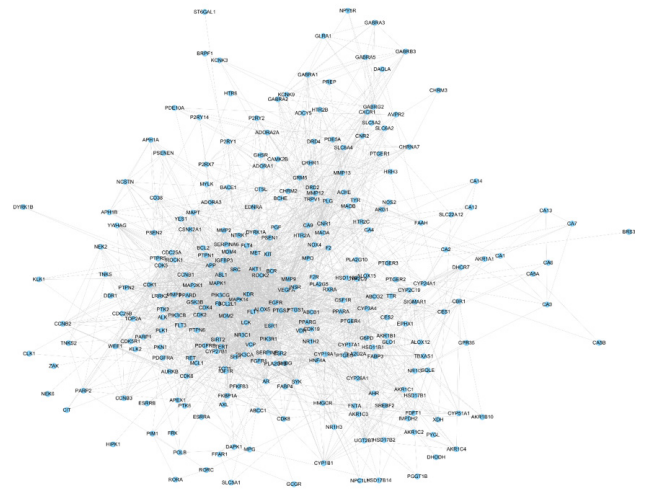


Figure 3: PPI Network of Wumei's potential targets. The blue nodes in the PPI network represent potential targets of Wumei, and the mapping relationships between targets were obtained through the STRING database.

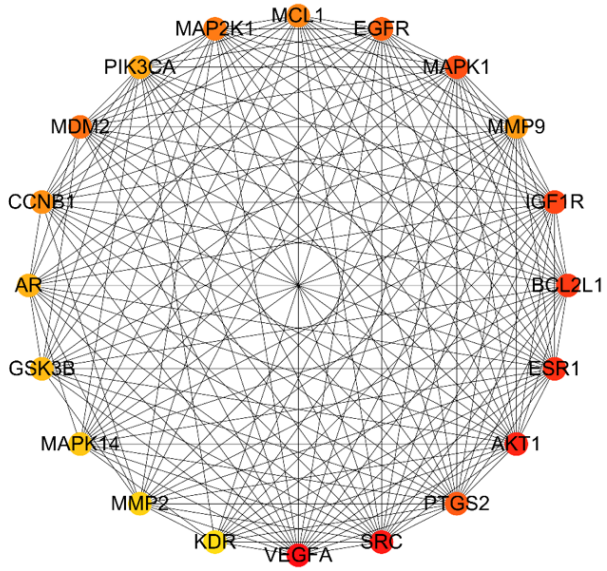


Figure 4: Key targets of the PPI network. Screen key targets in the PPI network through the CytoHubba plugin and associate the color of the target with the MCC value. The closer the color is to red, the higher the MCC value of the node.

Table 3: Ranking of key targets. This table displays the names of the 20 most critical targets in the PPI network, and ranks the top 20 targets based on MCC values.

Rank	Name
1	VEGFA
2	SRC
3	AKT1
4	ESR1
5	BCL2L1
6	IGF1R
7	MAPK1
8	PTGS2
9	EGFR
10	MDM2
11	MAP2K1
12	MCL1
13	CCNB1
14	MMP9
15	PIK3CA
16	AR
17	GSK3B
18	MAPK14
19	MMP2
20	KDR

1.4 Prediction of the anticancer mechanism of Wumei Enrichment analysis of the KEGG pathway:

KEGG pathway enrichment analysis showed that there were thirteen signaling pathways: pathways in cancer, PI3K-Akt signaling pathway, Rap1 signaling pathway, Ras signaling pathway, Central carbon metabolism in cancer, MAPK signaling pathway, Steroid biosynthesis, Neuroactive ligand-receptor interaction, Arachidonic acid metabolism, Nitrogen metabolism, Calcium signaling pathway, Steroid hormone b-Steroid

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biosynthesis, Neuroactive ligand-receptor interaction, Arachidonic acid metabolism, Nitrogen metabolism, Calcium signaling path, Steroid hormone biosynthesis and Ovarian steroidogenesis.

Table 4: KEGG Signal Pathway. According to the KEGG pathway enrichment analysis results, the table displays the top 13 pathways closely related to cancer, sorted by P-value.

HSA05200	**Pathways in cancer**	-41.27604898	-37.62417411	56/531
HSA04151	**PI3K-Akt signaling path**	-28.09546047	-25.22451062	38/354
HSA04015	Rap1 signaling path	-23.35571358	-20.72364582	28/210
HSA04014	Ras signaling path	-22.14156524	-19.59967412	28/232
HSA05230	**Central carbon metabolism in cancer**	-17.46310856	-15.26425657	16/70
HSA04010	**MAPK signaling path**	-14.07898933	-12.12536991	23/294
hsa00100	Steroid biosynthesis	-7.660813539	-6.23129697	6/20
HSA04080	Neuroactive ligand-receptor interaction	-36.17845309	-32.87177467	45/362
HSA00590	Arachidonic acid metabolism	-16.92690069	-14.77195419	15/61
HSA00910	Nitrogen metabolism	-20.68530566	-18.25104841	12/17
HSA04020	Calcium signaling path	-24.16113838	-21.4662798	30/240
hsa00140	Steroid hormone biosynthesis	-13.86315093	-11.9233198	13/61
HSA04913	Ovarian steroidogenesis	-11.87762713	-10.07733073	11/51

1.5 DisGENET disease enrichment analysis:

The findings of the DisGENET disease enrichment analysis indicate that Wumei drug targets have the potential to impact a range of diseases, including but not limited to adult medulloblastoma, childhood medulloblastoma, diabetes, memory disorder, neuralgia, childhood astrocytoma, non-Hodgkin's lymphoma, cognitive impairment, amyloidosis, mesothelioma, benign prostatic hyperplasia, endothelial dysfunction, hormone-resistant prostate cancer, pre-senile dementia, secondary bone malignant tumor, enzyme inhibition disorder, fatty liver, breast cancer stage IV, meningioma, and head and neck cancer (Figure 6). It can be seen from the above that tumor diseases account for 50% of the top 20 diseases.

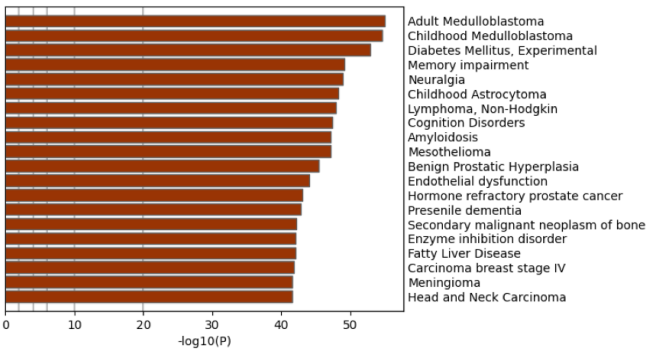

Figure 5: DisGENET enrichment analysis. This figure shows the enrichment analysis results of DisGENET disease, with the x-axis representing - logP. The larger the value, the higher the correlation between Wumei's targets and the disease.

1.6 Analysis of cell and tissue enrichment of PaGenBase:

The findings from the PaGenBase enrichment analysis indicate that the effective components of Mume plum primarily target various tissues, including the liver, placenta, uterus, kidney, caudate nucleus, breast, small intestine, lung, and others. At the cellular level, Wumei appears to affect dorsal root ganglion cells, adipocytes, prostate cells, liver cancer cells, hepatocytes, ovarian cancer cells, brain cells, Burkitt's lymphoma cells, human umbilical vein endothelial cells, bronchial epithelial cells, and myocardial cells (Figure 7).

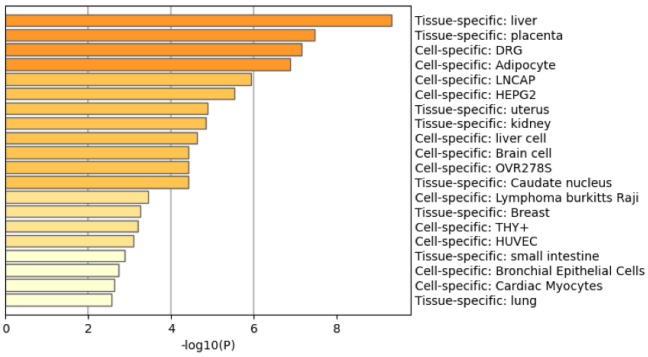

Figure 6: PaGenBase enrichment analysis reflects the tissues and organs involved in the target of Wumei's targets. This figure shows the results of PaGenBase disease enrichment analysis, with the x-axis representing - logP. The larger the value, the higher the correlation between Wumei's targets and the tissue or organ.

2. Discussion:

Wumei, a traditional Chinese medicine derived from the same source as food and medicine, is recognized for its safety and reliability, as well as its diverse clinical health and therapeutic benefits. Originating in China, Wumei is particularly renowned for its superior quality when produced in Zhejiang, although the most significant production of Wumei occurs in Sichuan. Various processed forms of Wumei, including purified Wumei, Wumei meat, vinegar Wumei, and Wumei charcoal, offer distinct therapeutic effects. The historical significance of Wumei in traditional Chinese medicine dates back to ancient texts such as *Shennong's Classic of Materia Medica*, which highlights its medicinal properties, including astringent lung qi, astringent intestines to stop diarrhea, generating fluids to quench thirst, relieving roundworms, and alleviating restlessness. Wumei's diverse clinical effects, particularly in the realm of cancer prevention and treatment, have garnered significant attention within the traditional Chinese medicine community. Currently, there is a growing body of research on the anti-tumor properties of Wumei. However, the majority of findings are constrained. This study aims to employ network pharmacology techniques to comprehensively investigate its anti-tumor mechanism.

However, this study has some limitations. This study primarily utilizes literature reviews and database analysis to ascertain the active components and potential targets of Ume. The reliability and precision of these databases may be constrained by the current state of research and data gathering, potentially leading to the exclusion or misinterpretation of specific active ingredients or targets. Furthermore, while network pharmacology techniques offer valuable insights into predicting drug mechanisms, this study lacks direct experimental verification to substantiate its predictive findings. Without experimental support, the proposed mechanisms remain hypothetical, highlighting the necessity for experimental studies to validate the computational predictions and bridge the gap between theoretical models and biological reality. Experimental validation plays a critical role in confirming the mechanism of drug

action and therapeutic potential and is imperative for the advancement of drug development.

Conclusion

This study conducted a preliminary analysis to predict 282 drug action targets of *Prunus mume*. The protein-protein interaction network topology analysis results identified key targets such as VEGFA, SRC, and AKT1. Among these targets, VEGFA, a vascular endothelial growth factor, was found to be involved in the pathogenesis of lung cancer, colon cancer, breast cancer, and other tumors, making it a significant drug target for anti-tumor therapy.[29,30] The SRC family of non-receptor tyrosine kinases plays a crucial role in the interplay between inflammation and cancer by facilitating communication and signaling between immune cells and tissue cells, thereby contributing to the initiation and advancement of cancer.[31] AKT1 has been identified as a carcinogen and is a critical component of the PI3K pathway, playing a significant role in developing and advancing diverse tumor types.[32,33] Pathway enrichment analysis reveals that Wumei is implicated in pathways associated with tumorigenesis, including *Pathways in cancer*, *PI3K Akt signaling pathway*, *Central carbon metabolism in cancer*, and *MAPK signaling pathway, et al*. However, this study still has certain limitations. Firstly, network pharmacology only relies on database mining and prediction, which may miss some components and targets. At the same time, the results of related mechanism research need to be further verified through experiments.

In conclusion, Wumei, a widely used traditional Chinese medicine in clinical settings, demonstrates notable medicinal properties, particularly in the realm of anti-tumor activity. Wumei has the potential to modulate various pathways and targets associated with tumors, thereby exerting therapeutic effects on diverse types of malignancies. Nevertheless, additional investigation and clinical trials are imperative to validate the anticancer efficacy and clinical utility of Wumei.

Future research should prioritize experimental validation of key compounds identified through network pharmacology, such as ursolic acid, which has shown pro-apoptotic and anti-proliferative effects in cancer models.[34] For instance, studies have demonstrated that ursolic acid inhibits NF-κB signaling and induces apoptosis in human cancer cells, supporting its predicted role in tumor suppression pathways. Similarly, oleanolic acid has been reported to exhibit anti-inflammatory and anti-cancer properties, which align with the bioinformatics-based findings.[35] These existing experimental results provide the foundation for designing targeted experimental studies to confirm the predicted molecular mechanisms and therapeutic roles of Wumei's active constituents.

Acknowledgment

The completion of the thesis is attributed to the support and encouragement of many people. I would like to express my sincere gratitude to my mentor, Professor Li, for his helpful guidance, valuable suggestions, and constant encouragement. He gave me much help and advice during the whole writing process, making my accomplishments possible.

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■ RESEARCH ARTICLE

The Effects of Micro and Nanoplastics on the Brain and Gene Expression

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ABSTRACT: In today's world, it is impossible to avoid coming into contact with micro- and nanoplastic (MNP) particles: from 1950 to 2015, the world produced approximately 6.3 billion tons of plastic in total. MNP particles infiltrate the air, water, and food. As a result, we ingest plastics every day throughout our lives by using ordinary things, such as tea bags, paper cups, water bottles, or toothpaste, and by eating vegetables and meats. In experimental rodents and fish, plastic particles have been detected within the brain, muscles, lungs, liver, kidneys, heart, and GI tract. MNPs that enter the body and breach the brain-blood barrier (BBB) inhibit crucial enzymes and neurotransmitters, in particular acetylcholinesterase (AChE), and induce oxidative stress in cells, leading to Alzheimer's and Parkinson's diseases. We analyze the mechanisms behind the neurotoxicity of MNPs by examining the causes of neurotransmitter inhibition and cellular damage, and their effects on the brain and gene expression. This review article highlights the existing research gaps and the urgency for more research on this topic to gain a more comprehensive understanding of the potential health risks of MNP exposure. Moving forward, potential solutions, including the use of probiotics, enzymes, and vitamins, must be explored and utilized.

KEYWORDS: Cellular and Molecular Biology, Neurobiology, Neurotoxicity, Micro and Nanoplastics (MNPs), Blood-Brain Barrier (BBB), Oxidative Stress; Acetylcholine (ACh).

■ Introduction

Throughout the past two decades, plastic has been continuously infiltrating our daily lives - through our food, drinks, air, and contact with the skin. On average, the world annually produces approximately 359 million tons of plastic, only about 15% of which has been recycled over the last 30 years.[1] Polystyrene and polyethylene, two of the most widespread types of plastic used in manufacturing, have the highest environmental pollution levels and can absorb a broad range of environmental pollutants. They are often used in plastic-related studies.[2] MNPs are extremely small fragments of plastic that can be categorized as either primary or secondary. Primary MNPs, which are added to cosmetics, fabrics, and paints, were originally manufactured in a very small size on purpose. Secondary MNPs come from larger pieces of plastic, such as car tires and fishing nets, that are degraded through natural means including photodegradation, sand and water abrasion, and erosion.[3] Plastic particles are considered microplastics (MPs) when they are less than 5 mm in size, and nanoplastics (NPs) when they are smaller than 0.1 μm.[4] The extent of plastics' impact on human health has only started to be uncovered. MNPs have been found in several major organs, including the heart, kidneys, gut, lungs, liver, placenta, and brain.[5,6] In multiple studies using adult zebrafish and in one study with tilapia fish, MNP accumulation was present in the intestines, gills, liver, and brain.[3,7-9] It is estimated that humans consume around 80 grams of plastic per day via fruits and vegetables that have been contaminated through the soil.[5]

Information on how plastics impact human organs, especially the brain, is still scarce. Currently, existing experiments observed that interactions between MNPs and brain tissue trigger neurodegenerative processes. These processes included elevated acetylcholine (ACh) levels, which suggests that the MNPs are inhibiting acetylcholinesterase (AChE) activity, and increased malondialdehyde (MDA) levels, which is a marker for oxidative stress.[3] These results indicate that MNPs can be dangerous for brain health and gene expression. This review article aims to explain the potential neurotoxic and genotoxic health risks MNPs pose to human health and emphasizes the critical need for more research to be done on this topic by highlighting research gaps in current studies.

Factors that Influence the Neurotoxicity and Cytotoxicity of Plastic Particles:

Physical Characteristics:

The shape and size of a particle can tell a lot about its neurotoxic potential. Sarasamma *et al.* noted that based on experimental results, the neurotoxicity of polystyrene nanoplastics (PS-NPs) on aquatic biota heavily relies on the size, shape, and composition of the PS-NPs. An experiment on wild-type mice found that only two hours after exposure, the 0.293 μm particles (smallest sized particles out of all the experimental groups - 9.55 μm, 1.14 μm, and 0.293 μm) were detected in brain tissue. This indicates that the smallest-sized particles were able to directly cross the BBB.[2] This suggests that the smaller the particle size, the easier it is for it to permeate the BBB.

Another study that tested the cellular uptake of MNPs in HeLa cells of 10 nm, 15 nm, 25 nm, 40 nm, 50 nm, and 500 nm radii found that only the smaller particles - 10 nm, 15 nm, and 25 nm, were able to penetrate the cell membrane.[10] This

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is similar to the findings of Kopatz *et al*., which stated that NPs less than 0.5 μm in size could come into and out of the cell through the process of transcytosis and potentially interact with intracellular organelles. Size can even influence how deep into an organ an MNP can go. MNPs less than 20 μm in size can penetrate organ barriers and those less than 10 μm in size can cross the cell membrane, cross the BBB, and reach brain tissue.[5]

Intrinsic Additives:

Additives are supplementary chemicals added during the process of plastic manufacturing that give plastics new, unnatural characteristics such as inorganic pigments and increased shininess. Stabilizing additives make plastic more durable by making it more resistant to UV radiation, humidity, bacterial degradation, mold, and weathering. Most of the time, additives are not polymerized with plastic molecules, so they easily contaminate the soil, water, and air in high amounts.[5] When in the natural world, MNPs can reach plants and aquatic biota, which humans consume later on up the food chain. This also means that these toxic additives can be left behind in plastic-packaged food and cause neural and cellular issues (Table 1).

Table 1: Neurotoxic and Cytotoxic Effects of Heavy Metal Additives in Plastic Particles. Adapted from Campanale *et al*., Table 1. "Main use of heavy metals as additives in polymer products and their effects on human health". Heavy metal additives, especially lead, manganese, and mercury, exacerbate the neurotoxic and cytotoxic effects MNPs pose to humans.

Heavy Metal Additive	Purpose of Additive	Neurotoxic/Cytotoxic Effects	References
Lead	Inorganic pigments, heat stabilization, and the prevention of UV degradation	Oxidative stress, Central Nervous System (CNS) disturbance (including damage to motor functions), harm to DNA reparation system, excess Reactive Oxygen Species (ROS) production, changes in apoptosis related genes, blocks neurotransmitter release, lowers Brain-Derived Neurotrophic Factor (BDNF) expression, impaired pre- and postsynaptic signaling systems, increased Blood-Brain Barrier (BBB) leakage, gastrointestinal (GI) tract issues	11, 12
Bromine	Flame retardant	Apoptosis, genotoxicity	5
Manganese	Inorganic pigments	Accumulation in the mitochondria of brain cells and impaired Adenosine Triphosphate (ATP) synthesis, harm to dopaminergic system, emotional dysfunction, neurodegenerative diseases, memory dysfunction, motor incoordination	11, 13
Mercury	Biocide (used to preserve materials by protecting from viruses and stopping microorganism, insect, and animal related degradation)	AChE inhibition, excess ROS formation, changes in DNA structure, interaction with brain macromolecules, changes in activity levels of energy related enzymes, lipid peroxidation, neurobehavioral changes, reduced motor neuron function, neuroinflammation	11, 14, 15
Barium	Inorganic pigment, UV stabilization	Metabolic, mental, and neurological alterations; bioaccumulation in the gut	5, 16
Cadmium	Inorganic pigments, heat stabilization, and prevention of UV degradation	Lipid peroxidation, DNA damage, apoptosis, altered gene expression	11, 17
Cobalt	Inorganic pigments	Development of excess ROS and problems with regulation of bodily senses	5
Chrome	Inorganic pigments	Free radical generation, severe issues throughout the body including in the GI tract and in the brain, potential death	5, 11
Copper	Biocide	ROS formation, excess oxidation, and DNA structural damage	11
Arsenic	Biocide	Neurological disorders, issues with GI tract, inhibited DNA reparation, increased ROS production, oxidative stress	11, 18-20

Extrinsic Co-contaminants:

Particle surface chemistry and charge both influence the neurotoxic potential of MNPs. Several studies have demonstrated that oxygen-containing groups on plastic surfaces make oxidized MNPs more neurotoxic than non-oxidized MNPs. The oxidation aggravates the existing neurotoxic problems the MNPs present.[21] MNPs can also bring co-pollutants such as pathogenesis microorganisms, extrinsically attached heavy metals, pharmaceuticals, and persistent organic pollutants.[3,15] The physicochemical qualities of plastic particles allow co-pollutants to latch onto MNPs through chemical interactions such as hydrophobic interactions and Van der Waals forces.[6]

The biomolecular corona, a layer of proteins, organic matter, biomolecules, and chemical and biological contaminants, that form on the surface of MNPs as they interact with the environment before they enter the body, can also increase or decrease their neurotoxic potential.[2] It contributes to the toxicity of MNPs as it may contain co-pollutants that latch onto the plastic particles and enter the body with them. Depending on the composition of the biomolecular corona, it can also make it easier or harder for the plastic to cross the BBB.[2]

The Entrance and Distribution of MNPs into the Body, Brain, and Cells:

Entrance Pathways:

The three main ways MNPs enter the body are through ingestion, inhalation, or dermal contact.[6] We ingest plastics by using ordinary things, such as tea bags, paper cups, or toothpaste, and by eating vegetables and contaminated meat (especially seafood).[3,6] Bottled water is estimated to contain 0.09 MPs/g, while sugar contains approximately 0.44 MPs/g.[5] Paper cups also have an internal plastic lining that releases MPs and toxic, heavy metals when exposed to hot beverages. Inhalation of MNPs occurs whenever we breathe in polluted air or dust.[6] Lastly, when we come into contact with items that contain primary MNPs, such as personal care products, the plastic particles can enter deep into the skin through dermal contact.[3] Usually, only MNPs less than 20 μm in size are able to enter organs and that 10 μm and less can penetrate the cell membrane, BBB, and brain tissue.[5] People working in nanoparticle manufacturing industries and people living around factories are most at risk for plastic inhalation and dermal contact.[6]

After entering into the body, MNPs immediately join blood circulation which distributes these particles to various organs.[6] For the plastic particles to reach brain tissue, they have to breach the BBB, a semipermeable membrane that lies between the blood and the brain to protect the brain from harmful pathogens and toxic substances. It makes it very difficult for any ions or molecules to pass through without any help. However, there are a few possible ways for a plastic particle to cross this seemingly impenetrable barrier or even enter into a cell.

Blood-Brain Barrier (BBB) Permeability:

The composition of the biomolecular corona of an MNP plays a big role in its ability to cross the BBB. A study conducted at the Medical University of Vienna, Austria, in 2022 simulated four different models of polystyrene plastic transfer through the BBB. Each plastic particle had a different biomolecular corona: pristine plastic, particles with 100 and 150 cholesterol molecules corona, and particles with 40 protein molecules corona. The simulation used a dioleoylphosphatidylcholine (DOPC) membrane (common substitution for BBB in studies) and observed that when an MNP with a cho-

lesterol corona is approaching, the DOPC molecules rearrange by pointing their hydrophobic tails toward the hydrophobic cholesterol particles.[2] Slowly, the plastic is engulfed, and the cholesterol molecules diffuse throughout the membrane, which disentangles the membrane's polymer chains. The plastic gets stuck in the membrane and cannot travel any further because the cholesterol corona diffuses once the MNP is in the BBB. This alters the structure of the membrane, harming its functioning potential as well.[2] After conducting calculations, it was found that pristine plastic particles can be absorbed into the membrane, but the driving force of cholesterol-coronated MNPs was stronger. This suggests that cholesterol molecules facilitate MNP entrance into the BBB, but once the MNP is in, it cannot get out into brain tissue. MNPs with a protein corona necessitated a large amount of energy to enter, which hampered their ability to enter the membrane.[2]

BBB leakage is another potential route for MNPs to pass into brain tissue. This can be caused over time through aging or by neurodegenerative disorders. A study conducted in 2021 at the Department of Life Sciences at the National Central University of Taiwan found that after eight weeks of PS-MP exposure, fragments of polystyrene were detected in the hippocampus. After comparing PS-MP mice and control mice, an increase in BBB leakage was found in the treated mice.[7] This suggests that the BBB becomes more easily permeable when exposed to higher concentrations of MNPs and for longer time periods. One more potential way the MNPs can breach the BBB is by using portions of the brain where the BBB is absent as a pathway. In some brain regions, such as in circumventricular organs, the BBB does not exist to allow for the exchange of hypothalamic hormones between the blood and the brain. MNPs can exploit this function as a way to interact with brain tissue.[7]

Entrance of MNPs Into the Cell and DNA/Gene Expression Damage:

MNPs are also capable of causing DNA damage and changes in gene expression.[2,22] Mediterranean mussels exposed to polyethylene and polystyrene MPs experienced DNA damage and changes to the nucleus.[3] Plastic particles can potentially enter the cell via endocytosis, a process where the cell membrane engulfs and absorbs its target particle.[2] MPs that are 0.5 μm or larger can potentially penetrate the cell membrane by binding to a cell surface receptor that eventually participates in phagocytosis, a process where the cell digests a particle enclosed by part of the cell membrane. NPs 0.5 μm or smaller can be transported across the cell in a vesicle formed by the cell membrane through a process known as transcytosis (Figure 1).[2] Particles that enter the cell can diffuse through either passive or active diffusion. Passive diffusion is when particles have two areas of different concentrations, and the particles from the more highly concentrated portion naturally flow to the less concentrated portion. In this case, it allows the plastic particles to directly enter the cell. This process usually pertains to smaller MNPs, which is dangerous because it could allow them to interact with intracellular organelles.[10] Active transport is when ATP is required to push particles from the lower concentration to the higher concentration, going against the natural flow. Caveolae-mediated endocytosis is a type of active transport that allows cells to select and regulate the substances being brought into the cell and can occur when large MNPs try to enter the cell. The large MNPs form aggregates that are engulfed by the cell membrane and carried into the cell.[10]

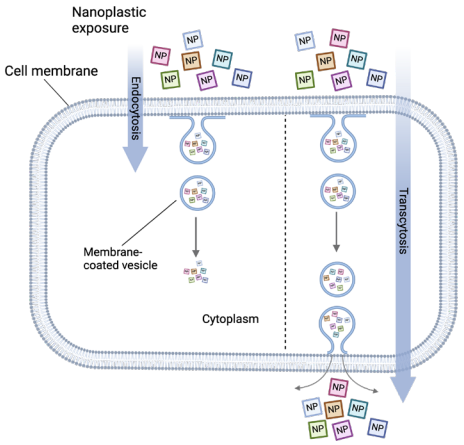

Figure 1: This diagram shows how MNPs penetrate the cell membrane and enter the cell, causing damage and potentially cell death. MNPs 0.5 μm or larger enter via endocytosis, while those 0.5 μm or smaller can enter through transcytosis (Created in BioRender. Ogai, V. (2025) https://BioRender.com/z14k960).

In the previously mentioned study that tested the cellular uptake of MNPs in HeLa cells, they discovered that smaller-sized MNPs were found in the cytoplasm, but not in the nucleus. This means that there was most likely no direct interaction between the MNPs and DNA fragments. However, cell death was nearly 100% at higher concentrations of 10 nm and 15 nm MNPs,[10] indicating that at higher concentrations, MNPs make the cell membrane more easily permeable, which is similar to the findings of Lee *et al*. ROS levels increased as smaller-sized (10 nm and 15 nm) MNP concentrations increased.[10] Elevated ROS levels cause cellular dysfunction and oxidative stress, a common sign of neurodegeneration. This can lead to cell death and the downregulation of anti-apoptotic genes and ROS protection genes. The MNPs in the cytoplasm most likely broke apart after being internalized, which allowed them to directly contact cellular organelles.[10] This demonstrates the obvious cytotoxicity and harmful health effects of the cellular uptake of MNPs. The ability of MNPs to permeate the cell membrane of neuronal cells specifically also has to be investigated to gain a more comprehensive understanding of how the cytotoxic effects of MNPs in other cells like HeLa cells are transferable to the effects of MNPs on brain cells.

Concluding Paragraph:

Plastic particles are able to cross organ barriers and even cellular barriers. Once these MNPs successfully breach the BBB and cell membrane, they can cause a variety of cytotoxic and neurotoxic problems by damaging the natural structure and functioning of cellular components and the organ tissues they interact with. This can cause many chronic issues as MNPs bioaccumulate in the body over time. For example, MNPs in the brain can damage neuronal tissue and thus the natural flow

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of brain activity by inhibiting important neurotransmitters and eventually leading to neurodegeneration.

Neurodegenerative Effects:

Intro Paragraph:

The global number of dementia diagnoses has been rapidly growing over the past two to three decades. Some researchers hypothesize that it is caused by the worsening worldwide plastic pollution crisis.[7] The broad results of research on MNP consequences on the health of the human brain suggest that plastic particles that interact with neural tissue inhibit neurotransmitter activity and contribute to the emergence of ROS and oxidative stress, which causes neuronal cell death.[23] This can cause behavioral changes, neuroinflammation, and the reduced expression of certain genes and proteins involved in brain function. This also contributes to memory loss and can accelerate the development of neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, Huntington's disease, and amyotrophic lateral sclerosis (ALS).[3,24] MNPs have even been found to alter brain structure and thus affect neurobehavioral patterns. Crucian carp exposed to MNPs exhibited decreased brain mass and morphological changes in the cerebral gyri. This structural change caused the behavior patterns of the carp to alter.[3] Additionally, the gut-brain axis has also been found to play a role in MNP-induced neurotoxicity. MNPs that damage brain tissue can also damage the gut microbiome, and vice versa.[21]

AChE and Oxidative Stress:

ACh is an excitatory neurotransmitter that helps an electric signal send a certain message throughout the brain. AChE is an enzyme that accelerates the hydrolysis of ACh.[21] AChE breaks ACh down into choline and acetate so that post-synaptic nerves do not get overstimulated (Figure 2). The overstimulation of post-synaptic nerves results in a process known as excitotoxicity, which severely damages neuronal functioning and leads to apoptosis. Since MNPs inhibit AChE activity, this allows cholinergic neurons to secrete ACh unchecked, which leads to a buildup of ACh in the synaptic cleft (Figure 3). This excessively promotes neuron excitability, hampers neurotransmission, and alters neurobehavioral patterns. Cholinergic neurons and cholinergic synaptic signaling have exhibited susceptibility to these problems after MNP exposure.[17] When AChE inhibition is greater than 30%, it disturbs the overall workings of the CNS, making it a reliable indicator for neurotoxicity.[3]

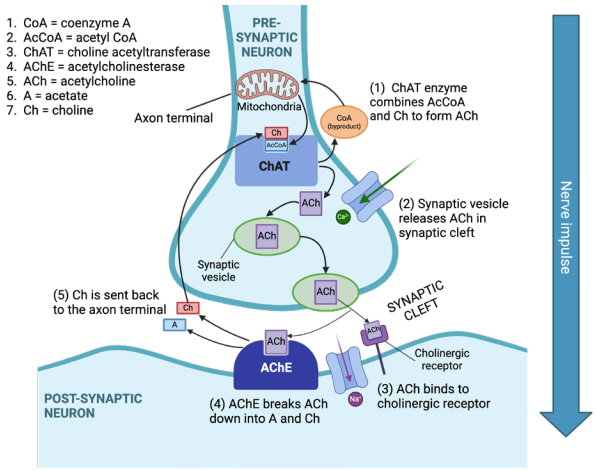

Figure 2: Visual representation of how the ACh (purple) neurotransmitter works within the brain: The ChAT enzyme (light blue) receives an AcCoA (blue rectangle) molecule and combines it with a choline molecule (red rectangle) to create a molecule of ACh. Next, a synaptic vesicle (light green) takes the ACh to the synaptic cleft, where it binds to its cholinergic receptor. While ACh is bound to the cholinergic receptor, sodium ions (dark purple circles) enter the pre-synaptic neuron, while calcium ions (dark green circles) enter the post-synaptic neuron. When the ACh neurotransmitter finishes sending its message to the post-synaptic neuron, it binds to the AChE enzyme (dark blue), which breaks it apart back into acetate (light blue rectangle) and choline. The acetate diffuses into the surrounding medium and the choline gets sent back to the axon terminal. The process repeats (Created in BioRender. Ogai, V. (2025) https://BioRender.com/y29b366).

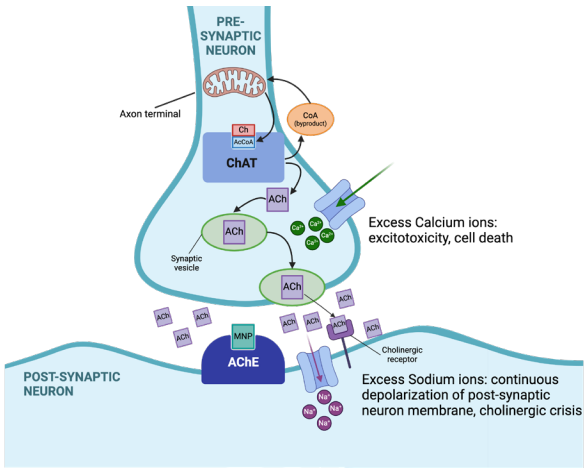

Figure 3: Visual representation of MNPs (turquoise) inhibiting AChE (dark blue) activity. The buildup of ACh (purple) in the synaptic cleft as a result of AChE inhibition causes excitotoxicity, damaging neuronal functioning. While the ACh neurotransmitter is attached to its cholinergic receptor, there is an influx of calcium (dark green circles) into the pre-synaptic neuron (to mobilize synaptic vesicles) and an influx of sodium (dark purple circles) into the post-synaptic neuron to depolarize its membrane. When there is too much ACh in the synaptic cleft, excess calcium ions enter the axon terminal of the pre-synaptic neuron, which can also cause excitotoxicity and cell death. However, when there is too much sodium continuously depolarizing the post-synaptic membrane, it can result in a cholinergic crisis. A cholinergic crisis occurs at the neuromuscular junctions between motor nerves and muscles and can cause paralysis, convulsions, and hypercapnia. This means that MNP inhibition of AChE activity can indirectly trigger a process that results in a cholinergic crisis (Created in BioRender. Ogai, V. (2025) https://BioRender.com/c69w522).

The inhibition of AChE activity has been noted in many organisms participating in studies focused on MNP neurotoxicity and is associated with neurobehavioral changes and neurological disorders.[2,25,26] Juvenile Common Goby fish were exposed to polyethylene MPs, and although there was no actual evidence of MP uptake, the fish experienced significant levels of AChE inhibition.[3] Similarly, a study using European seabass showed that fish exposed to MNPs demonstrated a 50% inhibition of AChE activity in the brain,[15] which means the overall functioning of the CNS was disturbed. This consequence was aggravated by the addition of mercury into the treated fish. The fish displayed decreased predatory performance and signs of oxidative stress.[15] A lack of antioxidants can worsen oxidative stress, cause protein damage, and contribute to cancer and neurodegenerative disease development (Figure 4). In studies surrounding MNP neurotoxicity, growing levels of oxidative stress are often correlated with lowering levels of AChE activity. However, in one study, earthworms exposed to polyethylene MNPs were found to experience increased AChE neurotransmitter activity, MDA levels, and catalase activity.[3] Increased MDA levels and catalase activity are suggestive of oxidative stress, but increased AChE activity is usually not correlated with elevated oxidative stress in the context of MNP neurotoxicity, which poses more questions.[3]

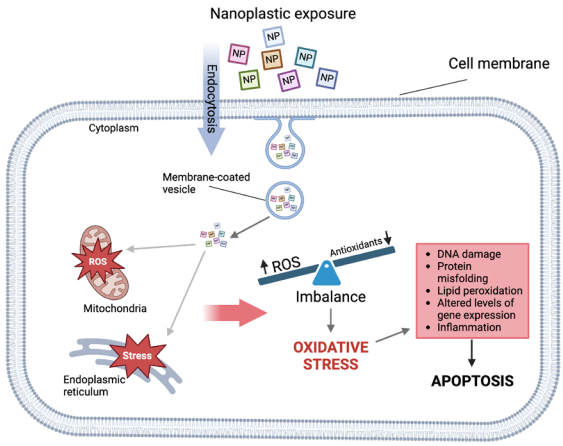

Figure 4: How NPs (multi-color squares) can enter the cell and cause oxidative stress: First, NPs are brought into the cell via a form of endocytosis. Subsequently, a membrane-coated vesicle (light blue circle) brings them to the cytoplasm of the cell. NPs' interaction with intracellular organelles leads to an influx in ROS levels. This triggers oxidative stress within the cell, which results in a variety of negative consequences that all eventually end in apoptosis. If this occurs in too many neurons, it will result in neurodegeneration (Created in BioRender. Ogai, V. (2025) https://BioRender.com/s64b548).

Effects of MNPs on the Behavior and Gene Expression:

Several biomarker analyses of zebrafish exposed to high Bisphenol-A (BPA) levels for one week showed that the plastics accumulated in brain tissue, causing issues including decreased locomotion activity and a dysregulated circadian rhythm.[8,27] Their speed was significantly reduced and they exhibited notable hypoactivity during both the light and dark cycles. At higher concentrations, NP exposure changed predator-induced fear responses, reduced aggressiveness, and altered predator avoidance patterns. The lowered levels of several neurotransmitters including dopamine and Gamma-aminobutyric acid (GABA) contributed to abnormal shoal formation.[8] NPs also elevated ROS levels, an indication of oxidative stress and pro-inflammatory responses. AChE activity was also significantly inhibited at higher NP concentration levels, which was found to correspond with anxiety-like behavior in the fish.[8] It was also found that oxytocin and vasopressin neurotransmitter levels, which both regulate learning, memory formation, and emotional processes, were reduced due to MP exposure in zebrafish.[8]

Similarly, S. Wang *et al.*, and J. Wang *et al.*, both noted that MP exposure in mice damaged learning, memory, and locomotion abilities while promoting depression-like behaviors. In the former study, MP exposure caused oxidative stress and inhibited cAMP-response element binding protein (CREB) phosphorylation which lowered ACh production.[28] In the latter study, it was also found that ACh content was significantly reduced and AChE activity was much higher in the mouse cortex/hippocampus than in the controls. Most other MNP neurotoxicity studies report an increase in ACh levels, as previously mentioned in the study focusing on earthworms, which presents more questions.[3] A reduction in ACh levels can lead to memory, thinking, and concentration problems. Additionally, MP-treated mice exhibited an increase in ROS and MDA levels, along with lowered glutathione levels.[28] It was also discovered that MP exposure inhibited the CREB/BDNF pathway, which causes neuronal death.[28] Hippocampal nerve cells became loose and disordered after MP exposure, which is similar to the irregular arrangement of nerve cells that J. Wang *et al.* observed.

In addition to neurobehavioral impairment, MNPs also hamper the expression of certain genes elicited by brain activity. Based on the findings of J. Wang *et al.* genes (Slc5a7, ChAT, Slc18a3) and proteins (ChAT and Slc18a3) that are important to cholinergic synaptic signaling pathways were damaged and downregulated after being exposed to low-density polyethylene (LDPE) MPs and oxidized-low-density polyethylene (Ox-LDPE) MPs. This disruption lowered ACh levels, induced oxidative stress, and caused neuroinflammation by up-regulating the expression of inflammation-related genes including interleukin 1 beta (IL-1β) and tumor necrosis factor α (TNF-α). This is similar to the biological mechanism that allows Alzheimer's disease to develop.[21] This means that MPs have the potential to cause Alzheimer's disease and other diseases with a similar pathogenesis.

In another study exploring the effects of MNPs on gene expression, fear conditioning, and behavioral tests were run on PS-MP-treated mice. The tests showed that the hippocampus-dependent memory of the mice was damaged and there were lower expression levels of immediate early genes (IEGs - control synaptic plasticity, learning, and memory), especially in the dentate gyrus, CA2, and CA3 subregions.[7] The dentate gyrus, CA2, and CA3 subregions of the hippocampus all play a major role in memory formation and encoding, demonstrating PS-MP's detrimental effects on neuronal activities. Several indicators of neuroinflammation were also found in PS-MP treated mice, including increased tumor-necrosis factor-alpha

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(TNF-α), interleukin 1 beta (IL-1β), and microglia activation levels in the hippocampus, as compared to control mice.[7,29]

The Gut-Brain Axis:

The gut-brain axis is the system through which the gut microbiome communicates with the brain. The vagus nerve and the connection between the BBB and the intestinal immune system are two pathways the gut microbiome uses to communicate with the brain (Figure 5).[7,21] Inflammation caused by MPs in the brain can potentially harm the gut microbiome as well.[21] Once BBB permeability is damaged, endotoxin lipopolysaccharides (LPS) can travel via peripheral circulation, enter the CNS, and accumulate in the brain, which can cause problems in the CNS and the gut.[21] When MPs invade the gut microbiome, they can travel up the gut-brain axis and cause hippocampal and cortical inflammation. In one study, it was found that the gut-brain barrier, including the gut epithelial barrier, the BBB, and the cerebrospinal fluid barrier (CSF), were destroyed in MP-treated mice.[21]

Lipopolysaccharides (LPS) (or endotoxins) are bacterial toxins and proteins that make up the outer membrane of gram-negative bacteria. LPS helps create a barrier around the pathogen to prevent antibiotics from coming into the molecule. MPs have been found to raise LPS levels, which aggravates BBB disruption and in turn, increases the movement of LPS from the blood to the brain.[21] Since the BBB became more easily permeable, this also means that it became easier for other substances including MNPs to pass through, which could worsen the existing neurotoxic effects presented by MNPs.

Since PS-MPs enter the GI tract when ingested, the vagus-nerve pathway is the most probable route PS-MPs travel through to get to the brain.[21] In a study where mice were experiencing MP-induced learning and memory disorders, vagus nerve ablation reversed and stopped any further MP-related issues.[21]

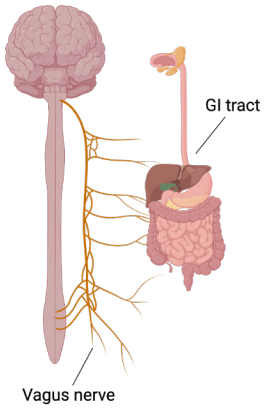

Figure 5: Visual representation of how the vagus nerve connects the brain and the GI tract. The gut microbiome communicates with the brain by sending it signals through neurotransmitters. This is a pathway MNPs can exploit to reach the brain if they originally enter the gut, and vice versa (Created in BioRender. Ogai, V. (2025) https://BioRender.com/x14h240).

In a separate study where Juvenile Discus Fish were exposed to NPs, brain ACh and dopamine concentrations increased while decreasing in the gut.[30] This demonstrates that the brain and the gut communicate to regulate the levels of neurotransmitters traveling through the gut-brain axis. The repression and overexpression of multiple genes in the neuroactive ligand-receptor interaction pathway and the serotonergic synapse pathway (both play important roles in behavioral processes, including appetite, aggression, and eating behavior) caused changes in behavioral patterns by weakening swimming capabilities and predatory instincts.[30] This indicates that ingested MPs that enter the GI tract and then travel to the brain can cause behavioral toxicities.

Concluding Paragraph:

The two main effects of MNPs on the brain are the abnormal AChE and ACh levels, and the triggered oxidative stress. In addition, MNPs have been found to downregulate the expression of genes important to neuroplasticity, memory formation, and behavioral patterns. The GI tract is also involved in how MNPs impact the brain, learning, memory, and BBB integrity via the vagus-nerve-dependent pathway. Further research still must be conducted on all the major effects of MNPs on the brain, especially on how plastics influence the ACh neurotransmitter since there have been some inconsistencies on this throughout the field. All these studies, especially the ones involving aquatic biota, suggest that predators at the top of the food chain such as humans are consuming MNPs and their co-pollutants through meat, especially seafood products.

Probiotics - A Potential Solution?

Probiotics' Reduction of Inflammatory MNP Effects:

Cellular-level probiotics can act as antioxidants, which gives cells and the GI tract the necessary properties to mitigate oxidative stress. When the gut microbiome is imbalanced, it causes an overreaction in the immune system and increases oxidative stress levels.[4] In one study, Wistar rats were exposed to BPA at high concentrations along with a combination of the *Saccharomyces boulardii* (yeast) and Lactobacillus probiotic strains.[4] The combination exhibited antioxidant characteristics by preventing lipid peroxidation (linked to neurodegeneration and free radicals). In a separate study, polybiotics were given to Parkinson's disease patients. The polybiotics were able to improve cognitive function and minimize signs of nerve injury and lipid peroxidation.[4] This is similar to the findings of Cheon *et al.*, who cultured neuroblastoma cell lines, induced neuronal damage similar to Parkinson's disease, and tested three Lactobacillus strains' effectiveness in treating brain damage. The Lactobacillus was able to upregulate the BDNF neurotrophic factor, a protein that helps maintain neuronal growth and survival.

This is comparable to the findings of J. Wang *et al.* also noted that probiotics can help prevent some of the neurotoxic effects MNPs have in mice. A combination of a probiotic strain known as *Lactobacillus plantarum* DP189 combined with galacto-oligosaccharides (GOS) (a prebiotic that increases the number of bacteria in the gut to help the immune system) was tested to see if it could reverse the neurotoxic effects of LDPE-MPs. It was found that DP189&GOS prevented neuron death and mitigated BBB and intestinal destruction.[21] It also restored ACh levels, reduced inflammation, and oxidative stress, and helped increase ChAT and Slc18a3 protein expression to different extents depending on the concentration of

MPs. It also reduced MDA content but not to a significant extent. This suggests that at higher concentrations, DP189&GOS could reverse the cognitive dysfunction caused by MP exposure.[21] Similarly, a separate study was conducted to determine the effectiveness of DP189 in treating Parkinson's disease. It was observed that α-synuclein (α-SYN) aggregation in the substantia nigra was delayed because oxidative stress and inflammation levels were reduced.[31] The Lactobacillus bacteria have been able to stimulate the antioxidant and immune systems, which aids in lowering oxidative stress, inflammation, and intestinal barrier destruction caused by MPs.[21]

Similar to probiotics, vitamins that act as antioxidants can potentially be incorporated into drugs to treat MNP-induced neurotoxicity. Vitamin E, an antioxidant that neutralizes the production of excess ROS, restored neurotransmitter levels and learning/memory functions in MP-treated mice.[28] This indicates that Vitamin E and other vitamins that can act as antioxidants including Vitamin C and Coenzyme Q10 (CoQ10) can be used to treat the harmful neurological effects of MPs. However, more research still must be conducted to corroborate the practicality of their use.

Probiotics and vitamins could potentially help with limiting the genotoxicity of MNPs as well. There have been many studies done solely on how probiotics/vitamins limit DNA fragmentation when being exposed to general pathogens, but few specifically focused on MNPs. This is an area in the field that also must still be further researched.

Discussion

Since the effect of MNPs on human neural health is still a new and developing field, there are many limitations in current experiments that prevent us from gaining a full understanding of how plastic particles act within our bodies. Several important research gaps were noticed while research was being conducted:

(1) The most notable research gap there is right now is that most experiments lack real-life MNP exposure. Virgin particle types, not the ones that are inhaled or ingested, are used in these studies. Most studies order manufactured spherical polystyrene or polyethylene particles when examining the neural effects of MNPs, instead of plastics of variable shapes, sizes, and surface qualities (that all have different levels of neurotoxicity).[3]

(2) Most studies expose their experimental groups to MNPs for short time periods but at high concentrations, which is the opposite of what we undergo in the real world. Humans and animals are exposed to relatively low plastic concentrations but are continuously exposed over their lifespans. This lack of realistic circumstances hinders our acquiring a complete understanding of plastic neurotoxicity.

(3) Some researchers suggest that when we ingest MNP-contaminated food, the plastic comes out through the GI tract, as plastic particles are detected in human feces.[32] Other than that, there is very little research on MNP bioaccumulation in organs and the bloodstream, especially in brain tissue. The way the body expels plastic particles (or what happens with them within the body) is a topic that must be further explored.

(4) NPs can further degrade into monomers once they enter the body. PS-NP monomers have demonstrated even more detrimental effects on several bodily systems, including neural ones, than PS-NPs themselves.[6] The immune system's response and the neurological response to invading MNPs and monomers must be researched further.

(5) Exposure to higher temperatures in the environment allows plastic particles to more easily adsorb co-pollutants, which broadens their range of potential health risks.[3]

(6) Another question that still has to be answered is what groups of people: age/race/gender/medical records - are more vulnerable to the harmful effects of plastic particles.

(7) More research must be conducted to corroborate the MNP neutralizing capabilities of probiotics. This analysis could also help find other substances or make new medications that could be even more effective.

(8) Since coming into contact with plastic is inevitable, another angle on this problem that has to be considered is if there is any way to prevent plastic particles from coming into our bodies in the first place. For example, we know chemicals and enzymes that can break down plastic exist, such as PETase and MHETase.[33] We must conduct other studies to understand whether such enzymes can safely be used in natural ecosystems and if they can be consumed to break down plastic in the body.

Conclusion

In 2022 alone, the global level of plastic manufacturing reached 400.3 million tons, a number that is expected to increase exponentially in the years to come. It is also approximated that by 2035 the amount of plastic in the ocean will equal the amount of fish in the ocean by weight.[1] These alarming numbers combined with the recent discovery that MNPs can enter the brain by crossing the BBB highlight the critical need for more research to be done on the topic of MNP neurotoxicity. There are also a variety of factors that can influence the neurotoxic potential of a plastic particle, including the size and shape of the particles, its manufactured company additives, and its environmental co-pollutants. MNPs that breach the cell membrane can damage DNA and gene expression, mess with AChE levels, and cause oxidative stress, all of which contribute to the development of neurodegenerative diseases and memory loss. The gut-brain axis and its role with regard to MNP ingestion is also a newly developing area of study. MPs that travel to the brain through the gut-brain axis can impair learning, memory, and behavioral patterns. As demonstrated through multiple different studies, probiotics, and vitamins that act as antioxidants have been showing promising results in terms of reversing or at least reducing the neurotoxic effects of MNPs. However, we still have yet to learn how we can prevent MNPs from entering the body in the first place using plastic-dissolving enzymes released into the environment and if they can be used in the human body along with probiotics.

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■ RESEARCH ARTICLE

Investigating the Influence of Tears-relevant Visual Cues on Contingency Judgments and Perceived Agency

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ABSTRACT: Emotional crying refers to a unique human behavior of shedding tears in response to emotional events. Prior findings have suggested interpersonal benefits of eliciting social support can be triggered by emotional crying. We hypothesized that crying has a communication value in signaling distress or helplessness to others. Specifically, we investigated whether tear-related visual cues influenced how individuals learn about and assess others' helplessness. Participants (N = 40) were presented with tearful and non-tearful facial stimuli during a learning sequence involving a contingency judgment task – assessing the correlation between an individual's actions (studying) and outcomes (passing a test). Results revealed that in both zero and positive contingency conditions, the presence of tears decreased the mean contingency rating; this suggested that the participants viewed the individual as more helpless. However, participants were also unable to discriminate between positive and zero contingency conditions when exposed to tear stimuli. We propose that the emotional salience of tears interferes with cognitive processes related to learning. We discuss these results in relation to alternative hypotheses involving potential carryover effects during learning. However, these findings suggest that tears function as a potent nonverbal cue for helplessness, highlighting their role in fostering social support.

KEYWORDS: Behavioral and Social Sciences, Clinical and Developmental Psychology, Emotional Crying, Contingency Learning, Helplessness.

■ Introduction

Background:

Auditory crying is a universal behavior found across different species. For the majority of the species, these behaviors are limited during infancy to vocalize distress or physical pain – which elicits an instant response from the caregiver.[1] *Homo sapiens* exhibit a unique type of crying that spans throughout their entire life span: emotional crying.[2] Emotional crying refers to the shedding of tears from the lacrimal apparatus in response to emotional events, in the absence of any irritation of the eyes. Research suggests that, in the United States, women cry an average of 3.5 times per month and men cry an average of 1.9 times a month.[3]

Despite the ubiquitous nature of emotional crying across human cultures, empirical research on its functional value remains limited. Darwin, the pioneering evolutionary biologist, concluded that "We must look at weeping as an incidental result, as purposeless as the secretion of tears from a blow outside the eye, or as a sneeze from the retina being affected by a bright light."[4] Other perspectives suggest that emotional tears function as a natural 'handicap.'[5] Handicapping aggressive or defensive actions by blurring vision, or tears have been proposed to make an individual more vulnerable – functioning as a sign of appeasement. Research on the impact and value of crying and emotional tears remains incomplete.

Motivation:

The ambiguity behind crying has been countered by the public belief that crying has an intrapersonal benefit – leading to a cathartic experience of distress relief. This notion, known as the 'catharsis' hypothesis, has been supported by Vingerhoets; among thirty countries, both men and women reported feeling better after crying.[6] However, the intrapersonal benefits were hardly found in controlled laboratory studies, those which artificially induce crying by mood induction techniques or standardized crying stimuli. Rather, these laboratory studies find that people report emotional impacts that are consistent with the feelings experienced while crying, that is, those related to sadness and depression. People who cried while watching a sad film felt more depressed relative to the participants who did not.[7] Similarly, Kraemer and Hastrup, examining the psychophysiological effects of crying such as heart rate and skin conductance, concluded that crying does not seem to reduce depressive symptoms as might have been expected given the catharsis hypothesis.[8]

Recent studies have shifted focus to the interpersonal effects of crying. They propose that crying, rather than directly influencing the individual, promotes prosocial behaviors of others that secondarily benefit the crier – therefore improving the crier's socio-environment. An investigation across forty-one countries analyzed the interpersonal effects of emotional crying, for instance, suggesting tears evoke the intention to offer social support.[9] Empirical findings have shown that individua-

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ls who have less frequent crying reportedly received less emotional support,[10] suggesting crying may play a role in triggering help from others. However, a research gap remains in understanding the intermediary step between crying and the social support it elicits – how does the crier communicate the need for help?

Human beings are ultrasocial animals that rely heavily on complex forms of cooperation, coordination, and division of labor for survival.[11] It is therefore plausible that the communicative role of infant crying –calling for help by displaying helplessness – has extended into adulthood. In this extension, the roles of caregivers could have simply shifted from parents to other socially interacting individuals. Based on this social-functional perspective, the current project aims to explore the tear-related cues' influence on how helpless an individual appears to others.

Hypotheses:

- **H_0:** There will be no statistical difference in observers' implicit assessments of helplessness between individuals presented with tear-related visual cues and those without such cues.
- **H_1:** Tear-related visual cues will significantly influence observers' implicit assessments of an individual's helplessness, which is posited to be a key factor in eliciting social support behavior.

Although we present this as a two-directional hypothesis, our intuition was that tear stimuli would impair contingency sensitivity.

Agency, as the opposite of helplessness, refers to one's capacity to perform an action (input) to derive a desired outcome (output). In other words, agency is proportional to the degree of correlation between one's input and desired outcome. Observers evaluate this correlation—the contingency between input and output—to infer an individual's level of helplessness. If a subject is perceived to be more helpless, the observer's assessed contingency would be lower than the actual contingency, reflecting cognitive bias. Therefore, participants' sensitivity to contingency-related information in a fictitious learning scenario was assessed to measure how helpless a subject appeared. This methodology was chosen as an alternative to directly asking the participants to rate the figure's helplessness to minimize explicitness; in explicit tasks, participants may consciously respond in ways to meet perceived expectations rather than being based on their instinctive, underlying cognition.

Methods

Participants:

A sample of forty participants composed of twenty-three male and seventeen female participants and aged 21 to 52 (M= 34.08, SD= 6.97) were recruited through MTurk for English speakers. The age cap was based on the general trend of decline in cognitive function and crystallized intelligence starting from age 45-60.[12,13] Participants completed a consent form and were given an honorarium for $4 for maximum 20-minute participation. The study was approved by the University of Oxford Central University Research Ethics Committee: Ethics Approval Reference: R88481/RE001.

Stimuli:

Four faces of individuals from Chicago Face Database9 were used as stimuli. All individuals, spanning from White, Asian, Black, and Latin, showed a neutral expression. To minimize the influence of stimulus-specific facial features on helplessness assessment, images with similar helplessness index – 2.336, 2.336, 2.336, and 2.277 – were selected. The helplessness index provided for each image was calculated from helplessness rating surveys across forty-one countries and 7,007 participants by Zickfeld. Three of the individuals were female, while the other was male. Imbalance in gender was disregarded as the variation due to their gender would have already been taken into account in the helplessness index. For each individual, an additional image with tears digitally added by using a Photoshop action by Küster was utilized.[8] Thus, the final stimulus pool was composed of eight pictures: four tearful and four non-tearful pictures of four different individuals of four different ethnicities. Stimuli for each condition were presented prior to the condition's contingency task with instructions. Each participant was exposed to all four facial stimuli, while the combination between the facial stimuli and condition was randomized.

Contingency task:

Stimulus presentation and response measurement were controlled by Gorilla Experiment (www.gorilla.sc), running on each participant's computer. Each cue-outcome pair was presented with a total duration of 1200 ms, displayed in the bottom half of the white screen. The facial stimuli were one each trial as shown in Figure 1.

The two types of predictive cues were 'STUDY' and 'Not STUDY', while the two possible outcomes were images written 'PASSED' or 'FAILED'. Each predictive cue with its paired outcome was shown simultaneously for 600 ms, which was followed by a fixation screen that appeared for 600 ms prior to the next trial (Figure 1).

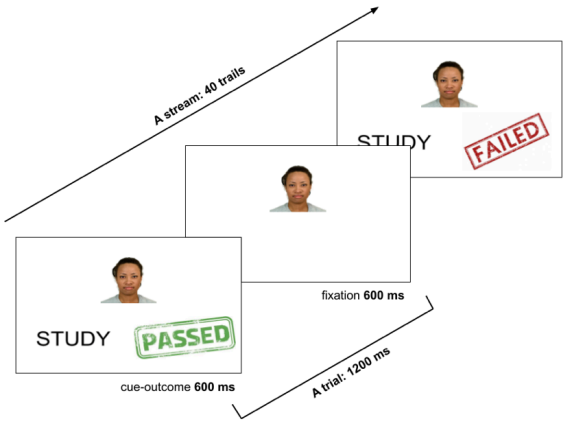

Figure 1: Example trial for the rapid presentation of predictive cues and outcomes in streaming trial procedure, along with facial fixation.

How predictive each action - STUDY or NotStudy - was for its consequences varied by altering four types of events. The events refer to the number of times that preemptive action 'STUDY' (Cue) has resulted in a positive outcome 'PASSED' (Outcome); b events refer to the number of times that pre-

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emptive action 'STUDY' (the cue, C) was present but the outcome, 'NotPassed', was neutral (~O); c events were when the preemptive action was absent (~C) but a positive outcome was resulted (O); and d events were when both preemptive action and positive action was absent, meaning both cues and outcomes were neutral (~C and ~O). As demonstrated in Table 1, the number of a, b, c, and d events were altered to create a positive and zero contingency, judged based on Allan's (1980) metric Delta P – a measure for contingency that ranges between -1 and +1.

Table 1: The distribution of trials for each cell along with statistical relations for conditional probability (P of O) and Delta P (ΔP). a= Cue (+C) and Outcome (+O), b= C+ and No Outcome (–O), c= No Cue (–C), and +O, d= –C and –O).

Zero				Positive			
	O+	O-	P		O+	O-	P
C+	10	10	0.5	C+	15	5	0.75
C-	10	10	0.5	C-	5	15	0.25
sum of +O			ΔP	sum of +O			ΔP
			0				0.5

The experiment followed a 2 x 2 within-subjects design, composed of two contingency levels (P= +.50, 0.0) and two stimulus types (tearful image, non-tearful image). Combined, this experiment had four conditions: (1) PT (Positive contingency, tearful image), (2) ZT (Zero contingency, tearful image), (3) PnT (Positive contingency, non-tearful image), and (4) ZnT (Zero contingency, non-tearful image). Each participant viewed each of the four contingency conditions, with one face assigned for each condition. Faces were randomized for assignment to each condition. Randomization was used to minimize the influence of facial-specific features on helplessness assessment (Table 2). Each condition consisted of forty trials with a total duration of approximately 48 seconds.

Table 2: F1, F2, F3, and F4 indicate distinct facial images of different individuals. Each participant was randomly assigned a trial sequence stream among $\alpha,\beta,\gamma,\delta$.

No tears		Tears		
Positive	Zero	Positive	Zero	
F1	F2	F3	F4	α
F4	F1	F2	F3	β
F3	F4	F1	F2	γ
F2	F3	F4	F1	δ

Procedure:

After participants read the information sheet and consent form, they completed an anagram task to exclude involvement by bots. The anagram task was followed by a demographic questionnaire - questioning age and ethnicity – followed by the contingency task with four conditions. On each of the four conditions, participants were instructed to imagine a specific scenario: "IMAGINE that (Name) has currently failed a test. They are very keen to do well, but as we know people sometimes have the best intentions but do not always follow through. Over a series of images, you will see on the left side of the screen, whether (Name) actually does study for future tests, and then on the right side of the screen you will be given information about whether (Name) has passed each test." In conditions with positive contingency, studying resulted in an increased likelihood of passing, while in the zero contingency conditions, studying had no impact on performance. They were also given the following instruction: "The left image indicates that the figure studies for future tests, while the picture on the right suggests he/she does not." Every participant received the same order of conditions, which was presented sequentially as PnT, ZnT, PT, and ZT. After viewing each stream of cues and outcomes, participants were asked to rate the relatedness between the subject's studying action and passing. Particularly, they were asked to respond on a rating scale from -10 to +10, where -10 indicates a 'strong negative relationship' (i.e., contingency), 0 indicates 'no relationship', and +10 indicates a 'strong positive relationship. Following the final condition, the experiment ended with a thank you statement and an offer to answer any questions.

Results and Discussion

The descriptive statistics suggest that the presence of tears in facial stimuli reduced the perceived contingency among participants, as shown in Figure 2. For both positive and zero contingency levels, the participant's mean judgment for non-tearful facial stimuli was lower than that of tearful facial stimuli. In the zero-contingency condition, the presence of tears decreased the mean contingency rating by 2.63, while in the positive contingency condition, the value was 4.23. This effect of tears was more pronounced in positive contingency conditions than zero contingency conditions; The error bars represent the 95% confidence intervals for each condition's mean.

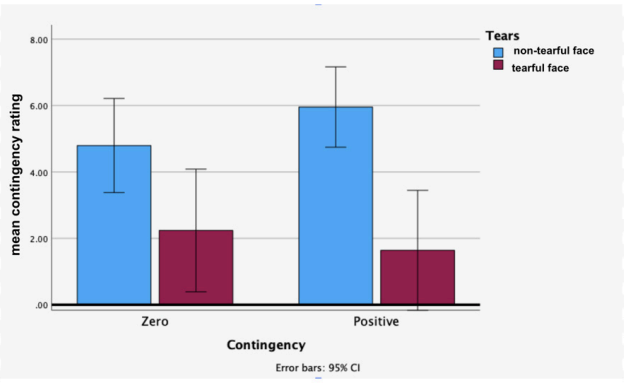

Figure 2: Participants' mean contingency rating along with contingency (zero, positive) and presence of tear in facial stimuli. For both contingency levels, the mean contingency rating decreased with the presence of tears. However, a lack of sensitivity to the contingency condition was observed.

The support for these observations come from the 2x2 factorial ANOVA with the within-subject factors contingency (zero, positive) and stimulus type (tearful, non-tearful). There was a significant main effect for tears [F (1, 36) = 22.360, p<.001]. This indicates that the presence of tears in facial stim

uli significantly reduced the perceived contingency judgment of participants.

However, the descriptive statistics also depict that the participants could not discriminate between positive and zero contingency conditions. While contingency ratings for non-tearful stimuli increased slightly as the condition changed from zero to positive, ratings for tearful stimuli displayed an opposite trend; the mean contingency rate increased from 4.77 to 5.85 in shifting from Condition ZnT to PnT, while it decreased from 2.15 to 1.62 in shifting from condition ZT to PT. Overall, the ANOVA analysis reflects the effect of contingency was not significant [$F(1, 36) = .148$, $p=0.703$]. This suggests that participants were incapable of discriminating between positive contingency conditions and negative contingency conditions.

The interaction effect between the combination of face type and condition type by tears was not significant in testing the within-subject effect [$F(3, 36) = 0.951$, $p= 0.462$], nor was the interaction between the combination and contingency [$F(3, 36) = 1.497$, $p= 0.232$]. Similarly, the combination did not have a significant between-subject effect on perceived contingency [$F(3, 36) = 0.832$, $p=0.485$]. This suggests that the combination of face type and condition was not a significant covariate for the contingency task and therefore can be disregarded from further analysis.

Our results have demonstrated that the presence of tears in facial stimuli and participants have a negative impact on contingency judgments. Specifically, the presence of tears significantly reduced the perceived contingency in both positive and zero contingency conditions. The analysis supported this trend.

A potential explanation for the participants' impaired ability to distinguish different contingency levels might be the tears' innate emotionality. Emotional tears facilitate the recognition of sadness, creating an emotional context for perceived information.[14] Humans show a priority of emotional over neutral stimuli in terms of processing information. For instance, emotional meaning captures orienting and engages attention early relative to neutral stimuli.[15] This prioritized perception of emotional stimuli could interfere with the attention in the process of executive functions that require cognitive flexibility, inhibition, and updating for manipulation of information.[16] In other words, the salience of tears could have interfered with the contingency learning process of participants. In fact, there has been evidence from ERPs and time-varying brain networks that suggest negative emotion interferes with cognition.[17] These aspects potentially provide an explanation why lack of contingency discrimination was only exhibited with exposures to tear-relevant cues.

Another possible explanation is the emotional carryover effect in serial tasks. The presence of tears, along with the given scenario of figures failing a test, could promote sympathy – eliciting negative emotional responses such as sadness. According to Morriss,[18] in studying emotional reactivity, it took 3500 ms for emotional recovery after offset of negative stimuli. However, this investigation lacked time intervals between each conditional task. The lack of time for emotional recovery from trial PT could have led to an emotional carryover effect, hindering the contingency rating in the subsequent trial ZT. This could suggest why the mean contingency rating was higher in zero contingency conditions than positive contingency conditions, but only in the presence of tearful faces.

A potential solution to this issue would be to randomize the order of tasks. Our study currently used a fixed sequence—PnT, ZnT, PT, and ZT—for all participants. Randomizing the order of conditions in future studies would reduce predictable emotional build-up or carryover effects, allowing each condition to be evaluated independently. Future research should also consider appropriate time intervals to further isolate the effects of trial order on observers' implicit assessments.

However, despite how the mean contingency rating was higher in the positive contingency condition than in the zero contingency condition without tearful stimuli, it appears that participants still exhibited an impaired ability to distinguish different contingency levels. For example, the mean rating for ZnT was 4.77, deviating substantially from the ideal value of 0, and the error bars for ZnT and PnT overlapped.

One possible explanation is that participants held a strong prior belief associating studying with success, and thus relied more on heuristics than on statistical information. In the no-tears condition, they may have simply defaulted to this prior belief of equating studying to passing a test.

Moreover, due to the serial design of the investigation, participants may have become skeptical about the purpose of showing them neutral and crying faces. Therefore, the participants could have relied on their common sense that passing a test requires being mentally stable (having confidence, being rational rather than emotional, etc.), guessing that crying people are more likely to fail. This compromises the reliability of the investigation as it becomes unclear whether the results are specific to the content of the contingency task.

A solution could be changing the content of contingency tasks so the outcome does not depend on the pictured person's emotional state. For instance, the input could be whether the person waters a plant and the outcome could be about whether the plant grew over a few weeks. While the content can still assess the individual's agency, it excludes the individual's emotional status as a factor that influences the outcome.

Conclusion

In conclusion, this study's findings underline the role of tears in making an individual appear more helpless. Tears, therefore, may be a potent non-verbal cue for communicating helplessness and subsequently eliciting social support– not only in infants but also in adults. One possibility is that the empathic response elicited by the tears allows observers to experience the sense of helplessness experienced by the crier. An alternative is that viewing tears activates memories in observers that activate helplessness. Either way, this form of empathy is activated by visual cues and has a direct effect on the cognitions of the observer. In this case, that effect was on the ability of the observers to process stimuli involving working memory and computational judgment.

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RESEARCH ARTICLE

The Earth's Rotation is Slowing Down: The Physical Reasons and How It Affects the Number of Days in a Year

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ABSTRACT: The tidal force of the Moon on the Earth can slow down the Earth's rotation, forming a "locked" or tidal synchronization phenomenon. Over the long course of the Earth's history, the Moon has gradually moved away from the Earth, and the energy of the Earth's rotation has been dissipated by the tidal forces on the surface and inside of the Earth, causing the Earth's rotation to slow down, resulting in an increase in the length of the day and a decrease in the number of days in the year. This study used Newton's law of universal gravitation and the law of conservation of angular momentum to derive a calculation formula for the rate at which the Earth's rotation slowed down, estimated the number of days in a year hundreds of millions of years ago, and verified the accuracy of the estimate using evidence from the Devonian tetracoral ridges.

KEYWORDS: Physics and Astronomy, Astronomy and Cosmology, Earth Rotation, Tidal Locking, Rugose Corals.

Introduction

Tides are the periodic rise and fall of ocean water caused by the gravitational pull of the Moon and the Sun. The Sun's gravitational pull on the Earth is almost 200 times stronger than the Moon's. However, the Moon's tidal effect is greater because the Moon's distance from the near side to the far side varies much more than the Sun's. The tidal force stretches the Earth along the line between the Earth and the Moon. Different points on the Earth's surface are at different distances from the Moon. Places facing the Moon experience a greater gravitational pull, and the ocean water bulges outward. On the far side, the Moon's pull on the Earth is greater than on the water, also causing high tides. Therefore, the two directions of the ocean surface perpendicular to the line connecting the Earth and the Moon correspond to low tides. As a result, the sea level rises and falls about twice a day.[1]

The growth of any organism on Earth is affected by environmental factors. Over the long years, as the Earth revolves around the sun, it produces seasons and tides. These changes have a periodic impact on the growth of the hard parts and bones of organisms.

Corals produced thickened growth bands once per year, like tree rings, due to seasonal changes in water conditions. Some scientists, using the isotope Calcium-45, show that in reef corals, the rate of calcium carbonate uptake in coral tissues falls at night or in darkness and rises during the day. John W. Wells from Cornell University investigated the effects of this on the coral skeleton. He observed there were tiny ridges that may be deposited daily throughout the life of the coral, as shown in Figure 1. In 1963, Wells published his landmark paper titled "Coral Growth and Geochronometry" in the journal, Nature. He counted the smallest ridges on modern corals and discovered that there were about 360 per year. This strongly suggests that the growth lines are diurnal or circadian in nature.[2] He then counted these growth lines on fossil corals and discovered that there were about 402 ridges on corals from the Silurian (430 million years ago) and 377 ridges on corals from the Jurassic (180 million years ago). These discoveries implied that the number of days in a year has been reduced over geologic time.[3]

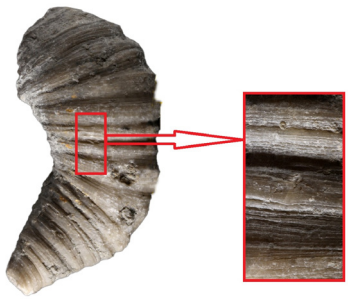

Figure 1: The Devonian rugose coral, with its fine growth lines in yearly skeletal deposition. Each fine growth line represents a day and night. By counting the number of growth lines included in each growth wrinkle, the number of days in a year can be inferred. (credit "photo": modification of work by the Field Museum of Natural History).

In this study, we first made a theoretical analysis of how tidal force affects the Earth's rotation, based on Newton's law of universal gravitation and conservation of angular momentum of the Earth-Moon system. The change rate of the Earth's rotation was derived from which we estimated how many days there were in a year hundreds of millions of years ago. The calculated results were compared with those found by Wells in rugose corals, verifying the rationality of the calculation model.

Methods

If the Earth-Moon system is approximately regarded as an isolated system, its total angular momentum is conserved,

$$L_{tot}=I_E\,\omega_E+I_M\,\omega_M+I_{orbit}\,\omega_{orbit} \quad (1)$$

where L_{tot} is the sum of the angular momentum of the Earth-Moon system; I_E, I_M and I_{orbit} are the moments of inertia

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respectively; ω_E, ω_M, and ω_{orbit} are the angular velocity of the Earth's rotation, the Moon's rotation, and the Moon's orbit, respectively.

The total kinetic energy of the Earth-Moon system is

$$E_K = \frac{1}{2}(I_E\omega_E^2 + I_M\omega_M^2 + I_{orbit}\omega_{orbit}^2) \quad (2)$$

From Eqns. (1) and (2), we can get the Cauchy–Schwarz inequality:[4]

$$2E_K(I_E + I_M + I_{orbit}) \geq L_{tot}^2 \quad (3)$$

When ω_E=ω_M=ω_{orbit}, the minimum total kinetic energy can be obtained:

$$E_{K,min} = \frac{L_{tot}^2}{2(I_E+I_M+I_{orbit})} \quad (4)$$

When this happens, the Earth-Moon system moves like a rigid body, a phenomenon called "locking" or tidal synchronization. In this case, the Earth-Moon system only moves as a whole, without relative motion, so the total kinetic energy is minimal. It has already happened to most moons in our solar system, including Earth's Moon. The Moon currently is always showing the same side to the Earth, and therefore its rotation period has locked into the orbital period about Earth. The same process is happening to Earth, and eventually, the Earth will become tidally locked to the Moon. If that does happen, we would no longer see tides, as the tidal bulge would remain in the same place on Earth, and half the Earth would never see the Moon. However, this locking will take many billions of years, perhaps not before our Sun expires.[1]

The Moon has an elliptical orbit in which the value of R varies by just over 10 %. It is reasonable to assume the Moon's orbit is circular. The I_E, I_M, and I_{orbit} in Eq. (1) can be calculated by

$$I_E = \frac{2}{5}M_E R_E^2 \quad (5)$$

$$I_M = \frac{2}{5}M_M R_M^2 \quad (6)$$

$$I_{orbit} = M_M R_{orbit}^2 \quad (7)$$

where the mass of Earth (M_E) is 5.97×1024 kg, the mass of Moon (M_M) is 7.35×1022 kg, the average radius of Earth (R_E) is about 6370 km, and the radius of Moon (R_M) is about 1700 km, and the average distance between the centers of Earth and the Moon (R_{orbit}) is 3.84×108 km.[1] The calculation results show the moment of inertia of the Moon's orbit is about 1.28×10^5 times of the Moon's rotation, and because the Moon's rotation has the same angular velocity as the Moon's orbit (ω_M=ω_{orbit}), thus the angular momentum of the Moon's rotation is negligible in Eq. (1).

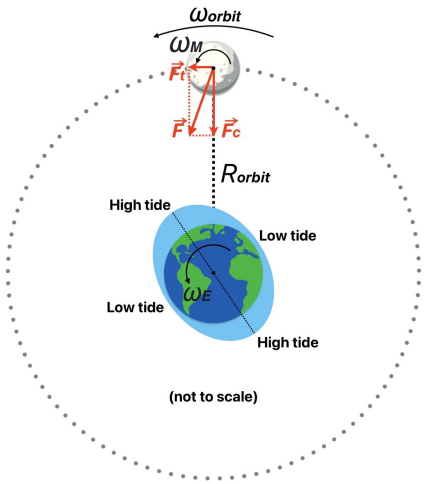

Figure 2: The high tides and low tides, and the Earth-Moon gravitational force on the Moon. The high tide level on the ocean surface is not on the line connecting the Earth and the Moon, which makes the gravitational force of the Earth on the Moon slightly point toward the high tide level.

Earth rotates approximately every 24 hours. Since the Moon orbits Earth approximately every 27.3 days, and in the same direction as Earth rotates, Earth rotates much faster than the Moon orbits Earth ($\omega_E \gg \omega_{orbit}$). This causes the high tide level on the ocean surface not to be on the line connecting the Earth and the Moon, but slightly offset in the direction of the Earth's rotation, as shown in Figure 2. The shape of the ocean surface makes the mass distribution of the Earth no longer spherically symmetric. The gravitational force of the Earth on the Moon is no longer along the Earth-Moon line, but slightly points to the high tide level, as shown in Figure 2. The Earth-Moon gravitational force has a tangential component to accelerate the orbital motion of the Moon. This acceleration of the Moon maintains the conservation of total angular momentum, while also causing the Moon's orbit move away from the Earth. The Moon is not moving away from the Earth at a constant speed. This speed will change over time and is affected by many factors. The Earth will eventually become tidally locked to the Moon after many billions of years, at which point the distance between the Moon and Earth will no longer change. Through the action of tidal forces, the angular momentum of Earth's rotation is slowly being transferred to the Moon's orbit, as described by

$$\Delta L_E = -\Delta L_{orbit} \quad (8)$$

The Moon's orbital speed can be obtained by,[1]

$$v_{\text{orbit}} = \sqrt{\frac{GM_E}{R_{orbit}}} \quad (9)$$

where G=6.67×10^{-11} Nm^2/kg^2 is the universal gravitational constant. Then the orbital angular momentum of the Moon around the Earth is

$$L_{orbit} = M_M v_{orbit} R_{orbit} = M_M\sqrt{GM_E R_{orbit}} \quad (10)$$

Then

$$\frac{\Delta L_{orbit}}{L_{orbit}} = \frac{1}{2}\frac{\Delta R_{orbit}}{R_{orbit}} \quad (11)$$

The angular momentum of the Earth's rotation is

$$L_E = I_E \omega_E \quad (12)$$

Then

$$\frac{\Delta L_E}{L_E} = \frac{\Delta \omega_E}{\omega_E} \quad (13)$$

From Eqns. (8), (11), (12), and (13) we get

$$\Delta \omega_E = -\frac{L_{orbit}}{2I_E}\frac{\Delta R_{orbit}}{R_{orbit}} \quad (14)$$

The Moon is spiraling away from Earth at an average rate of 3.8 cm per year, as detected by the Lunar Laser Ranging experiment.[5] As a comparison, the Earth spirals away from the Sun at a rate of about 1.5 cm every year, causing its orbital speed to drop by about 3 nanometers per second over that timescale.[6] Since the Sun-Earth system is much larger than the Earth-Moon system, the time it takes Earth to revolve around the Sun is generally considered constant in the units of hours or seconds, but not constant in days because the Earth's rotation speed is changing.

Results and Discussion

A day is the time it takes the Earth to make one revolution about its axis or to spin once around, so now a day is becoming longer than before, or now a year has fewer days than before. Based on the analyses above, we can estimate how many days were in a year when these corals were growing. Now the angular velocity of the Earth's rotation is

$$\omega_{E,now} = \frac{2\pi}{24[hours]\times 60[\frac{minutes}{hour}]\times 60[\frac{seconds}{minute}]} = 7.27\times 10^{-5}\ [s^{-1}] \quad (15)$$

Over the past 180 million years, the change in radius of the Moon's orbit can be calculated by

$$\Delta R_{orbit} = 0.038\left[\frac{m}{year}\right]\times 180\times 10^6[year] = 6.84\times 10^6[m] \quad (16)$$

From Eqns. (10), (14), and (16), the change in angular velocity of the Earth's rotation can be obtained:

$$\Delta \omega_E = -2.65\times 10^{-6}\ [s^{-1}] \quad (17)$$

Thus, 180 million years ago, the angular velocity of the Earth's rotation was

$$\omega_{E,past} = \omega_{E,now} - \Delta\omega_E = 7.54\times 10^{-5}\ [s^{-1}] \quad (18)$$

Now, a typical year has 365.25 days.[1] Let N represent the number of days that a year had 180 million years ago, then

$$\frac{N}{365.25} = \frac{\omega_{E,past}}{\omega_{E,now}} \quad (19)$$

From Eqns. (15), (18), and (19), we find that a year had 378.5 days 180 million years ago. This has good agreement with the findings of the ridges on corals (377 days per year, 180 million years ago). Table 1 lists our calculation results in comparison to those from counting growth lines on fossil corals for three different geologic times.[3,4] Our estimation errors are within ±2.0 %.

Table 1: Estimate of how many days in a year millions of years ago from counting ridges on corals and from the calculation based on tidal force effects on the Earth's rotation.

Geological time	Days in a year from counting ridges on corals	Days in a year from our calculation	Error [%]
180 million years ago	377	378.6	0.43
370 million years ago	400	393.0	-1.74
430 million years ago	402	397.6	-1.08

Wells mentioned in his paper that throughout Earth's history, a slowdown of about 2 seconds per 100,000 years has occurred according to recent estimates.[2] At the beginning of the Cambrian, the length of the day would have been 21 hours. Based on this information, he developed a simple relation between the geological time scale and the number of days per year. We cited his data in Table 2 and made our calculation in comparison to his results. Our calculation results are smaller than Wells' estimates, and the error increases with geological time. The largest calculation error, -3.10 %, nevertheless, occurred in the Precambrian period, which is about 600 million years ago (Figure 3). We do not adopt Wells' method in calculation because now the Lunar Laser Ranging experiment detects that the Moon is spiraling away from the Earth at an average speed of 3.8 cm per year. This gives another linear relationship with a smaller slope. Both lines in Figure 3 start from 365 days per year. This leads to our calculated results are always being smaller than Wells' results, and the difference becomes larger and larger as the geological age increases. Our calculations are more precise because they are based on observations from the more sophisticated Lunar Laser Ranging experiment.

Table 2: Number of days per year in past geological time: comparison between J. Wells' estimates and our calculation.

Geological period	Geological time [millions of years]	Days in a year, data from J. Wells, 1963	Days in a year from our calculation	Error [%]
Cenozoic-Cretaceous	65	371	370.1	-0.25
Jurassic	135	377	375.3	-0.46
Triassic	180	381	378.6	-0.62
Permian	230	385	382.4	-0.68
Pennsylvanian	280	390	386.2	-0.98
Mississippian	312	393	388.6	-1.12
Devonian	345	396	391.1	-1.23
Silurian	405	402	395.7	-1.56
Ordovician-Cambrian	500	412	403.1	-2.17
Precambrian	600	424	410.9	-3.10

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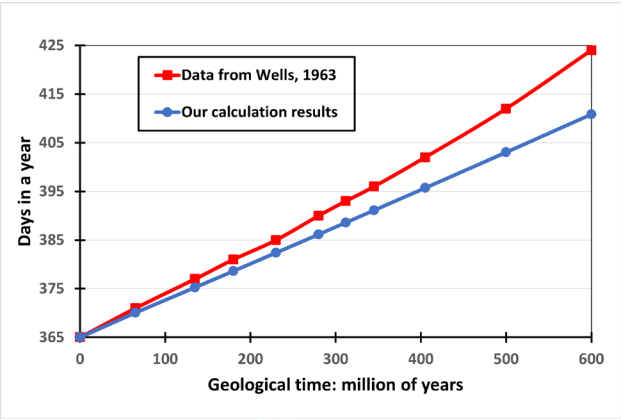

Figure 3: Relation between days in a year and geological time, comparison between J. Wells' results and our results. The difference increases with geological time, but in an acceptable range, with the largest calculation error of 3.10 %.

Conclusion

The tidal force of the Moon on the Earth slows down the Earth's rotation, eventually forming a phenomenon called "locking" or tidal synchronization. Throughout the history of the Earth, the Moon has been gradually moving away from the Earth. As the tidal forces on the Earth's surface and interior consume the energy of the Earth's rotation, the Moon's rotation slows down, resulting in a continuous increase in the length of the day and a continuous decrease in the number of days in a year. Using the Lunar Laser Ranging Experiment, researchers have determined that the Moon is moving away from the Earth at a rate of 3.8 cm per year. We assume that the orbit of the Moon is circular and the Earth-Moon system is considered an isolated system. We use Newton's law of gravity and the law of conservation of angular momentum to derive an equation that can calculate the rate of deceleration of the Earth's rotation. It can estimate how many days there were in a year hundreds of millions of years ago, but how to test this estimation over such a long time frame is a challenge. The growth of any organism on Earth is constrained by environmental factors. Over the long years, as the Earth revolves around the Sun, the seasons change, and the tides rise and fall, these changes have a periodic impact on the growth of the hard body and bones of organisms. The alternation of day and night in a day has a great impact on organisms, and this law must have a significant impact on organisms. Correspondingly, ancient organisms will show traces of regularity due to the alternation of day and night, and these effects are generally reflected in the hard parts of the organisms. These patterns could help determine Devonian storm frequency, changes in coral growth conditions, and potentially detect astronomical cycles like Earth's rotation rate and tidal periodicity.[7] Therefore, the fossils preserved by these organisms are the best ancient calendars, also known as "paleobiological clocks." Paleontologists have discovered that the calcium carbonate secreted by coral polyps every day can form tiny "day rings" that gather into a growth belt that is significantly different from other years, just like the annual rings of a tree. Scientists can determine how many days there are in a year based on the number of "day rings" in the growth belt. This prompted us to use experimental data from ancient corals to verify our hypotheses and computational models, and to use evidence from Devonian tetracoral ridges to verify the accuracy of our estimates. The calculation results are consistent with the actual observational data.

Many factors affect the Earth's rotation. In addition to the tidal force of the Moon, there are disturbances in the Sun's gravity, changes in the Earth's mass caused by cosmic dust, crustal movement, glacier melting, earthquakes, etc. The temporal variations in the atmospheric angular momentum and their contribution to the instabilities of the Earth's rotation are also non-negligible.[8] The decadal rotational variations likely arise from gravitationally driven electromagnetic coupling between inner and outer cores and the mantle.[9] These factors work together to cause small changes in the speed and direction of the Earth's rotation. In this paper, we show that it is possible to derive a predictable method to estimate how many days there were in a year in the geological past.

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RESEARCH ARTICLE

IoT-Based School Building Evacuation Guidance System with Audible Sound Wave Backup Communication

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ABSTRACT: Various IoT-based systems have been developed to guide people to evacuate buildings during emergencies like fires. However, research on environments where the internet is disrupted or the radio wave conditions are poor is insufficient. This study proposes an IoT-based system for guiding safe evacuations, utilizing both internet-based communication and a fallback audible sound wave mode for disruptions. This study presents an IoT-based evacuation guidance system that operates via MQTT and switches to sound-based communication when the internet fails. It receives digitally encoded emergency data through the internet or building sound broadcast systems and minimizes transmission by storing the building layout and calculating optimal evacuation paths using Dijkstra's algorithm. The system utilizes 18 kHz and 19 kHz audio frequencies with FFT (Fast Fourier Transform) and BFSK (Binary Frequency Shift Keying) modulation for sound wave communication. The test was conducted in a simulated school environment, where the system successfully guided users to exits during emergencies, including scenarios with network disruptions. Additionally, a noise interference test was performed to assess the robustness of sound wave communication. Results indicate that the proposed system effectively guides evacuation with minimal data transmission and maintains functionality during internet disruptions, showcasing its potential for enhanced emergency response systems.

KEYWORDS: Embedded Systems, Internet of Things, Networking and Data Communications, Evacuation Guidance System, Sound Wave Communication.

Introduction

In emergencies such as fires, safely and quickly evacuating a building is crucial to minimizing casualties. To facilitate this, all buildings above a certain size are required to display evacuation floor plans, guiding people to evacuate safely. However, this approach has limitations, as it cannot identify the exact location of hazards in real time. As a result, it may inadvertently direct people toward danger, causing confusion and potentially leading to more hazardous situations. This risk increases in complex buildings or for unfamiliar visitors, highlighting the need for a system that can intelligently guide individuals to optimal escape routes in real time.

Various methods utilizing IoT technology have been studied to assist in building evacuations during disaster situations.[1-4,7,10] Among these, certain studies utilize various sensors and LED guide lights to guide evacuees using pre-identified escape route data, demonstrating similarities to this study.[1,2] However, both rely on network connectivity for real-time information exchange between system entities, which means IoT devices may fail to function if the network is disrupted. Similarly, another study proposes a system that assists evacuation during fires using smartphone communication and frequency-based technology, but it also depends on Wi-Fi communication.[3] Despite these advancements, most IoT-based evacuation systems remain heavily reliant on internet communication. In disasters such as fires, where Wi-Fi may become inoperative or in areas with poor signal conditions, these systems risk becoming non-functional.

Therefore, research is needed on a system that can transmit emergency information to evacuation guidance devices even when the IoT-based network is disabled, assisting people in escaping safely. Additionally, by minimizing changes to existing building systems, this approach can reduce costs while also limiting communication between IoT devices, thereby reducing dependence on a central server that holds critical information.

To address these challenges, this study proposes an IoT-based evacuation guidance system that remains operational even in network-disrupted environments. The system is primarily based on the MQTT[6] (Message Queuing Telemetry Transport) protocol, which operates over TCP/IP. When the network is unavailable, it utilizes the alternative communication method on audible sound waves. Under normal conditions, evacuation guidance devices installed along passageways operate via MQTT. However, in emergencies where internet connectivity is lost, the system leverages existing building broadcast systems to receive situation codes and hazard location data. This information is then used to activate direction-indicating lamps, guiding people safely and efficiently toward proper exits.

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Methods

A. System Design:

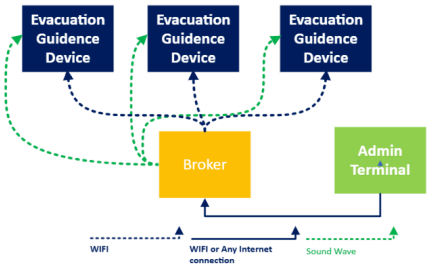

Figure 1: This figure illustrates the overall system architecture for the IoT system, including the interactions between the Admin Terminal, the Broker, and the Evacuation Guidance Devices.

The Evacuation Guidance Device is implemented on a Raspberry Pi and installed in each corridor of the building. This IoT device guides evacuees via escape route indicator lamps and is equipped with both a TCP/IP connection and an audio-band communication receiver. The Broker functions as a standard MQTT[6] broker, facilitating message transmission between the MQTT Publisher (Admin Terminal) and the Subscriber (Evacuation Guidance Device). Additionally, it converts the payload of messages published to specific topics into broadcast data suitable for the audio-band channel, which is then transmitted via speakers. The Admin Terminal is an IoT application installed on the administrator's system for managing and responding to emergencies. It publishes predefined disaster-related data to the broker, ensuring that the information is reliably disseminated to all Evacuation Guidance Devices (Figure 1).

Under normal conditions, the Evacuation Guidance Device receives emergency information through TCP/IP-based MQTT communication and periodically receives status signals from the voice band channel to verify communication connectivity. In an emergency, as long as the internet is operational, information for emergency escape route guidance is received via the internet-based MQTT protocol. If the internet is disconnected, information about the hazardous situation and location within the building is received from the Broker through the voice-band channel. Based on this information, the optimal escape route is calculated, and directional lamps are activated according to the user's current installation location.

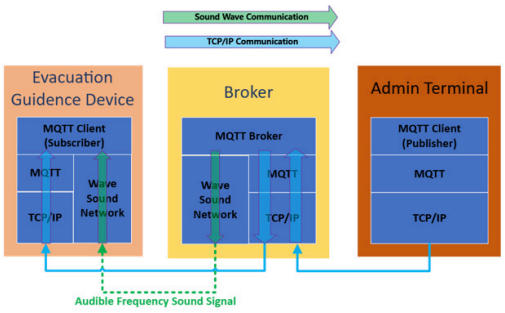

Figure 2: This figure illustrates the layered structure of the communication protocol between the Evacuation Guidance Device, Broker, and Admin Terminal.

The Evacuation Guidance Device is equipped with both TCP/IP-based MQTT protocol communication and data reception via audible frequency sound waves. In the event of a disaster where the network is unavailable, it utilizes the information transmitted through the sound wave-based communication channel. The Broker is responsible for relaying messages from the Admin Terminal to the Evacuation Guidance Device and transmitting published data through both communication channels: TCP/IP-based and sound waves. The Admin Terminal publishes information about disaster situations occurring in the building to Evacuation Guidance Devices by transmitting critical disaster information to the Broker via the TCP/IP-based MQTT protocol, which then forwards the messages to the devices through both communication channels (Figure 2).

B. Digital Data Transmission over Sound Wave:

In the system designed in this study, the sound wave-based communication process is carried out as follows. On the transmission side, digital data is converted into a bitstream and then modulated into sine wave tones at two carrier frequencies (18 kHz and 18.5 kHz). These tones are broadcast through a speaker. On the receiving side, a microphone captures the sound and processes it using the Fast Fourier Transform (FFT) to convert the signal into the frequency domain. The system employs Binary Frequency Shift Keying (BFSK) 5 digital modulation, where each of the two transmission frequencies represents a binary value: one frequency for '0' and the other for '1'.

Figure 3 illustrates this process, showing how the bitstream is transmitted from the sender to the receiver. The sender converts the digital bitstream into sound wave signals at the two designated frequencies, which are then broadcast via a speaker. The receiver's microphone captures these signals, undergoes the demodulation process, and reconstructs the original bitstream.

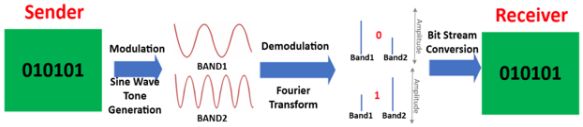

Figure 3: This figure illustrates the process of digital data transmission using Binary Frequency Shift Keying (BFSK) over sound waves.

C. Sound Wave Data Transmission Packet Format:

In the sound wave communication system implemented in this study, one synchronization channel (19 kHz) and one data channel (using two carrier frequencies) are utilized. Figure 4 shows the sound wave communication packet structure. Before data is transmitted in the data channel, a signal filled with '1's is first sent through the synchronization channel. Each synchronization channel's one frame consists of 40 bits. Data transmission through the data channel occurs while the sync channel is activated as '1' and is fixed at a length of 24 bits. A single data frame transmitted through the data channel consists of Preamble bits (4 bits), Data (16 bits), and Checksum (4 bits) for error detection.

In the experiment, each data bit has a duration of 0.1 seconds in the data channel and the sync channel. That is, the bit

transmission rate of the data channel, including all overhead while the data channel is active, is 10 bits per second.

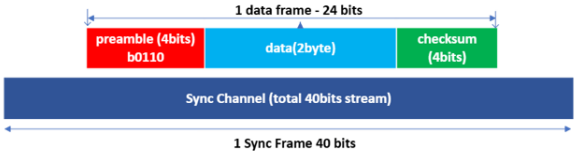

Figure 4: This figure illustrates the sound wave communication packet structure.

While one sync frame, as described in Figure 4, is active, one data frame is transmitted. There is a 1-second gap between each sync frame. As a result, one data packet frame is transmitted every 5 seconds (Figure 5).

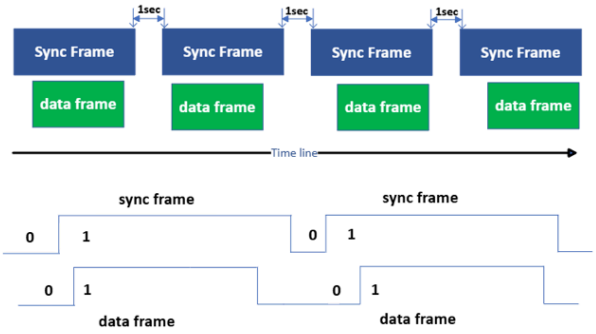

Figure 5: This figure illustrates the timing between the sync channel and the data channel in data transmission over the sound wave channel. The data frame, consisting of 24 bits, is transmitted during the 40-bit duration in which the sync frame signal remains at logic '1'.

D. Protocol:

Figure 6 illustrates the emergency evacuation guidance system protocol between the Admin Terminal, Broker, and Evacuation Guidance Devices. In normal (non-emergency) situations, the Broker periodically transmits a check message via the sound wave channel to indicate that the channel is active. When emergency-related information is published by the Admin Terminal, the data is transmitted through both the general IoT channel (TCP/IP) and the sound wave channel.

For emergency evacuation guidance, the messages transmitted via both the general IoT channel (TCP/IP) and the sound wave channel consist of a 1-byte status code and a 1-byte status parameter.

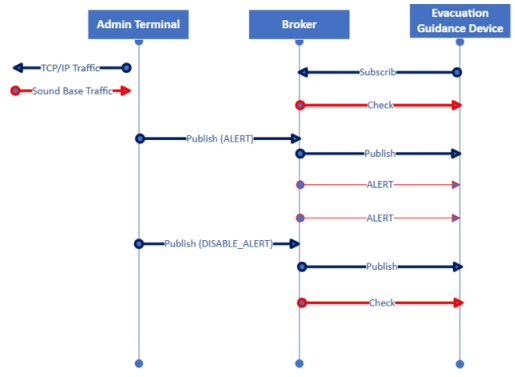

Figure 6: This figure illustrates the protocol between the Admin Terminal, Broker, and Evacuation Guidance Devices.

Table 1 provides examples of status codes and parameters used in ALERT or Check messages in Figure 6. For example, in a normal situation with no emergency, the Check Message transmits a 2-byte Emergency Code (Status code, region code): [0x00, 0x00]. When a fire drill is issued, the status code 0x01 is sent along with a region code indicating the risk area. For instance, [0x01, 0x04] indicates that a fire drill is in progress and the fire is in region 0x04.

Table 1: This table shows examples of status codes and parameters for emergency evacuation guidance.

	Status Code (1byte)	Parameter (region code, 1byte)
Normal	0x00	0
Fire Drill	0x01	0 ~ 255
Shooting Drill	0x02	0 ~ 255
Fire	0x80	0 ~ 255
Shooting	0x81	0 ~ 255

E. Simulated School Building Environment:

To test the operation of the system developed in this study, a school building layout like a real school environment was simulated. Figure 7 depicts a map of a virtual school building. Figure 7 (Left) shows the floor plan of the school building, and Figure 7 (Right) represents it as a graph, where each location is modeled as a vertex, and the movement cost between connected locations is represented as edges. Table 2 provides the data representation of the graph in Figure 7.

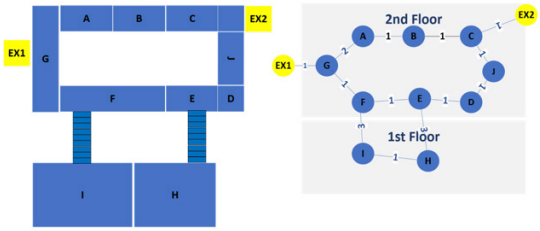

Figure 7: Left: Virtual school building floor plan, Right: graph data structure.

Under normal conditions, when no emergency has occurred, the evacuation guide device installed in region D determines the shortest evacuation route as D-J-C-EX2 using Dijkstra's algorithm8 (Figure 8).

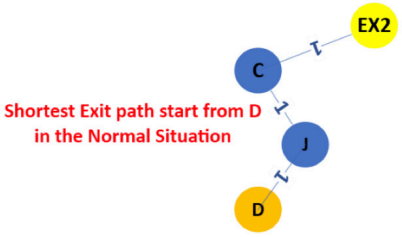

Figure 8: This figure shows the shortest path to the EX2 in a normal situation, starting from location D.

Each evacuation guidance device maintains a table that represents the cost of moving between vertices to calculate the optimal escape route from its location. Table 2 shows this

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vertex cost table based on the building structure illustrated in Figure 7. The intersection of each row and column indicates the cost of moving between two vertices, where a value of '0' denotes either an inaccessible path or the same vertex.

Table 2: This is the vertex data table that contains the cost of moving from every vertex to every other vertex in the building structure graph shown in Figure 7.

	A	B	C	D	E	F	G	H	I	J	EX1	EX2
A	0	1	0	0	0	0	1	0	0	0	0	0
B	1	0	1	0	0	0	0	0	0	0	0	0
C	0	1	0	0	0	0	0	0	0	1	0	1
D	0	0	0	0	1	0	0	0	0	1	0	0
E	0	0	0	1	0	1	0	3	0	0	0	0
F	0	0	0	0	1	0	1	0	1	0	0	0
G	2	0	0	0	0	1	0	0	0	0	1	0
H	0	0	0	0	3	0	0	0	1	0	0	0
I	0	0	0	0	0	3	0	1	0	0	0	0
J	0	0	1	1	0	0	0	0	0	0	0	0
EX1	0	0	0	0	0	0	1	0	0	0	0	0
EX2	0	0	1	0	0	0	0	0	0	0	0	0

F. System Testing:

To test the system developed in this study, a virtual school building layout defined in Figure 7 was used, and the following test scenario was configured:

- Two locations (A and D) were assumed to be equipped with evacuation guidance devices (Raspberry Pi[9]).
- The status codes and hazard area codes defined in Table 1 were transmitted via the MQTT channel and the Sound Wave Communication channel, respectively.
- Each of the two evacuation guidance devices calculated the expected evacuation route and was monitored to verify whether the guidance lamps were activated in the correct direction.
- The hazard status codes were changed sequentially (0x8003, 0x8006, 0x8007) and transmitted during the test.
- During the test, the internet connection was intentionally disconnected, and random noise was played to verify whether the Sound Wave Communication functioned correctly under such conditions.

Here, each region code 'A' to 'J', 'EX1', and 'EX2' from Figure 7 is mapped to numbers 1 to 10, 128, and 129.

■ Results and Discussion

A. Testing in Normal Situation:

To test the system developed in this study, the Evacuation Guide Device was implemented on a Raspberry Pi. Emergency codes were broadcast via sound waves using a regular speaker, along with background noise, and the system was designed to recognize these signals. A spectrum analyzer app and a noise level meter app on a smartphone were used to measure the frequency and noise levels. Figure 9 shows the test environment for this system.

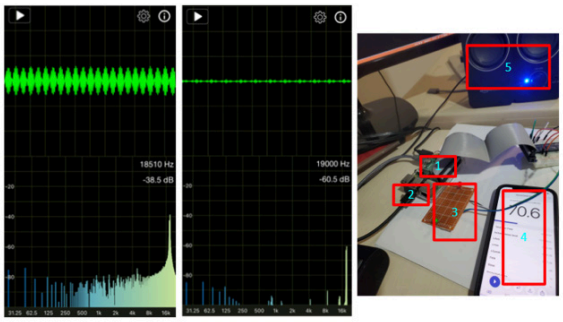


Figure 9: System testing environment: Left- 18.5 kHz, Center- 19 kHz sound wave signal measurement, Right–system testing picture (1: Raspberry Pi, 2: microphone, 3: evacuation direction lamp, 4: noise level measurement, 5: broadcasting speaker for sound wave).

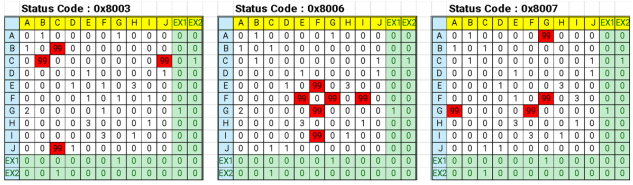
The status code and hazard location were transmitted via MQTT or sound wave to the Evacuation Guidance Device, and the system's response was recorded. Each scenario used a status code of 0x80 (Fire), with hazard locations designated as 0x03, 0x06, and 0x07, respectively. Table 3 presents the results of executing the evacuation guidance devices that are installed at two different locations of the building (A and D) when fire emergencies occur at three different locations.

Table 3: Test results of scenarios 1, 2, and 3 from locations A and D.

	Emergency alert packet Data (MQTT or Sound Wave)	Device at D Shortest Evacuation Path	Device at A Shortest Evacuation Path
Scenario 1	0x8003	D->E->F->G->EX1	A->G->EX1
Scenario 2	0x8006	D->J->C->EX2	A->G->EX1
Scenario 3	0x8007	D->J->C->EX2	A->B->C->EX2

Table 4 shows the updated Vertex Table according to the Emergency Scenarios in Table 3. When an Emergency Packet with a status code of 0x80 is transmitted, the Evacuation Guidance Device interprets the second byte of the payload (0x03, 0x06, and 0x07) as the hazardous area. It then updates the Vertex Table, setting the movement cost to or from the affected area to 99, effectively marking it as highly restricted.

Table 4: Vertex table updated by scenario 1(Left), scenario 2(Center), and scenario 3(Right).



As a result, the system generated different escape routes for each case based on location (A and D) and displayed directional guidance lamps accordingly (Table 3).

B. System Testing by Noise and Network Disruption Stress:

The test was conducted over approximately 30 minutes, alternating between Scenarios 1, 2, and 3, while applying random noise and intermittent internet disconnection to simulate stress conditions. To evaluate sound wave communication un-

der noisy conditions, continuous ambient noise ranging from 50 dB(A) to 80 dB(A) was introduced during testing. Additionally, the internet connection was intentionally interrupted five times during the test. As a result, a total of 300 sound wave sync frames and data frame transmissions were made over the 30 minutes. Among them, there were 2 instances where the sync frame failed due to the inability to maintain a continuous sequence of '1's for 40 bits. Additionally, there were 4 instances where the data frame had a checksum error, requiring a wait until the next transmission.

Conclusion

This study proposes a guidance system designed to ensure the safe evacuation of individuals from a school building in the event of a disaster. The system is based on IoT technology and operates using the MQTT protocol. Even in situations where the TCP/IP network is unavailable, it can guide people to the shortest evacuation route by broadcasting encoded emergency information via sound waves through the building's public address system.

When a disaster, such as a fire, occurs, the emergency code and the location of the hazard within the building, published by the management system via the MQTT IoT protocol, are transmitted to evacuation guidance devices installed throughout the building. Each device then activates a guidance light to indicate the nearest escape route based on its current location.

In this study, a virtual school building structure was created, and evacuation guidance tests were conducted under simulated emergency conditions. During testing, stress factors such as random noise and network disconnections were introduced to evaluate the system's stability. The results showed that the system generally operated reliably and demonstrated its potential to assist in the safe evacuation of school buildings during disasters.

In scenarios involving network disconnection, several synchronization and data transmission errors were observed in the sound wave channel, or data frame transmission errors occurred, requiring a wait for the next transmission. In the current implementation, the low transmission rate of 10 bps results in each data reception taking 5 seconds. If an error occurs during a transmission, the system must wait for the next attempt, causing a delay of up to 10 seconds. Therefore, improving transmission efficiency remains an area for future research.

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REVIEW ARTICLE

Effects of Epigenetics on miRNAs and the Function of T Cells in Allergic Diseases

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ABSTRACT: Especially with their inflammatory responses, immune T cells play a large role in the development and severity of allergic diseases. In recent studies, epigenetic modifications, specifically types that affect microRNAs (miRNAs), were shown to have the ability to significantly influence gene expression regulation in various T-cell subsets. Many epigenetic modification mechanisms, such as DNA methylation and histone acetylation, have been proven to successfully modify miRNAs along with T cell differentiation in allergic diseases. A secondary analysis was conducted using databases of ScienceDirect, Google Scholar, and PubMed to gather data. This article will explore the effects of epigenetic modifications that target miRNAs on T-cell function in allergic diseases such as atopic dermatitis, asthma, and chronic urticaria. Although these epigenetic methods can help noninflammatory regulatory T cells suppress proinflammatory T cells, the risks of these changes include mutations that worsen the allergic reaction. Because of this, researching the effects of epigenetics on T cells is crucial as it can lead to a deeper understanding of allergic inflammation and specific miRNAs affected by immune dysregulation, potentially aiding in future developments of more effective and longer-lasting treatments.

KEYWORDS: Cellular and Molecular Biology, Genetics, Epigenetics, Allergic Diseases, T cells.

Introduction

Allergies are becoming an increasingly widespread major health concern, with skin inflammation in allergic skin disorders creating significant risks to patients' health. One significant contributing factor to the adaptive immune system is T cells, specifically, regulatory T cells (Tregs), a subset of CD4+ T cells. These Tregs are responsible for maintaining immune tolerance as they suppress excessive allergic inflammatory reactions.[1] Another type of distinct immune cells, mast cells are involved with provoking immediate allergic reactions, releasing histamines and cytokines when they bind to allergen-specific IgE.[2] Other innate immune cells that also advance the pathogenesis of allergic diseases include eosinophils, basophils, and dendritic cells.

Micro-RNAs (miRNA) are a large subgroup of non-coding RNA that usually suppress gene expression. Additionally, they have been found to have the ability to influence regulatory systems involved in the inflammation of some skin diseases.[3,4] There are several major variational methods for epigenetic modifications, with the most prevalent being DNA methylation. Epigenetic processes can both directly and indirectly modify gene expression, such as blocking TFs (transcription factors) and preventing regulatory elements from receiving positive transcription signals;[5] helps sustain long-term gene expression in memory T cells, improving the enhanced effector response;[6] and regulate cytokine genes to affect the ability of CD4+ and CD8+ T cells to produce the necessary cytokines for immune responses.[6,7]

Epigenetic modifications have the ability to modify various miRNAs in allergic diseases to suppress immune responses. Although additional research still needs to be conducted, current evidence suggests that immunotherapeutic approaches targeting miRNAs in the immune system can be effective in stabilizing Tregs long-term for disease prevention and, along with many other potential outcomes of this treatment, in reducing skin barrier damage by inhibiting Th2 inflammatory cytokine.[7,8]

In allergic diseases, there is a clear difference in the number of regulatory T cells, the level of miRNAs, the balance of Th1 and Th2 cells, and the number of allergen-specific memory T cells between patients with and without the disorder. Because the majority of the outcomes of miRNA modulation are heavily dependent on individual circumstances and factors, including the miRNAs involved, the severity of allergic disease, and the cell types affected, there are several approaches to achieve a lowered severity of an allergic reaction. While a decrease in some miRNAs could benefit the situation, an increase in different miRNAs within the disorder could lead to the same overall effect.[4] Recent studies have primarily focused on the identification of Treg cell development and potential molecular treatments and suppression techniques. The continued discussion of Treg involvement will be important for developing treatments that target Tregs to alter its suppressive and restorative ability allowing it to better regulate allergic and autoimmune diseases.[7] Additionally, there have been studies conducted that identified several miRNAs not only as biomarkers but also as possible therapeutic agents for chronic skin disorders.[8] However, there are still many unexplored factors that need to be taken into consideration while developing treatments and drugs for clinical use. This article will explore the effects of epigenetic modifications that target miRNAs on T cell function in some of the most common allergic diseases,

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specifically atopic dermatitis, asthma, as well as chronic urticaria.

Discussion

DNA methylation and the function of T cells:

DNA methylation, being one of the primary methods of epigenetic editing, is capable of mediating the effects and risks within the development and progression of allergic diseases. This process can largely alter T cell function, and consequently, the immune system's response to external stimuli.[9] In addition to its role as a gene expression regulator, DNA methylation and imbalanced epigenetic gene regulations in general have been linked to several human disorders, especially observable in children. The epigenetic method of DNA methylation can be defined as a heritable epigenetic marking of a covalent transfer of a methyl group to the base cytosine ring in DNA-by-DNA methyltransferases (DNMTs). Different types of DNMTs, such as DNMT3a and DNMT1, each have a role in the methylation process that can determine the success of the epigenetic modification. Although able to work in cytosines anywhere within the genome, a large majority of DNA methylation takes place in CpG dinucleotides specifically.[10] Another product of DNA methylation is restricting gene promoters from transcription factors (TFs), which directly prevents those TFs from binding.[11]

T lymphocytes (T cells) are a subset of white blood cells responsible for immune responses and the surfacing of allergic symptoms. As T cell receptors (TCRs) recognize cognate antigens presented by major histocompatibility complex molecules (MHC) on antigen-presenting cells (APCs), T cells will undergo activation and clonal expansion to regulate immune responses.[12] These T cells can then be further categorized according to their specific function after having completed the process of T cell differentiation. This differentiation occurs after positive and negative thymic selection when naive cells are exposed to antigens of MHC molecules. During this, naive T cells are epigenetically reprogrammed and develop into either CD4+ T effector cells or CD8+ cytotoxic T cells, both of which are produced by the thymus. The two divisions of T cells are discerned based on their surface proteins and transcriptional programs throughout their development.[13] After their maturation in the thymus, naive antigen-specific CD4+ T cells are then distributed to peripheral tissues where their essential function is to support the immune system. When they interact with foreign antigens or APCs, they differentiate into various subsets of helper T cells and regulatory T cells depending on their surrounding environment and the polarizing cytokines involved.[14] The cells and their respective cytokines are connected to the development of several immunological illnesses including allergic diseases.

In allergic diseases such as asthma, urticaria, and atopic dermatitis, there is often a skewing of T helper 2 (Th2) cells that affects the immune pathway related to differentiation. Particularly with eosinophilic inflammation – which is related to eosinophils, white blood cells that can accumulate in patients' airways – some studies have theorized that the development of inflammation in allergic airway diseases can be associated with a dominant differentiation of Th2 cells, which produce IL-4, IL-5, IL-12, and IL-13 cytokines (Figure 1).[15] Given this hypothesis that Th2 cells and their cytokines have a strong influence on asthma pathophysiology, inducing a Th1 response was thought to be a promising solution. Surprisingly, Th1 cells and their IFN-γ cytokine were found to have potentially pro-inflammatory effects in the lung, contrary to the original theory. Unfortunately, due to a lack of consistent testing, results of studies on Th1 cells and their cytokines remain conflicting, with some results demonstrating dampening capabilities, while others find them to have pro-inflammatory effects on allergic diseases.[15]

Despite its known contributions, Th1 and Th2 are not the only cells that contribute to allergic reaction severity and sensitization towards treatments.[16]

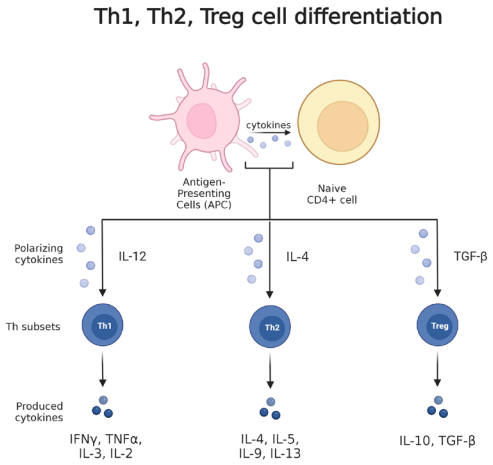

Figure 1: This figure illustrates the process of naive CD4+ T cell differentiation into Th1, Th2, and Treg Cells based on the cytokines involved. The polarizing cytokines are the determining factor for which subset the cell becomes: IL-12 induces CD4+ T cells into Th1 cells, the cytokine IL-4 promotes the differentiation into Th2, and TGF-beta cytokines into Treg cells. This diagram was created using BioRender.

Another subset of CD4+ T cells that also has the ability to inhibit the immune system is known as regulatory T cells (Tregs). By maintaining peripheral tolerance and immunological homeostasis, Tregs can contribute to the prevention of allergy disorders altogether.[17] There are two main subtypes of regulatory T cells based on where they develop: ones developed in peripheral tissues are known as pTregs, while ones in the thymus are called tTregs. Through cell-to-cell interactions and moderating inhibitory cytokines, regulatory T cells can prevent immunological responses. However, to maintain and manage Treg stability and function, epigenetic regulation mechanisms, including DNA methylation and histone modifications, are essential.[7] Although Tregs are important in allergic illnesses, it is unknown how and why a patients' tolerance can fail, but there are multiple treatments currently in trials for an eventual clinical application (Table 1).

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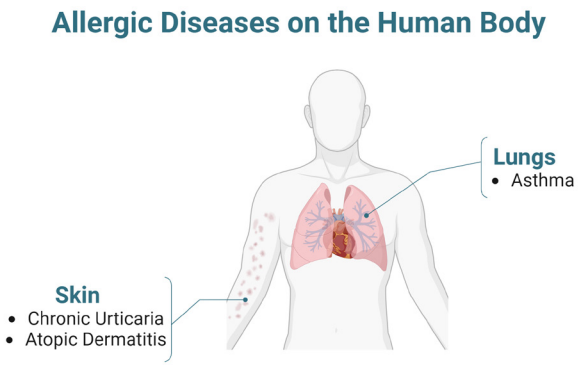

Figure 2: This figure illustrates where individual allergic diseases affect the human body. Both Chronic Urticaria and Atopic Dermatitis affect the skin, while asthma affects the lungs. This figure was made using BioRender.

Epigenetics on T cells in asthma:

By targeting Treg cells, there is potential for epigenetics to mediate the impact environmental factors have on the progression of allergic disorders like asthma.[5] Asthma is a clinically heterogeneous chronic airway inflammatory disease characterized by symptoms of wheezing, shortness of breath, chest tightness, and coughing (Figure 2).[7,18] miRNAs are one of the factors of the regulatory mechanisms that are involved with allergic diseases like asthma.[19]

For example, miR-155 and miR-221 have both been associated with Th2 responses along with several cellular elements of allergic responses including eosinophils, macrophages, and mast cells in asthma and allergic rhinitis. MiR-221 specifically targets genes involved in immune response and an increase in them would lead to an exacerbation of asthma symptoms including airway hyperresponsiveness and inflammation.[20] MiR-155 targets and suppresses genes that negatively regulate immune responses. This miRNA is often upregulated in airway tissues of asthma patients, and it has the potential to be used as a target therapeutic strategy in terms of reducing Th2-drive inflammation.

Allergic asthma disease is considered a T helper (Th) cell-mediated disease that has been thought to be brought on by a combination of environmental factors and some genetic predispositions.[9] Specifically, when there is a defective production of Th1 or T-bet cells, allergic asthma is actively present. Additional research examined genome-wide DNA methylation and gene expression patterns in both IL-13-treated and untreated airway epithelial cells. Produced by Th2 cells, the cytokine IL-13 is considered one of the main upregulated mediators in asthma. The findings demonstrated that IL-13 exposure can cause changes in DNA methylation in asthmatic airway cells, as well as contribute to asthma phenotypes.[19] DNA methylation methods in Th1 and Th2-produced cytokine genes were also explored, concluding that T cells can contribute to the development and eventual sensitization of asthma. After21 Results indicate that nasal DNA methylation has the potential to be a biomarker for IgE sensitization which suggests the individual is at a high risk of allergic respiratory disease.[22] Not only is this beneficial for asthma, but similar marks were also discovered in patients with rhinitis, showing the versatility of the efficacy of DNA methylation in various allergic diseases.

Furthermore, Th2 cytokine inhibition has been suggested as the most promising approach and anti-IL-5 treatment is the most successful for reducing asthma exacerbations.[23] As a response to allergen exposure, Tregs can inhibit pathways of allergic sensitization and IgE production. Patients with severe asthma tend to display comparatively lower levels of FOXP3+ Tregs and a reduced amount of circulating Tregs. Similarly, there was an increased frequency of CRTH2+ Tregs in patients with acute asthma. Overall, asthmatic patients inhabit a lower number of Treg cells, demonstrating a decreased lower functionality and a Th2 preference behavior.[7] With Tregs and Th cells playing such a large role in asthma regulation, modifications of miRNAs that impact those cells can, as a result, mediate the impact cytokines produced by those cells have on the pathogenesis of the disease.

MiRNA and Treg Function in AD:

Caused by genetic, environmental, and immunological factors, atopic dermatitis (AD) is a chronic relapsing inflammatory skin disorder that affects a significant number of adults and children (Figure 3). AD disorder has symptoms of severe skin dryness, itching, and rashes. These allergic inflammatory reactions can be brought on by a combination of the environment and the patients' genetics. Th2 cell hyperactivation in AD can cause persistent inflammation and a weakening of skin barrier functions. Other subgroups of T helper cell responses such as with Th22 and Th17 can also contribute to AD development through skin and blood infiltration.

Atopic dermatitis is also associated with Tregs, cells that typically aid in the regulation of immune responses by suppressing other immune cells. Treg dysfunctions have been connected to AD due to their observed frequency in other similar genetic illnesses that share skin abnormalities with AD such as immunodeficiency and Wiskott-Aldrich syndrome.7 In AD specifically, Tregs can have a reduced suppressive function. This reduced function could then lead to an inability to control Th2 cell activity and result in chronic inflammation and a worsening of symptoms. Tregs are also involved in maintaining the skin barrier. Their dysfunction in patients with AD can intensify skin barrier defects, allowing allergens to penetrate the skin which would trigger further immune responses (Figure 3).[8] Able to infiltrate the skin and regulate immune responses, Tregs are increasingly explored for their relevance in various genetic and allergic disorders. With a recent study showing a noticeable decline in the severity of AD after AIT treatment and vitamin D supplements, there is evidence to support that both methods contributed to either an increase in Tregs or an enhanced function in existing cells.[7]

MiRNAs have been known to be involved in multiple immunologic and inflammatory disorders. Due to its strong overexpression in several immune cell types such as mast cells, fibroblasts, and lymphocytes cells involved in the pathophysiology of chronic skin inflammation, the miRNA miR-155 has commonly been linked to inflammation in skin disorders. It was suggested to potentially have a regulatory effect on T helper cells based on its upregulation in multiple cell lineages.

Individuals with AD were shown to have varied expressions of miR-155 in their skin and peripheral blood vessels.[4] The research concluded that patients with AD had a higher expression of miR-155 in both peripheral CD4 T cells and various skin specimens and that there was a relatively positive correlation between the number of CD4 T cells present in a patient and the severity of AD disease.[24]

As studies have continued to investigate DNA methylation changes in AD, researchers demonstrated a strong correlation between CpG methylation changes in keratinocytes and innate immune cells and altered gene expressions causing skin barrier dysfunction and inflammation. Especially in immune cells, hypomethylation of the Il-13 cytokine was connected to an increased Il-13 expression, demonstrating epigenetic modulation of Th2 cell inflammation.[25] These results emphasize the role of DNA methylation in immune responses of Atopic Dermatitis, further implying its potential as a therapeutic target.

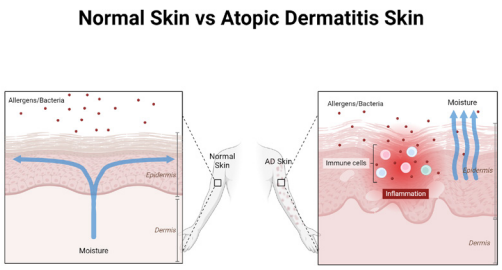

Figure 3: This comparison between normal skin and AD skin demonstrates a damaged skin barrier. On the left with the normal skin, a healthy barrier is intact which allows the skin to retain moisture. This prevents allergens and bacteria from entering the skin. On the other hand, atopic dermatitis skin on the right has a disrupted barrier function. This allows allergens and bacteria to penetrate the skin. The presence of immune cells reflects an active immune response which leads to inflammation from immune cell activation in response to the invading allergens/bacteria. The figure was made using BioRender.

miRNAs in Chronic Spontaneous Urticaria:

Chronic spontaneous urticaria is an allergic disease that can be defined as the spontaneous presence of itchy hives for six weeks or longer (Figure 2).[8] Despite its prevalence, the pathophysiology of chronic spontaneous urticaria (CSU) is largely unknown. However, two main pathogenetic mechanisms are thought to be responsible for the disease. The first of which involves the presence of autoantibodies to immunoglobulin E (IgE) on mast cells and basophils.[26] The autoantibodies can cause immune cells to release histamine and other inflammatory mediators, contributing to the development of hives on the skin. The second mechanism is related to the dysregulation of regulatory and immune signaling pathways within those cells, which results in an inflammatory response.[8,27]

Epigenetic research on skin conditions such as urticaria has contributed greatly to existing knowledge of the processes of gene regulation. Research has determined that there are five significantly upregulated miRNAs in CSU: miR-2355-3p, miR-4264, miR-2355-5p, miR-29c-5p, and miR-361-3p27. As a result, it can be implied that genes targeted by these miRNAs could be severely inhibited and can be used as biomarkers for urticaria disease.[3]

Overall, epigenetic processes like DNA methylation can regulate gene expression and immune responses in allergic diseases. These mechanisms can affect T cells, specifically T helper 1 (Th1), T helper 2 (Th2), and regulatory T cells (Tregs). As allergies are becoming increasingly prevalent and with inflammation causing increased health risks, there is a need for further advancements in treatment techniques. Currently, research has determined miRNAs as a reliable target to influence immune cell responses and reduce the severity of allergic diseases.[28]

In addition to miRNAs regulating immune cell functions, some genome-wide DNA methylation studies have been implicated in immune regulation through differentially methylated genes (DMGs). The study also identified several DMGs regulating the pathophysiology of CSU, which were heavily linked to immune pathways including cytokine signaling, mast cell activation, and inflammatory mediator regulation.[29] Thus, the studies concluded that epigenetic modifications, such as DNA methylation, are also suggested to be connected to the immunopathogenesis of CSU.

Despite the demand, further research and trials are necessary to better understand the complexities of epigenetic processes before an application of treatments for clinical approval. Some of the current miRNA-based treatments and therapies in trials are listed in Table 1.

Table 4: This table lists some of the current miRNA-based treatments and therapies directed at T-cell function in allergic disorders. Although there is constant progress being made toward miRNA-based therapeutics with some reaching clinical development, there are no therapeutics that have reached phase III human clinical trials or been approved by the FDA.[30] This figure was made using BioRender.

Altered miRNA	Treatment/ Trial	Function	Disorder	Status
miR-155	Anti-miR-155 Antagomirs	Regulate T-cell activation, Influence inflammatory responses	Atopic Dermatitis, asthma	Preclinical, early trials
miR-155	Toll-like Receptor 3 (TLR3) Agonist	Regulates inflammation and T cell responses	Atopic Dermatitis, asthma	Early-phase research
miR-21	MRX-21	Reduce inflammation and fibrosis	Atopic Dermatitis, asthma	Early clinical trials
miR-146a	miR-146a Mimics and Inhibitors	Regulate inflammatory responses and immune homeostasis	Chronic urticaria, asthma	Investigation, preclinical
miR-203	miR-203 Antagomirs	Reduce inflammation, regulate skin response	Atopic dermatitis, chronic urticaria	Preclinical
miR-34a	miR-34a Inhibitors	Modulate T cell differentiation, regulate inflammatory responses	Asthma, Allergic Rhinitis	Preclinical

■ Conclusion

Through an exploration of epigenetic processes, specifically DNA methylation and miRNA regulation, there is potential for its use in allergic illnesses such as chronic urticaria, atopic dermatitis (AD), and allergic asthma that can greatly contribute towards their understanding and development of treatments. As allergies become more prevalent in the world, there is an increasing risk of such diseases creating greater health risks due to the inflammation caused by their allergic reactions. From the research conducted, epigenetics of miRNAs has demonstrated potential for stabilizing noninflammatory regulatory T cells (Tregs) to reduce inflammation and, eventually,

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prevent the severity of allergic disease reactions from surfacing at all. Some studies recognize Allergen Immunotherapy (AIT) treatment to have the highest potential of fully suppressing allergic diseases as results have demonstrated a sustained healthy immune response to allergens. Because of miRNAs' involvement in regulating gene expression and inflammatory pathways, there is a higher likelihood of success if they are used for diagnostic purposes and treatments. Currently, the role of miRNAs in allergic diseases suggests that targeting and upregulating certain miRNAs could potentially have overall benefits. However, as a result of the complex process of miRNA modulation, there are many challenges presented for the further development of disease-specific treatments in addition to the opportunities for growth. This review discusses the effects of epigenetics, specifically DNA methylation, on allergic diseases as well as the various roles T cells can play in regulating their development. Some root causes for severe allergic reactions and the many areas for future development of treatments for specific allergic disorders were also identified.

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RESEARCH ARTICLE

Genome Editing: Breakthroughs in Double-Strand Break (DSB) Repair and What's Next

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ABSTRACT: Genome editing has revolutionized the field of genetics, offering remarkable possibilities for advancing medicine, agriculture, and microbiology. Optimizing double-stranded break (DSB) repair mechanisms is central to enhancing genome editing efficiency, which is crucial in ensuring precise gene integration. This review focuses on classical non-homologous end joining (cNHEJ), microhomology-mediated end joining (MMEJ), single-strand annealing (SSA), and homologous recombination (HR). It evaluates the current understanding of various DSB repair pathways, highlighting their strengths, limitations, and recent advancements to improve efficiency. Overall, there has been a significant enhancement in the efficiency of all four DSB repair pathways. However, we are not at a point where genome editing can be used for routine, safe medical therapy. Of the repair pathways reviewed, MMEJ emerges as the most promising due to its balanced propensity, precision, and relatively broad applicability. Lastly, while progressions on DSB-based genome editing are made, the potential benefits of switching to an alternative genome editing strategy, such as prime editor, should also be considered, which has the potential for high precision genome editing due to the avoidance of DSB creation.

KEYWORDS: Cellular and Molecular Biology, Genetics, Genome Editing, DSB Repair.

Introduction

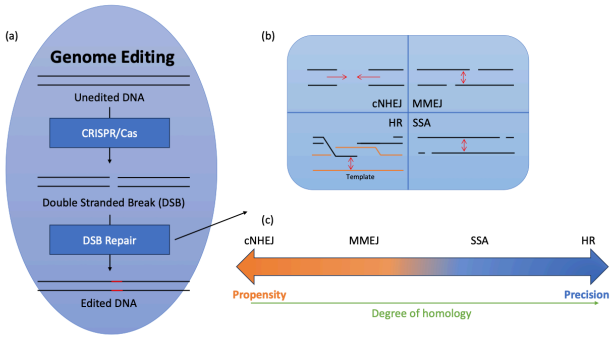

Figure 1: Graphical Abstract: (a) Overview of the process of CRISPR/Cas-based genome editing (arrows represent the proge; (b) Overview of 4 DSB repair pathways (cNHEJ, MMEJ, SSA, and HR); (c) Simplified comparison of 4 DSB repair pathways' propensity, precision, and degree of homology.

Genome editing aims to change organisms' characteristics by precisely manipulating a gene's DNA nucleotides.[1] This involves gene knock-out, gene knock-in, and single-nucleotide changes. Gene knock-out is a "loss of function mutation" in which the original gene is inserted with random DNA sequences or deleted, disrupting the gene function.[2,3] This is particularly beneficial to decipher the functions of a gene.[3,4] Gene knock-in, on the other hand, is a "gain of function mutation" that adds a new gene to an organism or replaces the original gene sequence with a correct or better-functioning allele.[5] This can be used to treat genetic disorders by correcting the mutated gene sequence.[6] Both gene knock-in and gene knock-out require the breakage of both strands of DNA. However, a single nucleotide change, also known as a point mutation or base editing, modifies a single nucleotide base by cutting only one DNA strand.[7] Single-nucleotide changes are highly beneficial in treating specific genetic mutations, offering precise and targeted therapeutic interventions.[8]

Genome editing is the cutting and gluing of DNA to create targeted changes in the genome, as illustrated in Figure 2. For DNA cleavage, sequence-specific nucleases—which include zinc-finger nucleases, transcription activator-like effector nucleases, and the clustered regularly interspaced short palindromic repeats (CRISPR)/Cas system—are used to cut the DNA and produce double-stranded breaks (DSB) at predetermined genomic sites.[9,10] These DSBs can then be glued back together through DSB repair pathways.

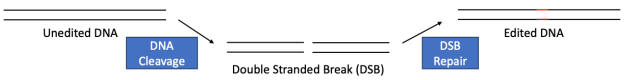

Figure 2: Simplified illustration of genome editing (arrows represent the progression of genome editing's stages): firstly, unedited DNA is cut into DSB through the process of DNA cleavage; subsequently, DSB is glued back together with the targeted edits via DSB repair.

The revolutionary power of genome editing traverses diverse biological domains, encompassing potential applications in bacteria, animals, and humans. In bacteria, genome editing has been used for industrial or medical purposes. For example, inserting the gene for human insulin into a plasmid of *Escherichia coli* bacteria cells can generate human insulin for therapeutic uses.[11] In animal trials, genome editing can serve agricultural and medical purposes. For medical purposes, genome editing techniques for treating human diseases are used on animals before human cells are used to ensure safety and efficacy.[12] For

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agricultural purposes, genome editing can be applied to expedite livestock breeding programs.[13] For example, improved meat production in Bama pigs has been achieved with genome editing.[14] Since genetic disorders are based on the fault of specific genes, there is hope that genome editing can treat genetic disorders in humans.

Despite all this potential, genome editing still faces many challenges in delivering efficient DNA editing. Efficiency depends on a precise and frequent editing of the targeted DNA sequence. For genome editing to initiate, all the biomacromolecules (such as sequence-specific nucleases and inserted template DNA sequences) must enter the cell.[1] This is particularly challenging in multicellular organisms because of the difficulty in reaching or editing every single cell necessary for changing the targeted phenotype. While nanoparticle-based delivery systems can potentially improve intracellular delivery of mRNA to the target organ and tissues, improving endosomal escape, transfect efficiency, and achieving targeted delivery remains challenging.[15,16] Another source of inefficiency is that off-target cleavage can result when sequence-specific nucleases bind to unintended genomic sites that share sequence similarity with the target site.[17] These unintended DSBs may undergo DSB repair, leading to unintended mutations or gross chromosome rearrangements. Thence, the on-target/off-target rate of DNA cleavage strongly correlates with the efficiency and safety of genome editing. Inefficiency also arises from the inclusion of unintended nucleotides during DSB repair, which can lead to the formation of indels that affect the functionality of the modified sequence.[18]

To achieve the greatest possible benefits that genome editing's potential applications can create, specific strategies and methods to improve the efficiency of the intermediate steps are necessary. Since genome editing is the cutting and gluing of DNA nucleotide sequences, DNA cleavage and DSB repair are the two most fundamentally important intermediate processes to enhance overall genome editing efficiency. Many reviews have addressed the challenges and advancements in DNA cleavage, especially in the CRISPR/Cas system.[19-21] Therefore, I will focus on the DSB repair challenges in this review.

Overview of recent genome editing:

In 1987, Clustered Regularly Interspaced Short Palindromic Repeats (CRISPRs) were unintentionally discovered in *E. coli*.[22] It wasn't until 20 years later that it was understood that bacterial cells with CRISPRs can resist virus infection and form the adaptive immune system of prokaryotes.[23,24] Meanwhile, the endonuclease Cas protein was identified, which, when working with particular single-stranded guide RNA (sgRNA), can target a specific genomic site and create a double-stranded break (DSB) by breaking the phosphodiester bonds between nucleotides.[25,26] Compared to previous DNA cleavage mechanisms, the CRISPR/Cas system was more precise and efficient at introducing DSBs.[25,27]

DSB occurs frequently in living organisms' cells due to various causes other than the cutting by an endonuclease, including mistakes in replication, ionizing radiation, chemotherapeutic agents, etc.[28] As DSB disrupts both strands of DNA if left unrepaired, it may lead to cell cycle arrest, genomic instability, cell death, and chromosomal abnormalities.[29,30] Given the deleterious nature of DSB, living organisms have a range of mechanisms to repair DSB and restore molecular function.[31]

Scientists applied and combined the natural adaptive immune system of prokaryotes, the CRISPR/Cas system, with the natural mechanisms of repairing the DSB of living organisms to develop genome editing. The four most commonly used DSB repair pathways are classical non-homologous end joining (cNHEJ), microhomology-mediated end joining (MMEJ), single-strand annealing (SSA), and homologous recombination (HR).[29] These can be seen in Figure 3.

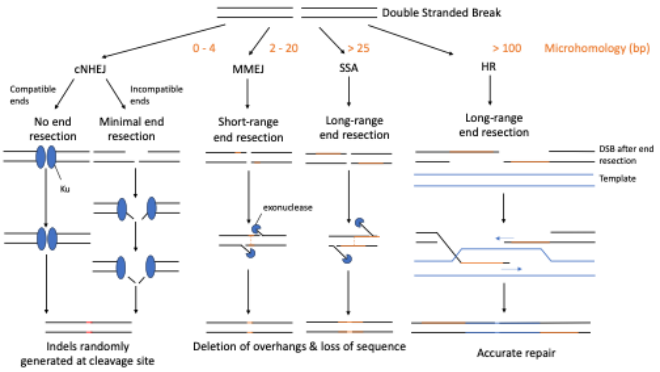

Figure 3: An overview of the four main DSB repair pathways: cNHEJ, MMEJ, SSA, and HR. cNHEJ repairs DSB (represented by black lines) regardless of microhomology (represented by orange lines), using Ku proteins (represented by blue ellipses); MMEJ and SSA repair DSB based on the annealing of microhomology (represented by orange dotted lines), using exonucleases (represented by blue ¾ circles); and HR repair DSB according to the homologous template (represented by blue lines). Black arrows indicate the progression of each DSB repair pathway. The outcomes of each DSB repair are shown at the bottom: cNHEJ – indels randomly generated at the cleavage site; MMEJ and SSA – deletion of overhangs and loss of sequence; HR – accurate repair.

cNHEJ is the dominant form of DSB repair, as it occurs with high frequency and repairs most of the DSBs formed in living organisms. This is because it is template-independent, and it can occur at any phase of the cell cycle.[18] As the name suggests, cNHEJ generally repairs DSB without needing a homologous sequence between the two exposed DNA ends of DSB; hence, it has the fastest processing of all repair pathways. However, when the broken DNA ends are incompatible, minimal end resection (cutting broken DNA ends by exonucleases) creates a low degree of microhomology (1 to 4 complementary base pairs).[32] Moreover, cNHEJ often results in the random generation of indels (unintended insertion or deletion of nucleotides) at the cleavage site. Due to its inability to maintain the original DNA sequences, cNHEJ repair is considered error-prone.[18,19,29]

DSB repairs through MMEJ and SSA depend on the annealing of homologous sequences between the two broken DNA ends of the DSB.[33,34] However, MMEJ requires a lower degree of homology (around 2 to 20 complementary base pairs), whereas SSA requires a higher degree of homology (>25 complementary base pairs).[35-37] Both MMEJ and SSA undergo DNA end resection to create sticky ends that expose

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homologous sequences as single-stranded overhangs. As a result of the DNA end resection, small sections of the original DNA sequences at the cleavage site can be deleted. Thus, these processes also fail to maintain the original gene sequences.[33,34]

HR is referred to as the error-free DSB repair pathway because, with the help of a homologous DNA template and a high degree of homology (>100 complementary base pairs), HR can precisely repair a DSB.[38] Despite the high fidelity, the HR pathways are limited to dividing cells because the required molecular components are only expressed in the S and G2 phases of the cell cycle.[39] Thus, it has low editing efficiency, and it often experiences extensive competitive pressure from cNHEJ, reducing the probability of cells undergoing HR.[40-42]

DSB repairs under molecular lenses:

Because inefficiency is present in every DSB repair pathway, I will now zoom in to the molecular level to understand where improvements can be made.

cNHEJ, as illustrated in Figure 4, is initiated as the ring-shaped protein heterodimer (Ku) detects and binds to a DSB. This protein prevents the DSB ends from extensive DNA-end resections and recruits other cNHEJ proteins to promote end ligations.[32] If the DSB has compatible end configurations, XRCC4-DNA ligase IV will bind to the Ku protein, forming a Ku-XRCC4-DNA ligase IV complex, ligating the two DNA ends. If the DSB has incompatible end configurations, cNHEJ proteins such as DNA-PKcs, Artemis, Pol μ, and Pol λ will bind to the Ku protein to create blunt ends via minimal end resections.[32] Once the DNA ends can be ligated, an XRCC4-DNA ligase IV complex will form, and the DSB will be repaired. These minimal end resections cause random deletion of DNA near the cleavage site, and free-floating nucleotides randomly attach to the exposed DNA ends, creating indels. Therefore, cNHEJ leads to mutations and error rates of up to 50%, reducing efficiency.[43]

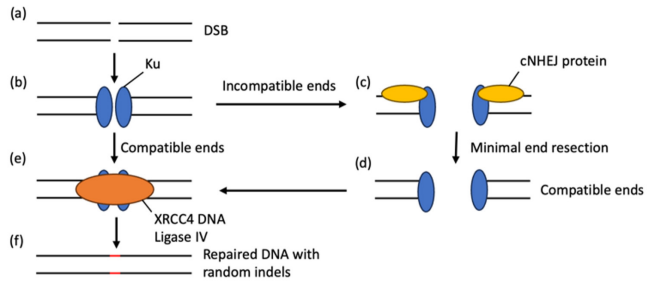

Figure 4: A simple schematic illustration of cNHEJ's molecular processes. Black arrows indicate the progression of cNHEJ. (a) A DSB, represented by black lines, is created. (b) Ku proteins, represented by blue ellipses, bind to DSB at the cleavage site. If the DNA end configurations are compatible, DSB processes to (e) in which XRCC4 and DNA Ligase IV, represented as orange ellipses, bind to Ku proteins. If the DNA end configurations are incompatible, DSB processes to (c), in which cNHEJ proteins, represented by yellow ellipses, bind to Ku proteins. (d) cNHEJ proteins undergo minimal end resection and change DNA end configurations to be compatible. Hence, DSB with compatible DNA ends undergoes (e). Finally, (f) cNHEJ completely repaired DNA, but indels, represented as red lines, are randomly generated at the cleavage site.

HR, as shown in Figure 5, is initiated by the long-range end resection of the 5' ends of the DSB site to produce 3' single-stranded DNA overhangs. This resection is carried out by a series of nucleases, including the MRN complex (MRE11-RAD50-NBS1), CtIP, EXO1, and Bloom helicase (BLM)-DNA2.[44,45] The activation of some of these key DNA end resection factors is restricted mainly to the S/G2 phases of the cell cycle, hence limiting HR's efficiency.[29] Following the extensive end resection, ssDNA overhangs are then coated by replication protein A (RPA), and eventually, the ATP-dependent DNA recombinase RAD51 replaces RPA bound onto the ssDNA overhangs, forming long helical filaments that search for a homologous sequence.[46] Once a homologous sequence is found, RAD51 facilitates strand invasion, where the ssDNA overhangs invade the template and pair with the complementary strand. This creates a displacement loop (D-loop) that starts generating new DNA nucleotides, with the help of DNA polymerases, along the 3' overhangs using the intact homologous sequence as a template.[47] This synthesis continues until enough DNA has been generated to copy the DNA nucleotide sequence of the template.

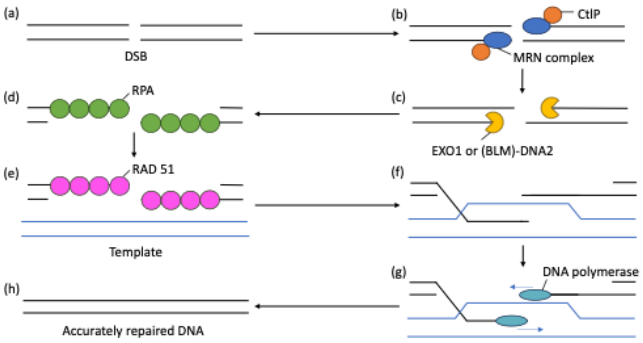

Figure 5: A simple schematic illustration of HR's molecular processes. Black arrows indicate the progression of HR. (a) A DSB, represented by the black lines, is created. (b) MRN complex and CtIP, represented by dark blue ellipses and orange circles, respectively, bind to DSB, removing DNA at the cleavage site. (c) EXO1 or (BLM)-DNA2, represented by yellow ¾ circles, replaces the MRN complex and CtIP, further removing DNA at the cleavage site. (d) After DNA end resection, RPA, represented by green circles, binds to the overhangs. (e) RAD 51, represented by pink circles, replaces RPA and searches for the homologous template, represented by blue lines. (f) DSB binds with the template according to homology. (g) DNA polymerases, represented as light blue ellipses, bind the DNA ends and repair DSB according to the homologous template. (h) Finally, HR completes with an accurately repaired DNA.

MMEJ and SSA share similar processes: end resections, annealing of microhomology, and ligation.[36,48] In particular, MMEJ resembles cNHEJ, as it involves short-range end resection, whereas SSA is more similar to HR due to its long-range end resection. According to Figure 6, both MMEJ and SSA initiate with the short-range end resection when MRN-complex (MRE11-RAD50-NBS1) and CtIP (C-terminal binding protein interacting protein) bind onto the DSB to prevent cNHEJ by removing Ku protein from the DNA ends and activate 5'-exonuclease activity, removing DNA sequences from 5' to 3' direction and generating 3' overhangs.[29,49] At this stage, the exposed microhomology suffices for MMEJ. SSA, however, requires additional steps to expose the microhomology further. Similar to the HR, exonucleases such as EXO1 or Bloom helicase (BLM)-DNA2 take over the job of MRN-complex and CtIP, continuing to remove DNA from the 5' to 3' direction

and elongating the exposed microhomology.[50,51] After end resections, MMEJ anneals the microhomology by DNA polymerase θ and fills any gaps via template-directed DNA synthesis.[29,49] Eventually, the DNA nucleotides are ligated together by DNA Ligase I and DNA Ligase III.[36] SSA, however, anneals the microhomology via RAD52: following the end resections, the resulting overhangs are bound by RPA, like HR; but unlike HR, RAD52 replaces the RPA instead of RAD51 and promotes the annealing of the microhomology.[37,52] Eventually, the DNA nucleotides are ligated together by an unidentified DNA Ligase, which some scientists have hypothesized to be DNA Ligase I.[37]

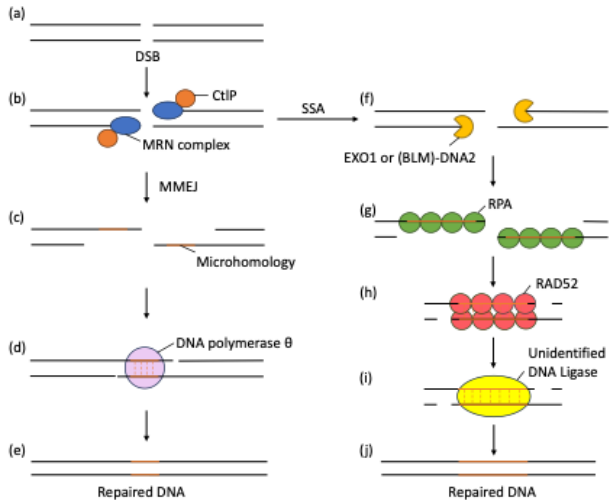

Figure 6: A simple schematic illustration of MMEJ and SSA's molecular processes. Black arrows indicate the progression of MMEJ and SSA. (a) A DSB, represented by black lines, is created. (b) MMEJ and SSA involve the attachment of the MRN complex and CtIP to DSB, as represented by blue ellipses and orange circles, which remove DNA from DSB at cleavage sites. Subsequently, MMEJ and SSA's processes separate into (c), (d), (e) and (f), (g), (h), (i), (j), respectively. (c) For MMEJ, microhomology, represented by orange lines, is exposed on overhangs after short-range end resection. (d) DNA polymerase θ, represented by pink circles, anneals the microhomology. (e) Finally, MMEJ is completed with repaired DNA. (f) For SSA, EXO1 or (BLM)-DNA2, represented by yellow ¾ circles, replace the MRN complex and CtIP, further removing DNA at the cleavage sites. (g) RPA, represented by green circles, binds to the overhangs after DNA end resection. (h) RAD 52, represented by red circles, replaces RPA and anneals microhomology. (i) Unidentified DNA Ligase, represented by a yellow circle, binds and ligases to the DSB. (k) Finally, SSA is completed with repaired DNA.

Comparison of the four DSB repair pathways:

Among the four main DSB repair pathways discussed, cNHEJ and HR were identified as the earliest and most extensively studied. cNHEJ has the least dependence on homology and the least restriction on cell phases; therefore, it occurs relatively fast and is the most frequently occurring DSB repair pathway in a cell (i.e., highest propensity). Despite having a high propensity, cNHEJ is less useful in genome editing due to its low precision. HR, on the other hand, has the greatest precision and would be preferable for genome editing. However, due to its complex mechanisms and restriction on cell phase, HR is generally slow and outcompeted by other pathways, occurring with low frequency. Therefore, among the current pathways used for genome editing, there is a trade-off between propensity and precision: the DSB repair pathway that involves more homologies receives higher precision, but its process also becomes more complex, reducing the likelihood of occurrence. Compared to cNHEJ and HR, MMEJ and SSA are more balanced in this propensity and precision trade-off, granting a more efficient approach overall. However, since SSA is also restricted to cell cycles like HR, MMEJ emerges as the more promising repair pathway for genome editing. Regarding their inefficiencies in nature, all DSB repair pathways require new strategies to improve. (Table 1)

Table 1: Detailed and comprehensive comparison of the four DSB repair pathways – cNHEJ, MMEJ, SSA, HR – over precision, propensity, degree of homology, phase dependency, and application scenarios.

	cNHEJ	MMEJ	SSA	HR
Precision	Low	Medium	Medium	High
Propensity	High	Medium	Low	Low
Degree of Homology	None	Low	Medium	High
Phase Dependency	None	Low	High	High
Application scenarios	Gene knock-out only	Both gene knock-out and gene knock-in	Both gene knock-out and gene knock-in	Both gene knock-out and gene knock-in

Possible improvements:

cNHEJ: dual-cutting for PAM-in configurations:

The CRISPR/Cas system is currently the most popular DNA editing mechanism. It often integrates with cNHEJ, known as CRISPR/Cas-based cNHEJ, to perform genome editing. According to Figure 7, the process starts with the Cas endonuclease, guided by its single-stranded RNA (sgRNA), detecting and binding to a region called Protospacer Adjacent Motif (PAM), which is a specific short sequence adjacent to the target sequence; then, it cuts the DNA at the target sequence, resulting in a DSB. According to the location of the PAM region, the two DNA ends of the DSB can be differentiated into the PAM-proximal ends and the PAM-distal ends.[19] After cleavage, the Cas endonuclease initially releases the PAM-proximal end but remains bound to the PAM-distal end for a prolonged period.[53,54] This prolonged attachment of Cas endonuclease prevents the Ku protein from binding onto the PAM-distal end, preventing cNHEJ from occurring, but it also limits the generation of indels.[54] Therefore, the precision of cNHEJ is relatively higher at the PAM-distal end than that of the PAM-proximal end, which contains the free generation of indels.[18,55]

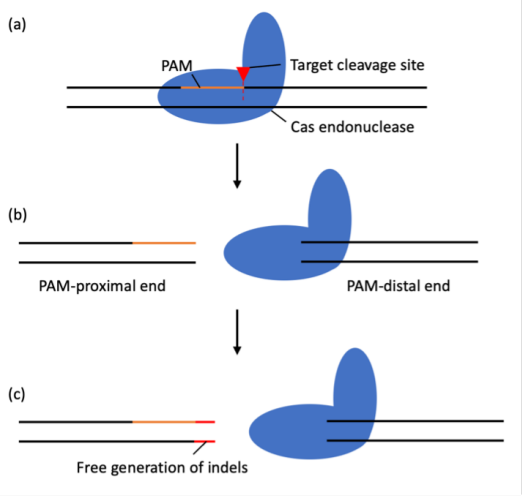

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Figure 7: A simple schematic illustration of the CRISPR/Cas system. (a) Cas endonuclease, represented by blue shapes, binds to the PAM region of DNA, represented by orange lines, and cleaves the DNA at the target cleavage site, represented by a red dotted line. (b) After cleavage, a DSB is created with a PAM-proximal and PAM-distal end, and the Cas endonuclease remains bound to the PAM-distal end. (c) As Cas endonuclease remains bound, indels are randomly generated at the PAM-proximal end, as red lines represent.

Since PAM-distal ends of the DSBs are more precise than PAM-proximal ends during CRISPR/Cas-based cNHEJ, a strategy of performing cNHEJ between two PAM-distal ends of the DSB can lead to higher precision. This is done by cleaving the DNA twice along a DNA sequence, generating two DSBs, and isolating a short sequence of DNA, which is released. Specifically, the two PAM regions from the dual cutting should be included in the target sequence through design (PAM-in), which is illustrated in Figure 8.[18,55] The two remaining ends of DSBs are both PAM-distal, which can perform cNHEJ more precisely. This strategy has produced a more precise gene knock-out in HEK 293T cells (human embryonic kidney cells) with ~79% accuracy.[18] However, this dual-cutting strategy is limited to gene knock-out, as the Cas endonuclease's attachment is too short for the inserted DNA sequence to stay attached. While restricted to gene knock-out, this dual-cutting strategy improves the precision (so as efficiency) of cNHEJ-based genome editing.

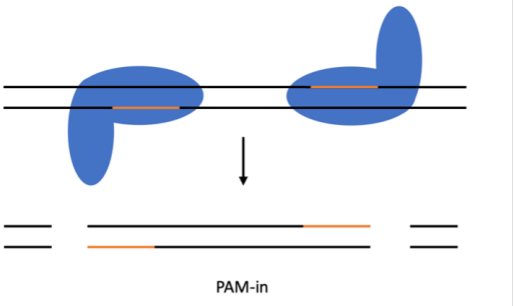

Figure 8: A simple schematic illustration of PAM-in configurations. Cas endonucleases, represented by blue shapes, bind to two PAM regions of DNA, represented by orange lines, cleaving the DNA at a certain target site. After dual cleavage, a short fragment of the DNA is isolated, which includes both PAM regions, as represented by orange lines.

HR: eliminating all its competitors:

The efficiency of HR is limited due to its low propensity, which is caused by multiple reasons. Firstly, as HR is significantly dependent on a homologous DNA template, the availability of a DNA template near the genomic cleavage site influences its efficiency. In yeast, the efficiency of HR drops by >50% when the homologous template is located more than 1 kb away from the break site.[56] Seconds, HR is limited to S and G2 phases of the cell cycle, as RPA, which is essential for recruiting RAD51; hence, HR depends on Cdk2, which has a low concentration in cells other than S and G2 phases.[57] Thirdly, HR experiences strong competition from other DSB repair pathways, especially cNHEJ, which occurs faster within the cell environment. Although the specific balance between HR and other alternative DSB repair pathways varies between species, cNHEJ always outcompetes HR. Lastly, the delivery of the large molecule of DNA template into cells also limits HDR application. Despite high precision in achieving target gene changes, HR is still far from prevalent in medical applications.

To improve the efficiency of HR, several strategies that assist HR to outcompete cNHEJ have been proposed, such as inhibiting the key components of cNHEJ, including DNA-PK and DNA Pol θ.[58,59] In addition, AZD7648 was identified as a selective DNA-PK inhibitor, which, combined with DNA Pol θ inhibitors, led to the development of the 2iHDR approach and significantly boosted the propensity of templated insertions in Jurkat cells (immobilized human T lymphocytes). Moreover, 2iHDR also reduces off-target effects of Cas9.[60] Overall, the discovery of this new treatment marked an innovative practice of combining multiple inhibitors to improve the overall efficiency of HR-based CRISPR/Cas genome editing.

MMEJ: computational algorithms:

MMEJ was once regarded as a backup DSB repair pathway of cNHEJ (alt-NHEJ), and it was not until recently that this pathway gained more attention in genome editing applications.[61,62] MMEJ is 10-fold more frequent than HR but still not as high as cNHEJ.63 Despite areas of improvement in its propensity, MMEJ's efficiency is mainly limited by its imprecision. As only a small degree of microhomology is used, the formation of indels at the exposed DNA ends may interrupt the original microhomology, leading to deletions and insertions at the cleavage side.[33] MMEJ uses microhomology between the two ends of the DSB that become exposed. Therefore, computational algorithms (such as MENTHU, inDelphi, and Lindel) were developed to assist in identifying the microhomologies and predicting the outcome of DSB repair across the genome. These computational algorithms achieved considerable success in correct prediction rates. Further improvement was obtained by combining MENTHU and Lindel to produce a new algorithm known as MENdel, which resulted in up to 90% successful prediction.[62]

SSA: microhomology elongation:

Similar to HR, the inefficiency of SSA is mainly attributed to its relatively lower propensity. Since SSA's mechanism is more complex than cNHEJ, it is outcompeted by cNHEJ to different degrees (for other cell and animal types). Moreover, like HR, SSA also undergoes extensive DNA end resection, experiencing similar limitations in cell phases. Specifically, RPA, an essential protein in recruiting RAD51 (so as extensive DNA end resection), depends on Cdk2 and has a low concentration in cells other than in the S phase. Therefore, SSA's efficiency is also limited to specific cell phases and non-dividing cells. One recent study identified that increasing the length of microhomology to over 500-2000 base pairs significantly enhances the frequency of successful SSA.[64] Thus, a strategy to improve SSA's propensity is to increase the length of microhomology, which can provide a better substrate for the annealing process, leading to greater efficiency overall.

HR & SSA: cell cycle synchronization:

As previously discussed, HR and SSA are limited to S and G2 phases. This restriction has reduced their propensity, hindering their overall efficiency. To overcome this challenge, scientists developed a strategy to synchronize the timing of

genome editing to specific cell phases (i.e., S and G2 phases) to maximize the efficiency of genome editing.[65] This is done by fusing Cas9 with the N-terminal region of human Geminin (hGem(1/110)). As a result, the Cas9 expression is synchronized with the cell-cycle progression, allowing Cas9 to be expressed at high levels during the S and G2 phases of the cell cycle, where HR and SSA are more active. Overall, this provides a potential for overcoming the limitation of the cell cycle and boosting the propensity of HR and SSA by 87%.[65]

DSB repair: Where do we go from now?:

Because cNHEJ and HR are the most studied pathways, they are still the most used DSB repair pathways in genome editing despite their low efficiency. cNHEJ's precision has been improved through a dual-cutting PAM-in approach, and HR's propensity has increased by actively inhibiting cNHEJ through the combined treatment: 2iHDR. However, they are still not ready for medical applications. For cNHEJ, the dual-cutting strategy is only viable for knock-outs; medical uses usually require knock-ins. Also, even in the same organism, different cell types vary slightly in their cellular compositions and biological mechanisms. This improvement strategy for cNHEJ has only been tested on HEK 293T cells; hence, its applicability in other cell types remains unknown. Similarly, for HR, 2iHDR has only been tested in a limited number of cell types, and its general applicability still needs to be determined. Confirming their reliability and adaptability under different cellular conditions is important for future investigations.

Regarding MMEJ and SSA, as the scientific community overlooked them in the past, they are still in the early stages of development. Although strategies such as MENdel and microhomology elongation improve the efficiency of MMEJ and SSA, respectively, their improvements are less significant compared to cNHEJ and HR's improvements via dual-cutting and 2iHDR treatment. Moreover, a strategy for controlling the timing of Cas9 expression has been developed to address the cell cycle restriction of HR and SSA. This benefits both HR and SSA by overcoming limitations in their propensity. However, concerns remain regarding their adaptability to non-dividing cells, leaving areas for future investigations. Collectively, there have been improvements in all DSB repair pathways, especially in HR. This constitutes one more step towards the ultimate goal of genome editing. However, none of these fields are yet mature, and more investigations are required in the future. I think more attention should be given to MMEJ in particular. Given the propensity and precision trade-off, DSB repair's overall efficiency can only be maximized if propensity and precision can be balanced. Hence, MMEJ and SSA deserve more focus than cNHEJ and HR. Between MMEJ and SSA, SSA's applicability is greatly restricted to dividing cells, whereas MMEJ can be applied to both dividing and non-dividing cells. Therefore, MMEJ should gain the most focus for DSB-based genomes in the future.

Primer Editing: another way of genome editing:

Despite the advancements in DSB-based genome editing, none are efficient enough for medical applications. Hence, DSB-based precise genome editing is particularly challenging and may not lead to an efficient stage. However, genome editing does not necessarily have to start with a DSB. Prime editing represents a revolutionary approach that circumvents many of the limitations of the DSB-based genome editing experience. Instead of cutting both DNA strands, prime editor, a fusion protein combining a catalytically impaired Cas9 and a reverse transcriptase, only cuts one DNA strand.[66,67] This nick in the DNA then allows the reverse transcriptase to synthesize a short strand of DNA, also known as a flap, according to the prime editing guide RNA, and replace part of the original DNA strand through a process called flap equilibration. Finally, the mismatch between the nucleotides of the edited strand and those of the original strand undergoes cellular mismatch repair to restore the complementary base pairing.[68] Compared with DSB-based genome editing, which frequently exhibits indel rates exceeding 20–30% or more, prime editing shows significantly improved precision, achieving indel rates as low as <1–10% at specific loci.[69] Moreover, prime editing exhibited much lower off-target editing compared to Cas9 nucleases at known off-target sites. For example, average off-target rates for prime editing were less than 0.1% at specific loci, while Cas9 with sgRNAs showed much higher frequencies of off-target activity, averaging from 16% to 60%.[69] Therefore, compared with DSB-based genome editing, prime editing allows for the direct and precise introduction of insertions, deletions, and substitutions without generating DSBs, circumventing the need for error-prone DSB repair pathways used in traditional CRISPR/Cas9 systems. As a result, prime editing significantly reduces the risk of off-target integrations and enhances the accuracy of genetic alterations.[70] However, prime editing also faces the risk of reverting the edits back to the unedited sequence, as the outcome of flap equilibration and cellular mismatch repair is not entirely predictable. Moreover, prime editing also faces challenges in scalability due to the complexity of delivering the larger prime editor complex and the design of prime editing guide RNAs, which requires careful optimization to ensure functionality. Overall, while the overall efficiency of prime editing is also limited, its lower indel rate and off-target rate make it a more promising tool, particularly in therapeutic settings where exact genetic corrections are critical.

Conclusion and Future Perspectives

As the scientific community continues to harness the power of genome editing, understanding the strengths and limitations of DSB repair pathways is paramount. cNHEJ, while fast and frequent, often sacrifices accuracy for speed. Therefore, it can only efficiently perform gene knock-out, where precision is not required, but not gene knock-in or other precise genetic modifications. HR, on the other hand, offers high fidelity but is constrained by its phase-specific nature and competition with faster repair pathways like cNHEJ. Hence, despite its potential for efficient and precise genetic modifications, HR's low propensity makes it an unreliable option for genome editing. Comparatively, MMEJ and SSA have a relatively balanced propensity and precision. However, given that SSA is also limited by specific cell phases, MMEJ emerges as a more promising pathway for future investigations.

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Recent advancements in the field of genome editing have focused on enhancing the overall efficiency of DSB repair pathways, yielding several promising improvements. Notably, the optimization of cNHEJ has seen developments like the dual-cutting strategy, which utilizes two Cas endonucleases to target close genomic sites, reducing the likelihood of unwanted indels at the repair site and enhancing precision for gene knockouts. In the realm of HR, the introduction of small-molecule inhibitors such as DNA-PKcs and DNA Ligase IV inhibitors has significantly improved the propensity of HR over the error-prone repair pathways. These inhibitors effectively increase the fidelity and efficiency of HR by blocking competing pathways, particularly cNHEJ. Additionally, advancements in MMEJ have included the development of computational tools like MENTHU and inDelphi, which predict the outcomes of MMEJ with high accuracy and help in designing genome editing strategies that leverage existing microhomologies. These tools enhance the practicality of MMEJ by allowing researchers to anticipate and mitigate potential errors in the genome editing process. Last, it has been suggested that increasing the length of microhomology between 500-2000 base pairs can significantly enhance SSA efficiency, providing a more effective substrate for the annealing process in genome editing.

Amid these advancements, prime editing stands out as a revolutionary alternative that bypasses the need for DSBs altogether, potentially offering a solution to the error-proneness of traditional methods. By only nicking one strand of DNA, prime editing allows for precise edits with reduced risk of unintended mutations, setting the stage for its future development and integration into therapeutic contexts.

As we look to the future, the field should pursue a dual approach: continue to refine DSB-based genome editing technologies, with a particular focus on optimizing MMEJ, while also advancing research into prime editor-based methods. This balanced approach ensures that as we improve existing technologies, we also explore new modalities that could redefine what is possible in genome editing. These developments pave the way for human health improvements via gene therapies. However, ethical considerations must be carefully examined as we approach the reality of efficiently changing genomes. Concerns regarding the equity of access, the potential for genetic discrimination, and the implications of germline editing raise significant questions about the responsible use of these powerful technologies. As such, a balance between the excitement for the bright future of genome editing and a thoughtful approach to its ethical implications is crucial to ensure these advancements are used responsibly, fairly, and for the benefit of all humanity.[71,72]

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■ RESEARCH ARTICLE

Predictive Analysis of Future Injury Using Machine Learning

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ABSTRACT: We performed predictive analysis on the athlete's physical injury by leveraging multiple machine learning algorithms with the historical features of the athlete's injury. Injury is a significant concern in professional sports. Preventing physical injury is beneficial to sustain the athlete's performance and to extend their career. Recent advances in computing technology have made significant progress in injury prevention. Unfortunately, such an application is not easy. The span of the player's physical conditions is extensive, and most importantly, the area is intensively engaged in the medical regime. Acquiring athletes' injury information is strongly restricted due to personal privacy. We hypothesized that synthetic data would be a feasible tool to elucidate the recent methodological applicability for injury prediction if the athletes' physical condition is classified with their performance. Given this assumption, we evaluated the models with various metrics and inspected which features are more important for the likelihood of future injuries. Our result shows that training intensity is the most important feature, and the average accuracy is about 0.5 regardless of the models used. Since the main goal of this study is to illustrate the capability of prediction using machine learning models, we demonstrated the whole analysis procedure, including the evaluation of results.

KEYWORDS: Biomedical and Health Sciences, Sport Injury, Machine-Learning Aid, Injury Prediction.

■ Introduction

Injury prediction is a trending topic in competitive professional sports[1] Injuries are common but can have physical, psychological, and financial impacts on the athlete's mental health and performance.[2] The prediction of its occurrence or occurrence frequency plays a crucial role in enhancing the safety and performance of athletes.[3] Various complicated risk factors are associated with injury prediction, so simple modeling cannot be implemented alone. An individual athlete's clinical conditions also broadly vary with the type of sport.[4] In addition, disclosing the players' clinical information is unfavorable, undermining the prediction's accuracy. If multi-dimensional datasets such as biomechanics, environmental conditions, and historical injury records are provided, we can make accurate predictions. However, secured predictive models should be established to identify high-risk scenarios and individuals more prone to injuries.[5] This "foreseeing ability" is a matter of preventive measures, from which specific training programs or real-time monitoring can be facilitated to prevent injury.

The application of machine learning (ML) methods, a branch of artificial intelligence (AI), is widely adopted to improve injury prediction.[6] ML offers several distinct advantages when it comes to predicting injuries. Firstly, it can analyze large and complicated data much more effectively than conventual statistical approaches by discovering unknown patterns and relationships of the variable that might not be apparent. This capability allows for the more accurate identification of risk factors and early warning signs about specific types of injuries. Secondly, machine learning models can adapt and improve over time as they get more data and learn from new observations, making them increasingly effective and precise in predicting future incidents. Thirdly, these predictive models can be applied across various domains, from sports and healthcare to industrial settings, providing specific insights and interventions to prevent injuries before they occur. Interestingly, deep learning is also broadly facilitated for desirable outcomes. While ML highly relies on algorithms to process data and make predictions, deep learning uses artificial neural networks to predict from learning from its errors.[7] Since deep learning requires much more datasets than ML, it has more computation power and can avoid overfitting.[8] However, the most impactful feature of the prediction performance of deep learning is unknown, so different metrics need to be applied for highly accurate prediction.[7]

Several reviews[7,8,10] characterized the application features of machine learning (ML) and deep learning (DL) to sports injuries. Of course, various factors influence the outcomes: sport type, the way of exercising, players' physical performance, injury nature, and so on. Unfortunately, the expected advantages are still debated, and the acquired accuracy likely remains below expectations. As mentioned, determining the high-risk factors for a solid model could be challenging since the injury is in the medical regime. Without validation of the athlete's physical information like muscle development, exercise intensity, or chronic illness, extracting the risk factors for reliable level prediction is difficult. In the worst scenario, a superficial model may be established based on unvalidated factors, such as inappropriate evaluation of an athlete's physical characteristics. This tendency could worsen further if multi-dimensional datasets of athletes' bio information are not provided. Therefore, evaluating the methodological feasibility of ML and DL before practical application is recommended. We hypothesized that well-classified injury data would be enough to evaluate the methodological applicability of the computing aid analysis.

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The term "well-classified" means that it should reflect an athlete's physical performance injury history, training intensity, recovery time, and the likelihood of the injury. These attributes should be independent of probing their contributions with the prediction models. We found a synthetic dataset to satisfy these conditions and then attempted to evaluate the predictive performance for the likelihood of future injuries using machine learning algorithms.

Methods

Data:

Table 1: The raw dataset structure with the attribute. A part of the athletes' injury data was applied in this study. This raw data describes the likelihood of injury for the athlete related to biometric information.

NO	Player_ Age	Player_ Weight	Player_H eight	Previous_I njuries	Training_Inte nsity	Recovery _Time	Likelihood_ of_Injury
0	24	66.25193 3	175.7324 29	1	0.457929	5	0
1	37	70.99627 1	174.5816 50	0	0.226522	6	1
2	32	80.09378 1	186.3296 18	0	0.613970	2	1
3	28	87.47327 1	175.5042 40	1	0.252858	4	1
4	25	84.65922 0	190.1750 12	0	0.577632	1	1

Analysis:

Our analysis aims to predict the "Likelihood of Injury" based on given historical information called features. The prediction target and features are as follows:

- Target: Likelihood of Injury
- Features: Age, Weight, Height, Previous Injury, Training Intensity, Recovery Time

The dataset is synthetic and pre-processed, so no noisy points or outliers exist. However, after mounting the data on our process notebook, we further preprocessed the datasets to clarify the application of the machine learning analysis procedure. We split the dataset into a 75:25 ratio for machine learning model training and test, and then evaluated the 25% data for the prediction. The analysis procedure is depicted in Figure 1. For each model evaluation, the divided dataset was trained and tested again.

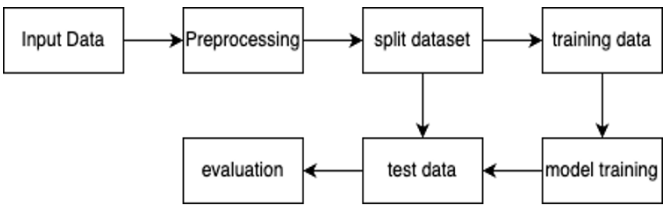

Figure 1: The procedure of analysis is depicted. The arrows indicated the flow of analysis. This sequence was utilized in the Python process applied.

First, all features were visualized to see if there were any strange data points in the distributions. Then, the correlation of features was inspected by plotting a correlation heatmap. This procedure helped us to determine if there are any features we can drop due to high correlation. If a strong correlation between the features appears, the machine learning models can learn the same information from the best-related feature. Figure 2 shows the Pearson correlation coefficient[12] among defined features and the variables for the target. The Pearson coefficient number is a measure that indicates the correlated degree of the two variables, as displayed in a color bar.

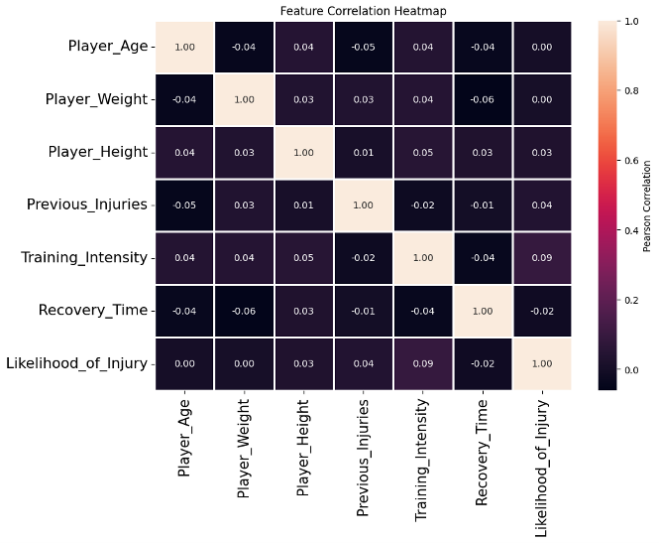

Figure 2: Correlation heatmap among features and the target (Likelihood of Injury). The values are Pearson correlation coefficients. The lighter the colors, the higher the correlation, as the color scale bar describes. Most values are less than 0.10, indicating no strong correlations between the attributes.

As shown in Figure 2, most coefficient values are lower than 0.05, implying that no highly correlated features exist, as expected. Hence, all features were applied for our analysis. We split the input data into two different sets: train and test sample sets. The training sample was implemented to train the models, while the test sample was applied to evaluate the models. This allowed us to minimize the evaluation bias since we were not using the same samples for training and testing. The splitting procedure used train_test_split from sklearn.[13] The ratio of train and test samples was set at 75:25 in stratified manners, meaning that the samples maintained the proportion of target portions in each sample. We tested the following 7 supervised models to see if there are any outperforming models:[7]

- Nearest Neighbors (NN)
- Linear SVM (LSVM)
- Naive Bayes (NB)
- Decision Tree (DT)
- Random Forest (RF)
- AdaBoost
- MLP

We selected the above models among others[7,8] because of the high-performance rate of multiple machine-learning algorithms. Some models were dropped due to technical difficulties. The default parameters are mainly used, as suggested in the example. Optimizing model parameters was challenging for this study. We propose such optimization as a future study. The confusion matrices for all models are presented in Figure 3. The values in the confusion matrices are close to 0.5 overall,

which means that the model did not learn much the features. This is expected because we did not see a high correlation between features and the target, as shown in Figure 2.

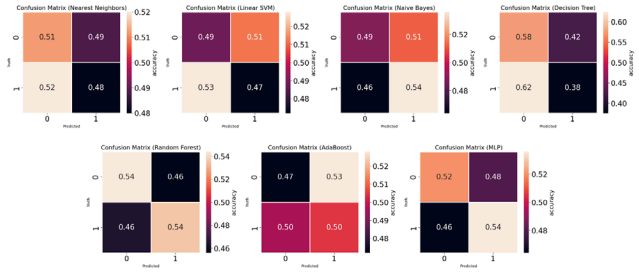

Figure 3: Confusion matrices for all models applied. The y-axis is truth information, and the x-axis is predicted values. The cell values are normalized. The values are mostly 0.50 ± 0.1-0.7, indicating 50% with 1-7% variation.

Results and Discussion

Table 2 summarizes the evaluation results using various metrics for all models applied in this study. Like the confusion matrices, the overall scores are around 0.5 with a standard deviation of ±0.025-0.58, regardless of the metrics. It also confirms our observation in confusion matrices: the models did not learn from the features. The Random Forest model shows better performance compared to others. Indeed, this trend is expected since ensemble models generally perform better. However, if we tune the model correctly, the AdaBoost and MLP would perform better than we currently see. However, tuning the model is out of the scope of this study and leaves it for our future study. The decision tree model shows poor performance compared to other models. We supposed that the training data is highly unbalanced and biased. Overall, Random Forest, MLP, and Naive Bayes perform slightly better, indicating slightly above 0.5 values.

Table 2: Summary of evaluation from the various metrics for all models applied in this study. These values completely reflect the acquired points from the confusion matrices.

Name	Accuracy	Precision	Recall	F1-Score	AUC
Nearest Neighbors	0.496	0.496	0.480	0.488	0.496
Linear SVM	0.480	0.480	0.472	0.476	0.480
Naive Bayes	0.512	0.511	0.536	0.523	0.512
Decision Tree	0.476	0.470	0.376	0.418	0.476
Random Forest	0.540	0.540	0.536	0.538	0.540
AdaBoost	0.488	0.488	0.504	0.496	0.488
MLP	0.528	0.522	0.536	0.5327	0.528
Std (each)	0.025	0.025	0.058	0.042	0.025

Figure 4 shows the importance of features for the two applied models (DT and RF), presenting the prioritization of which features are more critical for the model evaluation. This plotting was only available in those two models. Interestingly, the order of feature importance of both models is the same. The "Training Intensity" is the most utilized feature for both models' predictions. Then, the athlete's players' demographics (age, weight, height) are followed. The "Previous_Injuries" in DT is zero, meaning the feature was not used for learning. In contrast, RF shows that "Previous_Injuries" occupies a 0.025 rate.

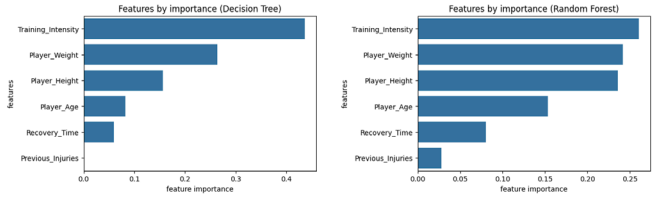

Figure 4: Feature importance of Decision Tree (DT) and Random Forest (RF). The y-axis shows the feature names, and the x-axis shows the importance score. The training intensity significantly contributes to the model prediction, but both models show a similar trend for the attributes.

Conclusion

In this study, we conducted a prediction analysis of the athlete's injury occurrence by applying various machine-learning models to synthetic data. The dataset is well-classified and strongly reflects the athlete's physical performance, including injury history, training intensity, recovery time, and the likelihood of the injury. Although the applied dataset is synthesized, its structural feature satisfied our hypothesis. We compared the performance with the possibility of injury for detailed analysis using various supervised models. The evaluation results did not appear to be well-performed. All prediction evaluated values are around 0.5, which is near 50%. We suppose this is due to the intrinsic characteristics of the synthetic data itself. The features applied for our evaluation are not highly correlated with the predicting variables, so the machine learning models could not learn valuable information from the features. This intrinsic feature is the exact characteristic we expected when choosing the synthetic data. Our study aimed to evaluate the capability of the methodological application to the athlete's injury prediction rather than examining how well machine learning aids injury prediction in predicting the injury likelihood. We found that the injury occurrence was highly relevant to the training intensity, as shown in the Pearson coefficient (Figure 2) and the contribution level plot (Figure 4). Nevertheless, the acquired accuracy of 50% does not mean machine learning aid prediction is not impractical. The review studies[7,8] exhibit that the overall performance accuracy ranges from 0.5 - 0.9 varying with factors such as type of sport, training intensity, model training method, location of injury, etc. However, the reviews addressed that implementing machining learning to injury prediction is challenging but an enabling tool to produce outstanding predictive projections from many sports-related datasets. Such technical applications could accelerate cost savings in the professional sports business because they can play a crucial role in enhancing the safety and performance of athletes. Considering this aspect, our study is meaningful because we showed how prediction analysis is generally done with various machine learning models, and this procedure can be directly applicable to actual data with similar features. From our modeling, we could prioritize the contribution of the individual features for prediction. However, the dataset should be uniformly balanced and classified to get acceptable injury prediction accuracy. This means that data collection must be

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carried out effectively using novel approaches. For example, it should be acquired by monitoring athletes' performance with highly sensitive player-worn sensors and video footage or by tracking individual athletes' biometrics with professional medical equipment and medical practitioners. This validation of the dataset is critical to making accurate predictions. Research on predicting injury occurrence is significantly committed to individual athletes' variability and appropriate training history. Time-dependent factors like fatigue, recovery rate, and training history also determine the prospective injury risk efficiency. Also, environmental factors like exercising gear and equipment, the opponent team, playing conditions, and team dynamics are considerable parameters. In this study, these effective factors were not taken into account in the original data, so our approach was not fully satisfactory. We speculate that these factors are the major contributors to the increase in uncertainty in our study. Importantly, various studies have claimed to make the correct decision on the risk factors predicted for injury occurrence.[6] However, the actual injury prediction's capability seems challenging unless the factors are compromised. Thus, as stated, we cannot determine which factors can lead to uncertainty in our approach to better strategic performance, even if we consider the intrinsic nature of our applied dataset.

Notably, at this moment, we cannot clarify how balanced data leads to reliable injury prediction at the level of our technical approach. As mentioned above, the prediction highly depends on various risk factors. We plan to investigate this issue for our future work, which will also further evaluate the different models by tuning the parameters. This proposed work would provide a better answer for the key uncertainty factors in the prospective injury predictive analysis.

Here, we can briefly narrate the characteristics of the methodological features of the applied models based on the literature that studied sports injury prediction (the script for this analysis is shared via the link 14): The details of the individual models refer to the reference.[8]

- Nearest Neighbors: easy to apply, but limited with data size and may be less accurate
- Linear SVM: as an ensemble model, applicable for high dimensional data
- Naive Bayes: simple probabilistic supervised classification with high accuracy
- Decision Tree: reasonable accuracy but limited with high dimensional data
- Random Forest: better performance accuracy but limited with high dimensional data
- AdaBoost: much better accuracy and possible with high dimensional data, compared to decision tree and random forest
- MLP: as a type of neural network, high accuracy with the capability of high dimensional data

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RESEARCH ARTICLE

Investigation of the Effectiveness of Trees as Natural VHF RF Antennas for Cosmic Ray and Neutrino Detection

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ABSTRACT: This study aimed to investigate and evaluate the effectiveness of trees as radio frequency (RF) antennas in the VHF range. The hypothesis is that a tree's structure and physical properties allow it to be an analog of RF antennas in the VHF range for neutrino and atmospheric shower detection. This paper provides and discusses the results of field tests conducted to evaluate the performance of the trees. Observations and analyses of various physical factors on which signal reception depends have also been conducted. The paper has proposed two techniques, one for measuring signal strength and the other for measuring VSWR. Two types of connection of measuring devices to the tree, nail, and induction coil were compared. The research was conducted in two stages: the first was experimental, and the second was to analyze the collected data and identify the correlations of different factors. The work results showed that the measured trees have indices very close to the dipole antenna, indicating trees' possible application in detecting neutrino particles. The readings and measurements we have collected are proper in astronomy and may also be helpful in other fields of science. Trees can also reduce the financial cost of producing commercially available neutrino detection antennas. So far, very few papers have been written on using trees to detect atmospheric showers and neutrino particles, indicating the need for further research.

KEYWORDS: Astronomy and Cosmology, Astroparticle Physics, Cosmic Rays, Neutrino Physics, Radio-detection of Neutrino, Ultra-High Energy Neutrinos.

Introduction

With the development of multi-messenger astronomy, scientists make observations using four fundamental forces: gravitational, electromagnetic, weak, and strong nuclear interaction forces; neutrino astronomy is an example of observations of the weak nuclear interaction force.[1] Neutrinos are nearly massless, chargeless particles that interact only via the weak nuclear force, allowing them to traverse cosmic distances without being absorbed or deflected, as shown in Figure 1. This unique property enables scientists to study extreme astrophysical phenomena, such as supernovae, gamma-ray bursts, and active galactic nuclei, which might otherwise remain hidden due to the limitations of electromagnetic observations.

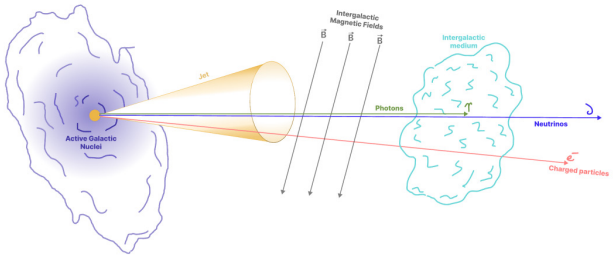

Figure 1: Cosmic ray flight from an astronomical object. Charged particles like electrons are deflected by intergalactic and planetary magnetic fields, photons are consumed by the intergalactic medium, making neutrinos a valuable astrophysical messenger.

Current neutrino detection techniques include the use of water and ice Cherenkov detectors, such as IceCube, Baikal GVD, and the Hyper-Kamiokande under construction, to detect the interaction of neutrinos with the medium (mainly ice).[2-4] Radiofrequency antennas such as ANITA, CODALEMA, TREND 50, and Tunka-Rex are also used to detect coherent radiation emitted from neutrino-atmosphere interactions.[5-8] Future projects such as GRAND and Hyper-Kamiokande are predicted to be the most significant area and volume detectors.[9] Thus, neutrino observations are becoming the most ambitious research and engineering projects.

The Ice-Cube Neutrino Observatory, located at the South Pole, utilizes more than 5,000 optical sensors embedded in a cubic kilometer of Antarctic ice to detect Cherenkov light produced through neutrino interaction with ice. Ice-Cube is known for being the first to identify a high-energy astrophysical flux, marking a major milestone in neutrino astronomy. In Russia, the Baikal-Gigaton Volume Detector (Baikal-GVD) operates in the depths of Lake Baikal and serves as one of the largest neutrino telescopes in the Northern Hemisphere. Similarly to the Ice-Cube, Baikal GVD uses strings of photomultiplier tubes to detect Cherenkov light, however, Baikal GVD aims to complement Ice-Cube by observing the opposite sky semisphere. Super-Kamiokande is a water Cherenkov detector in Japan located 1,000 meters underground, consisting of a 50,000-ton tank of ultra-pure water lined with over 11,000 photomultiplier tubes. Super-Kamiokande is highly sensitive to the lower-energy neutrinos. It has made precise measurements of solar neutrinos, helping to resolve the solar neutrino problem and confirming predictions of the Standard Solar

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Model. Collectively, these detectors play a crucial role in the current development of multi-messenger astronomy and our understanding of neutrino oscillations and interactions. However, building such detectors involves using expensive hardware that occupies a large area, resulting in time-consuming and labor-intensive work and limitations due to financial concerns.

In this paper, we present and discuss the results of an experimental approach to evaluate the feasibility of using trees as radio frequency (RF) antennas in the very high-frequency range (VHF) as a replacement for commercially available RF antennas. This work was inspired by Stephen Prohira, who suggested in his paper that trees could be a good and effective analog of antenna arrays for neutrino detection since less hardware is required and more area can be covered.[10] One of the issues raised in Stephen Prohira's paper was the lack of data on tree performance in the VHF band (30-130 MHz). Our main goal was to measure the performance of trees in the VHF band and evaluate their performance, as well as to learn about possible factors that may affect the strength of signal reception.

Neutrino Radio Detection:

The principle of operation of neutrino radio detectors is to detect radio emission of cascades of charged particles arising due to two phenomena under study - the Askaryan effect and the geomagnetic effect.[11] The Askaryan effect occurs when a high-energy neutrino interacts with a dense medium, such as ice or lunar regolith, producing a cascade of charged particles that generates coherent radio emission due to the net negative charge excess in the cascade. The geomagnetic effect results from a deflection of charged particles by Earth's magnetic field, causing them to emit waves through the synchrotron-like radiation. The astrophysical neutrino, in the case of its interaction with the atmosphere, results in the appearance of an atmospheric rain of charged particles, which in turn emit radiation similar to that of the Cherenkov, only in the radio frequency range of 10-100 MHz. Often, as a radio neutrino detector, arrays of antennas are used to cover the largest possible area and then, using the method of signal angle of arrival (AoA), reconstruct the trajectory of the neutrino and relate it to a space object.

The Tree as an Antenna:

The first mention of the idea of using trees as antennas was proposed in a paper by Major General Squire in the 1900s; in this paper, several field tests were carried out, which showed that a nail hammered at a certain height into a tree could significantly increase the strength of the received signal.[12] Further subsequent works also carried out tests using various instruments at very low frequencies (hereafter VLF), ranging from 100 Hz to 100 kHz and at higher frequencies from 500 MHz to 1.5 GHz.[13-17] The results indicated that trees, when coupled with HEMAC toroidal induction coils, outperformed conventional ground-based dipole antennas by 10–18 dBV across both low and high-frequency ranges. Additionally, shrubs operating in the 500 MHz to 1.5 GHz range demonstrated strong efficiency as both radio wave transmitters and receivers.

Due to their physical and electrical properties, trees can work as antennas and interact with electromagnetic waves. Trees are electrically conductive due to the water content inside the trunk. The electrolytes that are dissolved in water make it a fairly efficient conductor. It is assumed that wood and the water it contains work as dielectrics, interacting with electromagnetic waves, causing polarization and changes in the path of wave propagation. Also, wood has resonant frequencies at which it interacts most effectively with electromagnetic waves; these depend on the height, structure, and moisture content of the wood. Given these properties, when an electromagnetic wave passes through a tree, the current generated by the wave can be converted into a signal.[16,17]

Methods

To assess the feasibility of using trees as natural antennas for receiving and analyzing VHF signals related to neutrino and cosmic ray detection, we adopted a quantitative approach in our studies. The methods described in previous works were used to create experimental setups. In total, two types of setups were created.[13-16]

The experimental setup illustrated in Figure 2 was assembled to measure the voltage response of the tree coupled with either a brass nail or a toroidal induction coil in comparison to the dipole antenna. A tree coupled with a brass nail or toroidal conduction coil was connected to the 200MHz bandwidth KEYSIGHT MSOX2024A oscilloscope via direct connection through the oscilloscope kit's probe. KEYSIGHT MSOX2024A has a 200 MHz bandwidth, which, in combination with a 200,000 waveforms/second update rate, was highly suitable for RF measurements in the VHF range. For grounding, a steel rod with a diameter of 16 mm was used, which was buried at a depth of 50 cm and connected to the oscilloscope through a crocodile clip. The radio waves emitted by Astana's local FM stations were received by a tree-antenna and dipole at the same location. The choice to measure RF signals from local radio stations is justified by the fact that their operational frequencies correlate with the peak of Askaryan coherent RF emission produced by cascades of charged particles. Therefore, the efficiency of trees in receiving RF signals from radio stations can be extrapolated to estimate their efficiency as neutrino detectors. The recorded signals were then analyzed to evaluate the performance of trees as antennas relative to conventional dipoles. Differences between the brass nail and the induction coil were compared in the "Results" section.

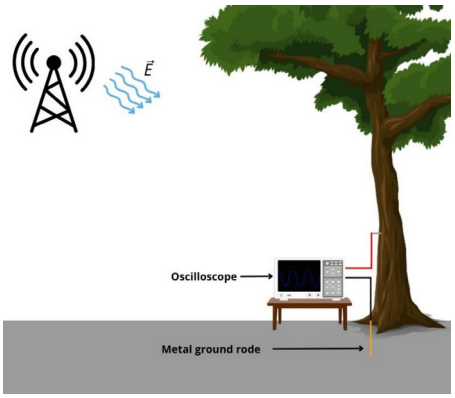

Figure 2: Experimental setup for signal amplitude measurement. FM signals transmitted by local FM stations are received by trees, and the signal strength is measured using an oscilloscope connected to a tree using either a brass nail or a toroidal induction coil.

The second setup was a similar system designed to measure the voltage standing wave coefficient (VSWR). An Amphenol coaxial cable was used for the measurements, and a Nano VNA was connected to a brass nail and ground. The Amphenol coaxial cable was calibrated using the calibration probes from the Nano VNA kit to minimize cable losses.

Before each test, measurements were taken with the meters (oscilloscope and Nano VNA) connected and disconnected from the tree to ensure that no excess signal was being received on the stylus or nail, which would distort the final results. Tests with nails and coils were conducted at different heights of the tree trunk relative to the ground to reveal a potential relationship between signal strength and nail/coil location. All measurements were carried out on different trees that differed from each other in terms of species, height, and trunk diameter (see below). To make sure that different temperatures, climates, and soil conditions do not affect our results, the tests were conducted at a specific location and under the same climatic conditions (temperature 27-32 degrees Celsius, humidity 57-59%).

The antenna designed as a reference was calculated using the dipole formula

$$L = \frac{468}{f} \times 0.3024 \quad (1)$$

Where v is the frequency of the received signal, which in our case was 100 MHz

The number of turns for an induction coil was derived from the vector circulation theorem and Faraday's law.

$$N = \frac{R}{\mu_0 a \nu \cdot \ln\left(1 + \frac{b}{r_0}\right)} \quad (2)$$

Results

Measurement of FM signals:

Figure 3 presents a graph showing the voltage level (dBV) as a function of frequency (MHz). This graph illustrates the differences in signal amplitude recorded from a tree when an oscilloscope is connected to either a brass nail or an induction coil. The results indicate that the induction coil method provides a more effective means of capturing FM signals than the nail method. The voltage amplitude detected using the coil was approximately 2-3 dBV higher than that measured using the nail. This difference remained consistent across all tested trees, suggesting that the induction coil offers a more efficient coupling mechanism for detecting FM signals from trees.

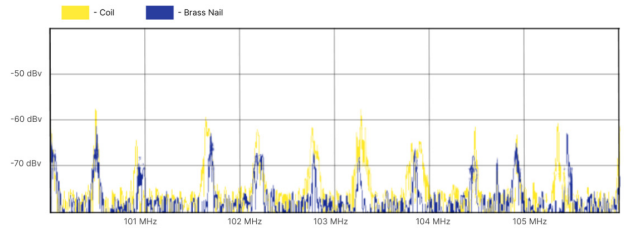

Figure 3: The graph shows a comparison of the voltage response between connection to wood using an induction coil and connection using a nail. The yellow graph represents voltage response values using a toroidal induction coil wrapped around the tree, while the blue graph represents the voltage response value using a brass nail. This graph demonstrates a significant difference between the nail and coil connection methods, indicating that the induction coil exhibits superior performance.

For comparative analysis, measurements were taken to evaluate the received signal strength from the nail and the induction coil relative to a self-designed dipole antenna, as shown in Figure 4. The dipole antenna was calibrated using a NanoVNA vector network analyzer to optimize reception in the FM radio broadcast band. According to the results, both the nail and the coil methods demonstrated acceptable performance when compared to the dipole antenna. However, Figures 4 and 5 indicate that while the induction coil approaches the signal reception capabilities of the dipole antenna, the nail connection is significantly less effective.

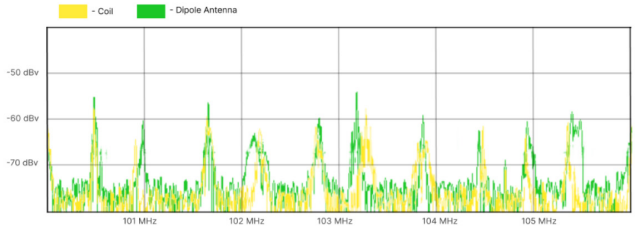

Figure 4: The graph shows a comparison of voltage response between connection to a tree using an induction coil and an antenna. The yellow graph represents voltage response values using a toroidal induction coil wrapped around the tree, while the green graph represents the voltage response value using a dipole antenna. This graph shows that the induction coil performs slightly worse than the antenna. However, the voltage response remains at a sufficient level, indicating that the induction coil can still be a viable alternative.

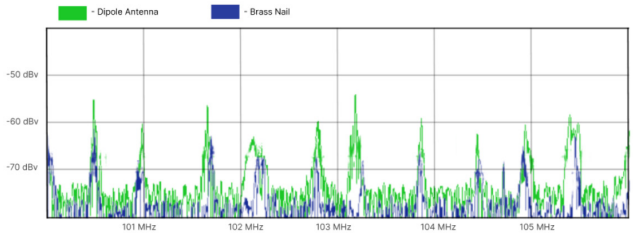

Figure 5: The graph shows a comparison of voltage response between connection to wood using a brass nail and antennas. The blue graph represents voltage response values using a brass nail as a conductor, while the green graph represents the voltage response values using a dipole antenna. This graph shows that the nail performs significantly worse than the antenna, as its voltage response is lower. This indicates that the nail cannot compete with the antenna.

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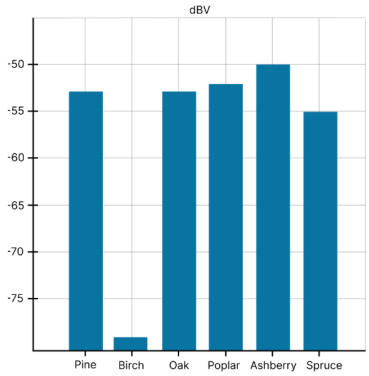

Figure 6: The bar chart represents the comparative average voltage response values among the various tree species. This chart indicates that tree species do not play a significant role, as all exhibit similar average performance. However, an anomaly is observed in birch trees, likely due to the age and humidity of most tested specimens.

In addition to measuring signal amplitude, the influence of tree species on signal reception was analyzed. Figure 6 presents a comparative bar chart of the average voltage response among different tree species. The results indicate that tree species generally do not have a significant impact on signal strength, except for birch trees, which exhibit lower amplitudes. This anomaly can be attributed to their relatively large trunk diameters. A larger trunk diameter increases the material through which the signal must propagate, leading to greater attenuation. Moreover, older trees typically have lower internal humidity, further reducing conductivity and signal transmission efficiency.

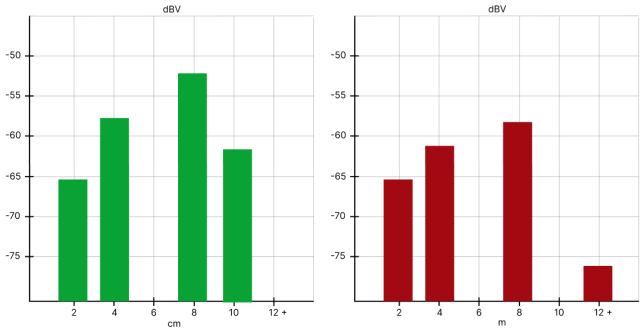

Figure 7: The green and the red bar charts are presented. Green bars represent the voltage response on trees with different radii. Red bars represent the voltage response of the trees with different heights. The graphs indicate that an optimal trunk radius is 8 cm, while the optimal trunk length is 8 meters.

Figure 7 investigates the effects of trunk radius and height on signal reception. The green bars represent voltage response as a function of trunk radius, while the red bars represent voltage response as a function of tree height. The optimal trunk radius for effective signal reception is approximately 8 cm, and the optimal tree height ranges between 3 and 9 meters. Beyond this range, either at heights below 2 meters or above 15 meters, the signal becomes significantly noisier and, in some cases, is completely lost. This suggests that tree dimensions play a critical role in determining the efficiency of signal coupling for both the nail and coil methods.

Measurement of VSWR:

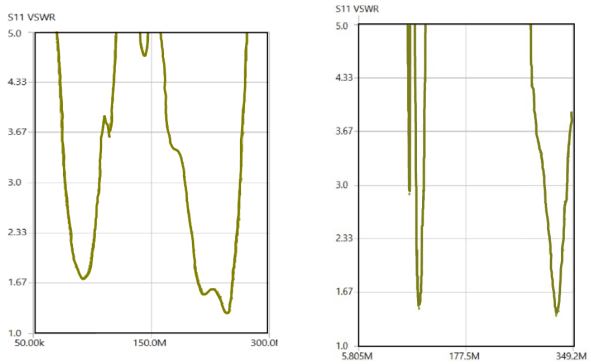

Figure 8: On the snapshot from Nano VNA, the left graph displays the tree's VSWR, and the right graph displays the antenna's VSWR. The graphs show that the tree's VSWR is higher than the antenna's, but it has a broader effective frequency range.

For a tree to function as a radio wave detector in the context of neutrino-induced radio emissions, it must exhibit a Voltage Standing Wave Ratio (VSWR) of less than 3 within the relevant frequency range. Most neutrino radio detection experiments operate within the frequency range of 1 MHz to 300 MHz.[5-8] Thus, the VSWR characteristics of the tree were analyzed over this range.

Figure 8 presents VSWR measurements for both the tree (left) and the dipole antenna (right) as recorded using the NanoVNA. The results indicate that while the tree's VSWR is higher than that of the dipole antenna, it maintains a broader effective frequency range. This suggests that, despite being slightly less efficient in terms of impedance matching, the tree may offer advantages in detecting broadband radio signals. This property could be particularly relevant in experiments aimed at detecting radio wave signatures from high-energy neutrino cascades, where broad frequency sensitivity is beneficial.

Overall, these findings support the hypothesis that trees can function as natural electromagnetic receptors, with their effectiveness being influenced by physical parameters such as trunk diameter, height, and species characteristics. Future research should focus on optimizing these natural detectors for improved performance in neutrino radio wave detection.

■ Discussion

The experimental analysis of tree-based antennas for RF signal detection highlights their potential as cost-effective, naturally occurring alternatives to conventional antennas in astroparticle physics, particularly for neutrino and ultra-high energy cosmic rays' detection. Although trees are subject to biological variability and environmental influences, the results suggest that, when properly selected and coupled, they can serve as effective electromagnetic receptors in the VHF range.

Measurements show that trees maintain acceptable performance in both signal amplitude and VSWR across a frequency range of approximately 20 MHz to 260 MHz. This range overlaps with the spectral window relevant to radio emissions produced by the Askaryan and geomagnetic effects. As seen

in Figure 8, the tree's VSWR is higher than that of a dipole antenna, yet it maintains a broader frequency range. This indicates that while trees may be less efficient in terms of impedance matching, their ability to respond across a wide spectrum could be beneficial for detecting broadband impulsive radio signals generated by high-energy particle cascades. The coupling method was found to be one of the most important factors affecting performance. Trees connected using a toroidal induction coil consistently demonstrated higher voltage responses, by about 2–3 dBV, compared to those using a brass nail. This performance increase is likely due to improved impedance matching and reduced degradation at the contact point. While the dipole antenna still produced stronger absolute signals, the induction coil-tree system yielded a stable and sufficiently strong response that may be suitable for deployment in remote or resource-limited areas. In contrast, the brass nail configuration showed significantly weaker signals, likely due to inefficient coupling, oxidation, and capacitive leakage.

Tree dimensions were shown to be a critical factor. The highest signal amplitudes were recorded for trees with a trunk radius of approximately 8 cm and a height between 7 and 9 meters, as shown in Figure 7. Trees that were significantly smaller or larger performed worse, possibly due to impedance mismatches or excess signal attenuation through the biomass. While tree species had only a minor effect overall, birch trees showed lower signal strength, likely due to their thicker trunks and lower internal moisture content. Foliage density had no consistent effect on signal amplitude or VSWR, suggesting that, in the VHF band, leaves do not contribute significantly to signal attenuation or impedance changes. This simplifies the criteria for selecting trees in field deployments. Environmental conditions during testing were typical of a temperate continental climate, with temperatures ranging from 27°C to 32°C and relative humidity between 57% and 59%. Under these moderately dry conditions, the trees still performed well. However, studies such as that of Ikraf *et al.* [15] have shown improved performance in tropical climates, where higher internal moisture content leads to better dielectric properties and signal conductivity. This suggests that tropical and subtropical regions may provide more favorable conditions for the use of tree-based antennas.

Overall, these results support the idea that trees, under optimized conditions, can serve as viable components of RF detection networks. Future research should focus on standardizing coupling methods, investigating long-term seasonal effects, and developing calibration protocols for comparing performance across species and climates. It may also be valuable to test tree antennas during actual cosmic-ray or neutrino events and explore integration into hybrid detection systems alongside traditional RF infrastructure.

While tree antennas will not surpass engineered RF arrays for high-precision monitoring campaigns, they represent a promising, innovative complement for large-scale, low-cost deployment, especially for remote or underdeveloped areas. To increase the utility of this pioneering detection strategy, some research avenues are worthy of further development. Extension of the operational frequency range to cover the HF and UHF ranges might extend their use to other astrophysical events, for example, solar radio bursts and atmospheric transients. Merging the use of tree antennas within hybrid detection systems involving conventional dipoles, log-periodic antennas, or low-noise amplifiers might extend their sensitivity at a reasonable cost. Real-time deployment as a complement to particle detectors during cosmic-ray or neutrino events might confirm their potential under real observation scenarios. Long-term monitoring on a seasonal, environmental stability basis is important to assess their long-term feasibility.

Combining them with advanced signal processing algorithms—for example, matched filtering, noise reduction, and machine learning—might extend detection limits within noisy environments. Geo-informatics data capabilities, such as satellite imaging and LiDAR, may determine optimal deployment planning and network modeling. Modeling electromagnetic coupling biophysically, while considering the physiology of the trees and environmental variability, might advance their predictability and detailed design. It is likewise important to examine the ecology of using trees as scientific infrastructure to determine their sustainability, with minimal disruption to nature. Additionally, these antennas have the potential for use within education and even citizen science applications, especially in rural, underrepresented communities, to promote wider participation in science development. Along with autonomous, solar-powered data systems and low-bandwidth telemetry, these antennas might perform as autonomous, scalable units for passive long-term scanning over vast environments.

Conclusion

In this paper, the goal was to evaluate the performance and measure the performance of trees as antennas in the VHF band, as well as to determine what factors may affect the signal reception strength. The goal was achieved using the above-mentioned solution methods. In the "Analysis of Results" chapter, the data from the tree measurements were analyzed. The obtained data showed that the performance of trees in the VHF range, although inferior to the dipole antenna, is still at a sufficient level, and the tree may well be a cheaper substitute for the antenna, depending on what conditions and what equipment it is used with. It has also been shown that certain characteristics of the tree, such as species, trunk radius, and height of the tree, affect the strength of the signal reception. During the research work, the main obstacle was the weather, which caused some experiments to be postponed.

It must also be said that with more advanced and expensive measuring devices, more accurate measurements can be achieved, with less error and more parameters for additional measurements.

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Authors

Denis Kim and Kirill Ilin are students at the Canadian International School Astana with a deep passion for physics and engineering. Kirill's interests lie at the intersection of cosmology and high-energy physics, while Denis is dedicated to aerospace engineering. Their work has garnered significant attention from scientists at the Joint Institute for Nuclear Research, leading to an invitation to collaborate in person with researchers at the Dzhelepov Laboratory. In March, Denis and Kirill won the Republican Competition of Science Projects, qualifying their project for the International Science and Engineering Fair.

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■ RESEARCH ARTICLE

Engineering Thermal Stress Resistance in Crops Using Protein Solutions and Synthetic Biology

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ABSTRACT: Climate change poses a major threat to global food security by increasing the frequency and severity of extreme temperatures, which can significantly damage crop productivity. To address this challenge, this study explores a synthetic biology-based approach to engineer thermal stress resistance in crops using protective proteins. Specifically, we investigate the roles of heat shock protein HSP70 and trehalose biosynthesis enzymes OtsA and OtsB in enhancing cellular stability under heat and cold stress. Using *E. coli* as a model system, we constructed two plasmids: pHot, a heat-inducible system for HSP70 expression, and pCold, a cold-inducible system for OtsA/OtsB expression. These plasmids were introduced into *E. coli* BL21 STAR (DE3), and protein expression was tested under thermal stress (42°C and 20°C). Western blotting and fluorescence microscopy confirmed successful expression of the target proteins, although heat-inducible dsRED expression was not detected—likely due to inefficient T2A cleavage. Cold-inducible constructs showed strong EGFP fluorescence, supporting the role of OtsA and OtsB in cold tolerance. This work provides a foundation for developing temperature-responsive genetic tools in agricultural biotechnology and informs future strategies for building climate-resilient crops.

KEYWORDS: Biomedical Engineering, Synthetic Biology, Heat, Thermal.

■ Introduction

Global agriculture faces increasing threats due to climate change, including rising temperatures, unpredictable weather patterns, and resource scarcity. These challenges have intensified food insecurity and underscored the urgent need for adaptive agricultural strategies. Econometric studies of Taiwanese agriculture, for example, highlight significant yield sensitivities to weather variability, reinforcing the importance of resilience-focused solutions.[1] Similarly, global studies, such as those modeling rice production under fluctuating temperatures and precipitation, illustrate the economic and environmental consequences of uncontrolled stressors.[2,3]

Synthetic Biology as a Solution:

Synthetic biology offers transformative solutions by engineering protein-based systems to mitigate abiotic stress. Heat shock proteins (HSPs) and trehalose biosynthesis enzymes are critical tools for stabilizing cellular structures under extreme environmental conditions. HSP70, a molecular chaperone, prevents protein aggregation during heat stress and supports protein refolding.[4] Prior research demonstrated that HSP70 plays a role in thermal tolerance across plant species, such as in cotton[5] and carrots.[6] Similarly, trehalose biosynthesis, mediated by OtsA and OtsB, mitigates cold stress by stabilizing membranes and preserving cellular integrity. Research on cold-inducible CBF genes and their regulation of osmolyte production further underscores the trehalose pathway's importance in freezing tolerance.[7]

To enhance scalability and precision in protein expression systems, this study employs an advanced plasmid-based strategy. Heat-inducible (pHot) and cold-inducible (pCold) plasmids were designed to drive targeted protein expression under thermal stress conditions in *E. coli*. These constructs enable precise regulation of HSP70, OtsA, and OtsB synthesis, ensuring controlled and efficient protein production under relevant environmental conditions.

Recent advancements, such as split intein-mediated plasmid selection systems, have significantly improved the efficiency of synthetic biology applications in microbial expression systems.[8] Studies indicate that trehalose biosynthesis pathways not only enhance cold tolerance but also influence metabolic processes that improve plant biomass accumulation.[9] The evolutionary conservation of HSP functions in cellular stress protection is further supported by studies on Theileria annulata[10] and heat shock protein mechanisms in plants.[11] By leveraging these technologies, this study validates protein expression and stability under thermal stress conditions, providing a foundation for future applications in mitigating climate-induced stress in agricultural settings.

Research Gap and Novel Contributions:

While previous studies have investigated the roles of HSP70 and trehalose biosynthesis in stress tolerance, most have focused on their natural expression in plants rather than leveraging synthetic biology-based solutions for controlled protein expression. Additionally, existing research lacks precise regulatory mechanisms for temperature-induced expression, which limits its scalability for agricultural applications. Without efficient, inducible expression systems, the potential for real-world deployment remains constrained.

This study addresses these gaps by engineering a dual-plasmid system (pHot and pCold), incorporating heat- and

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regulation of HSP70, OtsA, and OtsB expression. Unlike prior research that primarily examines trehalose biosynthesis in natural stress responses, this work harnesses synthetic biology to create a tunable, stress-responsive system. This approach builds on previous findings demonstrating trehalose's role in microbial and plant metabolic regulation, offering a more controlled and scalable platform for application in crop resilience.

Furthermore, this study bridges the gap between fundamental protein research and practical agricultural applications by using *E. coli* to validate stress-response protein expression and stability before eventual plant integration. Small heat shock proteins in *E. coli* have been shown to enhance stress resistance, reinforcing the value of microbial models for pre-screening candidate genes and regulatory systems for crop engineering. By optimizing construct design for future plant applications, this study paves the way for genome editing approaches such as Agrobacterium-mediated transformation or CRISPR/Cas9 to introduce stress resilience traits directly into crops. Research on trehalose biosynthesis genes in crops such as tomatoes and the role of HSP70 in stress and disease responses suggests further potential for genetic engineering applications in agriculture.

Methods

To evaluate the functionality and stability of heat shock proteins (HSP70) and trehalose biosynthesis enzymes (OtsA and OtsB) in mitigating thermal stress, a series of controlled experiments were conducted using *E. coli* BL21 STAR (DE3) as the expression system. The study aimed to optimize plasmid constructs, validate protein expression, and assess protein stability under controlled thermal stress conditions. The engineered plasmids (pHot for heat-inducible expression and pCold for cold-inducible expression) were designed to regulate protein synthesis in response to environmental stressors. Fluorescence microscopy and Western blot analyses were employed to confirm protein expression, localization, and cleavage efficiency. These approaches provided both quantitative and qualitative insights into the effectiveness of the constructs in driving protein production under heat and cold stress conditions.

Genomic DNA was extracted from *Pomacea canaliculata* (HSP70 source) and *Stutzerimonas stutzeri* (OtsA and OtsB sources) using a method to isolate DNA from cells. This involved breaking open the cells and using chemical reagents to separate DNA from proteins and other materials. Polymerase chain reaction (PCR) amplification of HSP70, OtsA, and OtsB genes was then performed using specifically designed primers incorporating restriction sites to facilitate plasmid integration. The amplified gene fragments were purified and inserted into the pHot and pCold expression vectors through restriction enzyme digestion and ligation, ensuring precise regulation of target protein expression. The constructs were verified via colony PCR and Sanger sequencing to confirm correct insertion and reading frame integrity.

The recombinant plasmids were transformed into *E. coli* BL21 STAR (DE3) cells, and protein expression was induced using isopropyl β-D-1-thiogalactopyranoside (IPTG) under optimized conditions. Induction parameters were set at 37°C for pHot (heat-inducible HSP70 expression) and 20°C for pCold (cold-inducible OtsA and OtsB expression), with 0.5 mM IPTG used as the optimal inducer concentration. Post-induction, bacterial cultures were harvested via centrifugation, lysed using a sonicator and buffer system, and purified using affinity chromatography. Protein purification was facilitated using His, Flag, and Myc tags to enhance specificity and improve detection efficiency in downstream assays.

To assess the functionality and stability of the expressed proteins under thermal stress, *E. coli* cultures were subjected to controlled temperature conditions. Cultures expressing HSP70 (pHot) were incubated at 42°C for 4 hours to evaluate their ability to withstand heat stress, whereas cultures expressing OtsA and OtsB (pCold) were incubated at 20°C for 16 hours to simulate cold stress conditions. Fluorescence microscopy was employed to track protein expression and localization, while Western blot analysis was conducted to evaluate protein integrity, cleavage efficiency, and expression levels under stress. Comparative fluorescence intensity measurements between treated and control groups were performed to determine protein stability and potential thermal resilience.

Quantitative fluorescence data were collected using a fluorescence microscope, allowing for the assessment of protein expression patterns and localization within *E. coli* cells. Fluorescence intensity measurements were normalized against non-induced control samples to provide a comparative analysis. Western blot analysis was used to confirm molecular weight accuracy and assess relative protein abundance across different stress conditions. Densitometry software was used to quantify Western blot bands, and statistical analyses including t-tests and ANOVA were performed to determine significant differences in protein expression levels. All experiments were conducted in triplicate to ensure reproducibility and minimize variability.

To control for confounding variables, a series of negative and positive controls was implemented. Negative controls consisted of *E. coli* cultures transformed with empty plasmids or non-induced cultures without IPTG to establish baseline fluorescence and protein expression. Positive controls included cultures expressing previously validated stress-tolerant proteins to compare expression efficiency. Experimental conditions, such as temperature variations (20°C vs. 42°C), IPTG concentration (0.1–1.0 mM), and induction duration, were carefully standardized to isolate the effects of the target proteins and minimize external variability.

The following equipment was used to ensure precision and reliability in data collection: a thermal cycler for PCR amplification, a NanoDrop spectrophotometer for nucleic acid and protein quantification, a fluorescence microscope for protein localization and expression analysis, and an SDS-PAGE and Western blot apparatus for evaluating molecular weight accuracy and protein integrity. Additionally, a shaking incubator was used to maintain bacterial cultures under controlled temperature and induction conditions, while a centrifuge and sonicator facilitated cell lysis and protein extraction.

By integrating synthetic biology-based approaches with rigorous experimental design and quantitative analysis, this study provides a systematic evaluation of protein-based thermal

stress mitigation strategies. The use of *E. coli* as a model system allows for scalable, cost-effective validation of genetic constructs, paving the way for future applications in crop engineering and environmental stress resilience.

Results and Discussion

Plasmid Transformation and Validation:

The transformation of the pHot and pCold plasmids into *E. coli* BL21 STAR (DE3) cells were successfully achieved, enabling the expression of heat-inducible HSP70 and cold-inducible OtsA and OtsB, respectively. The plasmid map for pHot (Figure 1) illustrates the inclusion of the ibpA promoter, which enables heat-inducible expression of HSP70, along with a HisTag for protein purification. Similarly, the pCold plasmid (Figure 2) features the cspA promoter for cold-inducible expression of OtsA and OtsB, with Flag and Myc tags for downstream protein analysis and tracking.

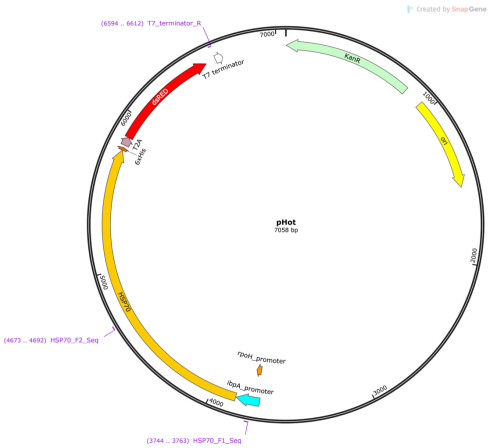

Figure 1: Plasmid map of pHot created using SnapGene Viewer. Illustrates the ibpA promoter-driven heat-inducible expression of HSP70, along with a HisTag for purification. The construct enables targeted expression under heat stress conditions.

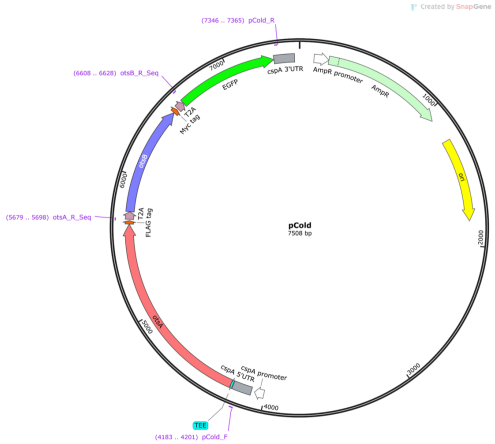

Figure 2: Plasmid map of pCold created using SnapGene Viewer. Shows the cspA promoter regulating cold-inducible expression of OtsA and OtsB, tagged with Flag and Myc, respectively. This construct facilitates protein expression under cold stress.

To confirm successful plasmid uptake, colony PCR was performed, followed by agarose gel electrophoresis. The expected fragment sizes of 2869 bp for pHot and 1516 bp for pCold were observed, confirming successful transformation. Additionally, the co-transformation of both plasmids was verified, as evidenced by the stability of bacterial colonies under selection pressure, indicating the retention of both constructs.

Colony growth patterns further validated transformation success. *E. coli* colonies harboring pHot formed distinct colonies on LB agar plates (Figure 3), demonstrating effective transformation. Similarly, transformation with pCold resulted in well-defined bacterial colonies, which were further validated using colony PCR (Figure 4). The stability of these constructs in transformed bacteria supports their potential for regulated protein expression under induced thermal stress conditions.

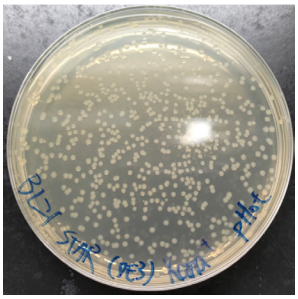

Figure 3: Colony growth on LB agar plates after transformation with pHot plasmid. Demonstrates successful transformation of *E. coli* with pHot, validated by colony PCR. The distinct colony formation indicates plasmid retention and expression capability.

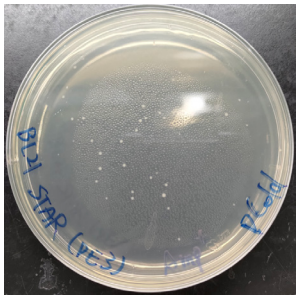

Figure 4: Colony growth on LB agar plates after transformation with pCold plasmid. Confirms successful pCold transformation in *E. coli*, verified through colony PCR. The stable colonies suggest the construct remains functional under selective conditions.

Protein Expression and Analysis:

Western blot analysis confirmed the successful expression of HSP70, OtsA, and OtsB, each tagged with His, Flag, and Myc, respectively, to facilitate detection and purification. The presence of distinct bands at the expected molecular weights validated the effectiveness of the plasmid constructs in driving protein synthesis under their respective heat- and cold-inducible conditions. Specifically, the pHot construct induced HSP70 expression at ~72 kDa (Figure 5), while pCold successfully expressed OtsA-FlagTag (~57 kDa, Figure 6) and OtsB-MycTag (~31 kDa, Figure 7) under cold-inducible conditions. These results demonstrate the specificity and efficiency of the designed plasmid systems.

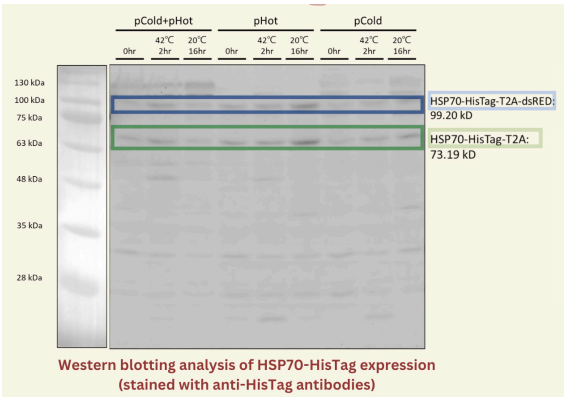

Figure 5: Western blot analysis of HSP70-HisTag protein expression in *E. coli* transformed with the pHot plasmid. HisTag was fused to the HSP70 protein to enable affinity purification and facilitate detection via anti-HisTag antibodies. Bands at ~72 kDa confirm successful expression under heat-

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inducible conditions. These results validate the pHot plasmid's ability to drive heat-inducible protein expression and demonstrate HisTag's reliability for protein identification and purification.

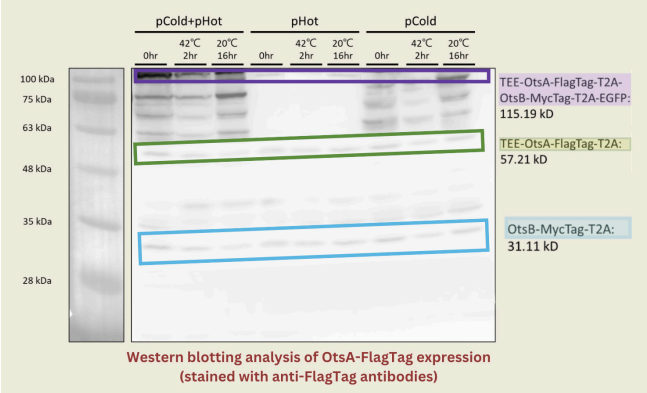

Figure 6: Western blot analysis of OtsA-FlagTag protein expression in *E. coli* transformed with the pCold plasmid. FlagTag was fused to the OtsA protein for detection using anti-FlagTag antibodies. A protein band observed at ~57 kDa confirms successful OtsA-FlagTag synthesis under cold-inducible conditions, validating the pCold plasmid's function. The precise banding pattern suggests efficient transcription and translation, with the FlagTag facilitating post-translational identification.

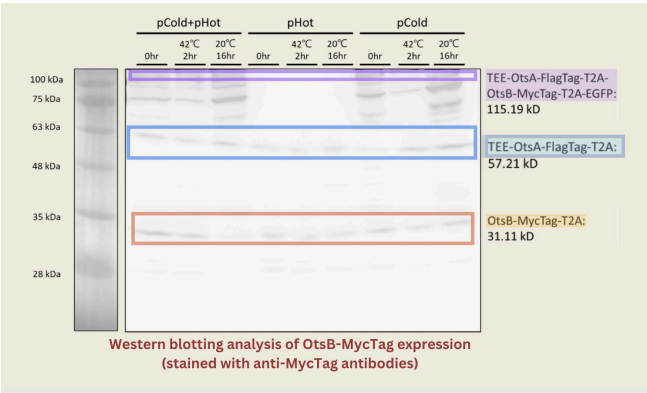

Figure 7: Western blot analysis of OtsB-MycTag protein expression in *E. coli* transformed with the pCold plasmid. The MycTag was fused to OtsB for characterization via anti-MycTag antibodies. The band at ~31 kDa confirms proper OtsB-MycTag expression under cold-inducible conditions. The observed molecular weight confirms the pCold plasmid's effectiveness in expressing OtsB, a critical enzyme in the trehalose biosynthesis pathway for cold stress adaptation.

To further confirm plasmid integrity, agarose gel electrophoresis was performed following PCR amplification of transformed *E. coli* colonies. The presence of the expected 2869 bp band for pHot (Figure 8) and 1516 bp band for pCold (Figure 9) validated the successful transformation and integration of the target genes. Minor background bands were observed in some samples, potentially indicating nonspecific amplification or residual genomic DNA contamination. However, the primary bands aligned with the expected fragment sizes, reinforcing the reliability of the cloning strategy.

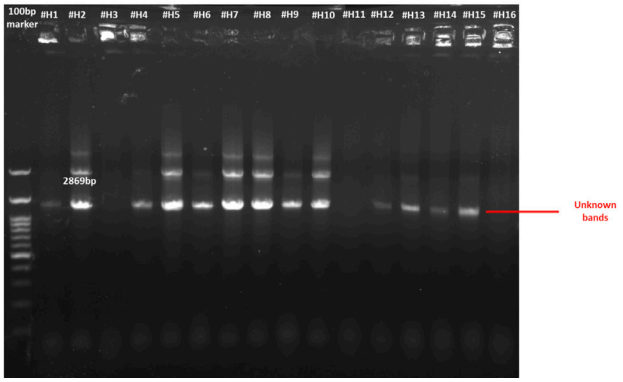

Figure 8: Agarose gel electrophoresis of PCR products for plasmid pHot validation. The expected band at 2869 bp confirms successful plasmid transformation. The presence of additional unexpected bands may be attributed to nonspecific amplification or experimental variability. Further optimization may be required to improve band specificity.

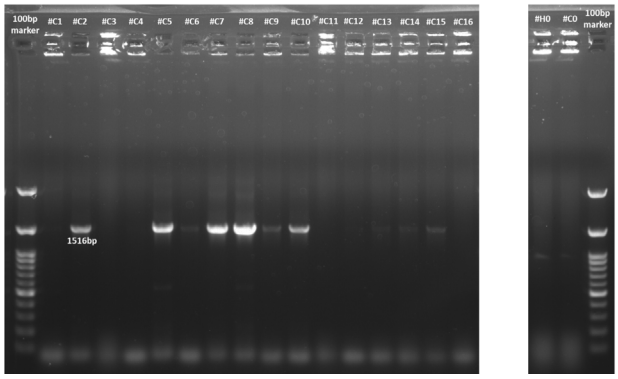

Figure 9: Agarose gel electrophoresis of PCR products for plasmid pCold validation. The presence of a distinct band at 1516 bp confirms successful plasmid transformation. Minor background bands may result from nonspecific amplification or residual genomic DNA, but the primary band aligns with the expected fragment size, supporting successful construct verification.

Fluorescence microscopy analysis provided additional insights into the protein expression profiles. EGFP fluorescence confirmed successful cold-inducible expression of OtsA and OtsB, indicating active translation and localization of trehalose biosynthesis enzymes under cold stress (Figure 10). However, dsRED fluorescence, which was intended to confirm the heat-induced expression of HSP70, was not detected. This absence suggests inefficiencies in T2A cleavage or structural challenges affecting dsRED folding and stability, necessitating further optimization.

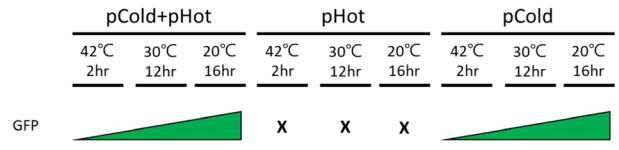

Figure 10: Fluorescence microscopy of EGFP. This shows a successful expression of EGFP under cold-inducible conditions but the absence of detectable dsRED fluorescence. There is an observed lack of prominent dsRED signal, which suggests inefficiencies in T2A cleavage or dsRED folding, requiring further construct optimization.

Thermal Stress Testing and Functional Analysis:

To assess protein stability under induced thermal conditions, *E. coli* cultures expressing HSP70 were subjected to heat stress at 42°C, while those harboring OtsA and OtsB were exposed to cold stress at 20°C for 16 hours. Western blot analysis confirmed that HSP70 maintained stability under heat stress conditions, reinforcing its role as a molecular chaperone that prevents protein denaturation and aggregation. Similarly, fluorescence microscopy revealed a significant increase in green fluorescence intensity (EGFP) under cold stress conditions, validating the cold-inducible activity of OtsA and OtsB (Figure 11).

Figure 11: Fluorescence microscopy comparison at 400X magnification. Shows the expression of fluorescent proteins under cold-inducible conditions. Samples include "pHot+pCold-20°C-16hr," "pHot-20°C-16hr," and "pCold-20°C-16hr." The relative fluorescence intensities highlight the cooperative effect of co-transformation (pHot+pCold) versus single plasmid transformations (pHot or pCold). These results demonstrate the functionality and stability of the constructs under low-temperature stress, with distinct localization patterns indicating successful protein expression and activity.

A comparative fluorescence intensity analysis across different experimental conditions provided additional insights into the effectiveness of co-transformation (Table 1). The highest fluorescence signals were observed in pHot + pCold co-transformed cultures, suggesting a cooperative effect between heat- and cold-inducible constructs in enhancing stress resilience. In contrast, cultures transformed with either pHot or pCold alone exhibited relatively lower fluorescence intensities, indicating that the presence of both constructs contributed to improved expression stability under stress conditions. The control group, which did not undergo induction, displayed minimal fluorescence, highlighting the specificity of the stress-inducible regulatory elements.

Table 1: Heat and cold stress response metrics in *E. coli*. Summarizes the stability and expression of HSP70, OtsA, and OtsB under heat and cold stress. HSP70 stability was confirmed via Western blot, while OtsA and OtsB exhibited strong EGFP fluorescence under cold conditions. The absence of dsRED fluorescence suggests inefficiencies in heat-inducible reporter expression, highlighting potential areas for optimization.

Treatment	Heat/Cold Stress Stability	Cold Fluorescence (EGFP, %)
HSP70 (Heat)	Confirmed via Western blot	-
OtsA + OtsB (Cold)	High stability under cold stress	observed
Control	Low stability under cold stress	observed

Note: "-" indicates that data was not applicable or not collected.

As summarized in Table 1, HSP70 stability under heat stress was confirmed via Western blot analysis, as dsRED fluorescence (heat-inducible) was not detected, likely due to inefficient T2A cleavage. In contrast, cold fluorescence intensity confirmed the expression of OtsA + OtsB, as these proteins were only induced under cold conditions. Notably, the control group exhibited low stability under cold stress, further reinforcing the functional significance of trehalose biosynthesis enzymes in preserving cellular integrity.

Discussion

Interpretation of Results:

This study demonstrates the effectiveness of synthetic biology-based thermal stress mitigation strategies through engineered HSP70, OtsA, and OtsB proteins in a microbial expression system. The successful expression of heat- and cold-inducible constructs validated the pHot and pCold plasmid designs, with Western blot analysis confirming target protein production at expected molecular weights. Additionally, fluorescence microscopy confirmed the expression and functionality of cold-inducible OtsA and OtsB, as evidenced by EGFP fluorescence signals under 20°C stress conditions.

However, dsRED fluorescence (heat-inducible) was not detected, suggesting potential challenges related to T2A cleavage efficiency, structural folding issues, or suboptimal expression conditions. This finding underscores the necessity for further optimization of ribosome binding sites, promoter sequences, and polyprotein expression elements to enhance the efficiency of heat-induced protein expression.

Overall, HSP70 exhibited robust expression under heat stress conditions, aligning with its established role as a molecular chaperone that prevents protein denaturation and aggregation.[4] Similarly, the stability of OtsA and OtsB under cold stress supports their function in trehalose biosynthesis, which is known to stabilize membranes and protect cellular structures from freezing damage.[7]

Comparison with Published Data:

The results align with previous studies demonstrating that HSP70 enhances thermotolerance in microbial and plant systems, such as in *E. coli* expressing plant-derived HSP70,[6] and in thermophilic bacteria expressing small heat shock proteins.[13] Additionally, the trehalose biosynthesis pathway has been established as a key regulator of abiotic stress adaptation in microbial and plant models, with Zhai *et al*. demonstrating its role in improving biomass accumulation and stress resilience in engineered crops.[9]

The lack of dsRED expression in this study is consistent with reports indicating that T2A cleavage efficiency can vary depending on sequence context, protein structure, and ribosomal stalling effects.[8] Alternative strategies, such as using separate transcriptional units or optimizing linker sequences, may improve cleavage efficiency and enhance heat-induced protein expression in future designs.

Errors and Unexpected Challenges:

A significant limitation in this study was the inefficiency of T2A cleavage, which likely impaired dsRED expression under heat-inducible conditions. Structural complexities of the T2A peptide or dsRED's inherent folding sensitivity may have contributed to this issue, requiring further optimization through alternative cleavage sequences or different fluorescent reporters such as mCherry.[13]

Additionally, fluorescence intensity variations were observed between experimental replicates. Potential factors contributing to variability include temperature fluctuations during protein induction, differences in IPTG diffusion rates, or instrumental

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sensitivity in fluorescence imaging. These challenges highlight the importance of standardized environmental controls and increased experimental replicates to minimize variability in future studies.

While this study successfully verified protein expression through Western blotting and fluorescence microscopy, it did not include densitometric quantification or fluorescence intensity measurements. This was primarily due to resource limitations during the research period. Nonetheless, the current findings establish a strong qualitative foundation, and future work incorporating quantitative analysis will help further validate and refine the conclusions presented here.

Future Research Directions:

The proposed future research directions build upon the findings of this study by optimizing construct design, expanding validation in microbial systems, incorporating advanced genetic techniques, assessing field applications, and exploring synergistic pathways. One primary improvement involves replacing the T2A sequence with independent transcriptional units or optimized cleavage sequences to enhance protein expression and functionality. Studies suggest that optimized ribosome binding sites and alternative cleavage sequences can enhance polyprotein expression efficiency.[8] Additionally, alternative fluorescent markers, such as *mCherry*, should be explored to address dsRED's potential folding issues and improve detection accuracy.[13]

To further validate the constructs, future research should extend their evaluation beyond *E. coli* to yeast or other bacterial strains, which could provide insights into their broader applicability in diverse expression systems.[6] One visual step forward is to observe whether these constructs, when transferred into yeast, still glow under cold stress or maintain HSP70 stability under heat, offering a clearer picture of cross-system reliability. Investigating the performance of these constructs under prolonged thermal stress would also be crucial in assessing their long-term stability and efficiency. Investigating the performance of these constructs under prolonged thermal stress would also be crucial in assessing their long-term stability and efficiency. This aligns with studies demonstrating that heat shock proteins and trehalose biosynthesis enzymes contribute to long-term stress adaptation in various organisms.[5,9]

Incorporating CRISPR/Cas9 technology could enable precise integration of the constructs into host genomes, ensuring stable expression and enhanced regulatory control.[14] For instance, one next step would be to use CRISPR to directly insert the cold-inducible OtsA/B genes into a test plant model, then subject the plant to cold chambers and monitor growth or fluorescence, giving real-world visual cues of success. Computational modeling and machine learning approaches may also help optimize plasmid design by predicting the ideal stress-inducible promoter efficiency and ribosomal binding site strength, which have been demonstrated to enhance metabolic regulation in engineered crops.[9]

For real-world applications, transitioning from controlled laboratory experiments to pilot-scale bioreactor studies would help assess the scalability and industrial feasibility of protein-based stress mitigation systems. Economic viability should also be evaluated to determine cost-effectiveness and production efficiency in commercial agricultural applications.[1]

Finally, exploring the potential interactions between HSP70 and trehalose biosynthesis pathways could identify additive or complementary effects in stress mitigation.[11] Studies suggest that HSP70 and trehalose biosynthesis proteins regulate cellular stress responses through different yet interconnected pathways, enhancing overall resilience to abiotic stressors.[7,12] Additionally, the co-expression of oxidative stress-resistant proteins should be investigated to further improve the resilience of engineered microbial and plant systems under extreme environmental conditions.[16] These future research directions will refine and expand the potential applications of this study, advancing scalable synthetic biology solutions for climate-resilient agriculture.

Conclusion

This study demonstrates the feasibility of utilizing synthetic biology to engineer protein-based solutions for mitigating thermal stress in microbial expression systems. The successful transformation and expression of pHot and pCold plasmids in *E. coli* confirm their ability to drive heat- and cold-inducible protein synthesis. Western blot analysis validated the expression of HSP70, OtsA, and OtsB, while fluorescence microscopy confirmed the successful localization of EGFP under cold stress conditions. These findings highlight the ability of HSP70 to enhance heat tolerance and OtsA/OtsB to stabilize cellular structures during cold stress.

Despite the successful expression of cold-inducible proteins, dsRED fluorescence was not detected under heat-inducible conditions, likely due to inefficiencies in T2A cleavage or protein folding challenges. This limitation highlights the need for further optimization of cleavage sequences or alternative fluorescent markers to improve heat-inducible reporter detection. However, the observed fluorescence intensity differences between treated and control samples confirm the functional roles of HSP70, OtsA, and OtsB in conferring stress tolerance.

This research provides a validated framework for scalable and precise protein expression systems, contributing to the advancement of biotechnological approaches for climate resilience. The study also identifies key areas for future optimization, including ribosome binding site engineering, CRISPR/Cas9 genome integration, and enhanced construct stability for long-term applications. By refining these systems, synthetic biology can pave the way for more robust agricultural and industrial applications, enabling the development of climate-resilient crops and sustainable bioengineering solutions.

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REVIEW ARTICLE

Utilizing the Therapeutic Potential of Stem Cells in Celiac Disease

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ABSTRACT: Stem cells have been one of the most significant advances in recent medicine. They are cells that can uniquely differentiate into other body cells and self-renew, unlike other cells. This review discusses how these stem cells can impact a severe gastrointestinal and autoimmune disease known as celiac disease. Many studies describe that the most common stem cells for the treatment of autoimmune and gastrointestinal diseases such as celiac disease include mesenchymal stem cells (MSCs), hematopoietic stem cells (HSCs), and induced pluripotent stem cells (iPSCs). Stem cells are a highly effective treatment for celiac disease and should be utilized to create current therapies for those suffering from this severe condition. This review paper gathers data from numerous sources to compare treatments and conclude which is the most effective. Through our research, we found that MSCs have great potential for celiac disease treatment, and they have proven to be the most effective method in the full treatment of this disease. This paper outlines how various stem cells tackle celiac disease in patients by interfering with their autoimmune response to gluten using many methods, opening up new windows of knowledge for further research..

KEYWORDS: Disease Treatment and Therapies, Disease Detection and Diagnosis, Mesenchymal Stem Cells, Celiac Disease, Hematopoietic Stem Cells.

Introduction

Celiac disease is a lifelong autoimmune condition of the small intestine that is activated by the ingestion of gluten and is widespread in genetically susceptible individuals. This disease affects millions of people around the world, and its rates are growing by around 7.5 percent every year.[1] Celiac disease is a harsh condition that impacts 1.4 percent of the world's population and can lead to catastrophic disorders such as enteropathy-associated T-cell lymphoma (EATL), which has a five-year survival rate of around 20 percent. Some common symptoms of celiac disease include abdominal distension, diarrhea, nausea, malaise, anemia, and weight loss. However, it is possible to develop more severe symptoms of this disease, such as osteoporosis, fatigue, and neurological problems. While most patients receive symptoms of this disease, around ten percent of those with celiac disease have an asymptomatic type of celiac disease and exhibit nearly no symptoms.

In patients with celiac disease, the immune system mistakenly causes inflammation in the small intestine by wrongly recognizing gluten to be a dangerous substance in the body. When a patient consumes gluten, the major histocompatibility complex HLA-DQ molecules attach themselves to gluten peptides, which then introduce themselves to CD4+ T cells, leading to an inflammatory response.[2] During this response, the continued presence of the T- and B-cells ultimately leads to the destruction of enterocytes, which then leads to villous atrophy and malabsorption syndrome.[3] Diagnosis can be made through a series of blood tests that look for anti-tissue transglutaminase and anti-endomysial antibodies since they are common in those with this disease. Another less accurate form of diagnosis would be genetic testing for human leukocyte antigens HLA-DQ2 and HLA-DQ8. Still, it can only be confirmed through an endoscopy or biopsy of the small intestine, as it allows for a more detailed view of damage done to the small intestine.[4]

Although most patients with celiac disease can be treated through a gluten-free diet, this lifestyle is difficult for many patients to maintain due to its permanent nature and the increased presence of gluten in a variety of foods. Additionally, up to five percent of those with celiac disease develop a more serious form of this disease known as refractory celiac disease, in which symptoms are prevalent despite being on a gluten-free diet. This refractory stage of celiac disease leads to a drastic increase of intraepithelial lymphocytes, which can put patients at high risk of developing enteropathy-associated T-cell lymphoma (EATL), which has an incredibly low survival rate.[2]

Although there are many traditional therapies for this disease, these do not show promising results and usually have very severe side effects. Stem cell therapy is an emerging idea that has shown encouraging results in the treatment of many autoimmune diseases, such as this one, and this paper will dive deeper into the impact of these cells on celiac disease. In many clinical trials, stem cells have shown great success in preventing villous atrophy and decreasing inflammation in patients.[5] This type of success with such severe symptoms can promote new fields of research in stem cells such as this one. This comparative literary review goes over three different types of stem cell therapy: MSCs, HSCs, and iPSCs, and it gives an overview of why MSCs are preferred over other traditional therapies and stem cell treatments. This paper will cover how celiac disease

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affects the body, the current restorative options available for treatment, and how different stem cells help tackle this illness.

Discussion

The Epithelial Barrier:

Celiac disease is a lifelong intestinal enteropathy that gets activated once a patient consumes gliadin, a glycoprotein found in gluten. Gluten is a protein found in many ingredients such as wheat, rye, barley, and triticale. The protein gliadin is made up of multiple single-chain polypeptides that are joined by intramolecular disulfide bonds.[6] Some of these peptides contain certain amino acid sequences called proline-glutamine motifs, which are resistant to gastrointestinal enzymes, making them harder to metabolize and digest.[7] In a normal person, all the peptides not containing proline-glutamine would be digested and then excreted as waste. As for the peptides containing this amino acid sequence, they would either be digested like the rest of the peptides or pass through the epithelial barrier. However, even so, their passage would be so limited that no harm would occur. On the other hand, patients with celiac disease have a much weaker capability to break down gliadin due to a damaged epithelial barrier. This means that a more significant amount of digestion-resistant peptides are likely to cross the barrier, leading to an immune reaction. Gliadin is a substrate for tissue transglutaminase (tTG) deamination, which makes the gliadin peptides more immunogenic. This deamination does not trigger an immune response in those without celiac disease. However, in those with this disease, the deaminated gliadin is able to bind to the HLA-DQ due to the damaged epithelial mucosa, leading to an inflammatory immune response.[8] Lastly, this protein also impacts the epithelial mucosa's permeability, further damaging the immune system. Gliadin is just one of the many proteins that can wreak havoc in a person's body due to the damage to the epithelial barrier since it is a crucial body function.

The epithelial barrier is a physical and chemical barrier that regulates the movement of substances across the barrier, helping to ensure that the correct nutrients are absorbed. It also protects the body from external materials that could be dangerous to the body, such as environmental toxins and microbes. This system is driven by the epithelial cells, tight and adherent junctions, and the mucus layer that all come together to help maintain the epithelial barrier's selective permeability.[9] Celiac disease attacks this system, damaging its overall function and weakening its selective permeability. Those with this condition have more pro-inflammatory cytokines such as TNF-α, IFN-γ, and IL-1β.[10] These cytokines bind to receptors on intestinal epithelial cells, triggering many intracellular signaling pathways such as the MAPK (mitogen-activated protein kinase) pathway and the NF-κB (nuclear factor-kappa B) pathway to stimulate.[11] This stimulation causes these pathways to control the phosphorylation of tight junction proteins by taking over soccludin, claudins, zona occludens (ZO) proteins, and other protein kinases responsible for this phosphorylation.[12] The pro-inflammatory cytokines can then regulate the phosphorylation of the protein kinases through the pathways, destroying the tight and adherent junctions. The intense damage done to these junctions due to this illness leads to the increased permeability of the intestinal epithelial barrier so more dangerous substances, such as gliadin and other immunogenic substances, can get through the barrier. This further damages the patient's body. Celiac disease completely damages the epithelial barrier, destroying a very crucial body function.

Intraepithelial lymphocytes (IELs) are a segment of oligoclonal T lymphocytes located within the intestinal epithelium.[13] In normal epithelial mucosa, these cells greatly help maintain the operation of this system by monitoring it for any sign of damage and destroying anything that could harm the function of this barrier. In celiac disorder, however, exposure to gluten causes the intestinal epithelial cells (enterocytes) to release increased levels of cytokine interleukin-15 (IL-15). Upon exposure to the IL-15, the CD8+ TCRαβ+ IELs enhance the expression of receptors such as NKG2D and CD94-NKG2A on their cell surface. On the other hand, the enterocytes start to display stress-related ligands, such as MHC class I-related chains (MICA and MICB) and HLA-E, as a result of the gluten-induced stress that occurs as a reaction to the consumption of gluten. The NKG2D and CD94-NKG2A receptors on the activated IELs recognize these ligands and bind to them, triggering a cytotoxic reaction that results in the apoptosis of enterocytes.[14] This process that leads to enterocyte destruction is known as direct cytotoxicity. The persistent activation of IELs through IL-15 and the stress-related ligands leads to a chronic state of IEL activation.[2] This chronic state of activation propels the proliferation of the CD8+ TCRαβ+ IEL subset. This increased amount of CD8+ TCRαβ+ IELs produces increased levels of cytotoxic effector molecules, such as interferon-gamma (IFN-γ), perforin, and granzymes.[14] These cytotoxic molecules cause apoptosis in enterocytes through Indirect cytotoxicity. This combination of indirect and direct cytotoxicity is known as the dual cytolytic effect, in which the enterocytes become targets for destruction by the IELs. The dual cytolytic effect is one of the leading causes of villous atrophy and malabsorption syndrome, which are the main factors for the rest of the symptoms of celiac disease.

Celiac disease is incredibly damaging to the intestinal mucosa's function, ruining much of the body's innate immunity. The deterioration in the innate immunity of patients with this disease is a main factor for many symptoms of this disease, such as malabsorption syndrome and abdominal distention, causing lots of distress for those with this illness. While there are some traditional treatments to help with these symptoms for celiac disease they may not all be effective.

Current and Traditional Treatments for Celiac Disease:

Due to the severity and permanent nature of the disease, celiac disease treatments and therapies are constantly being developed by scientists for patients with this illness. While the most common way celiac disease is prevented in patients is through a strict, gluten-free diet, this method does not work for all patients and is not easy to maintain. So scientists are currently trying to develop better options. There are a multitude of methods by which celiac disease can be treated, such as anti-inflammatory drugs, the blockage of cytokines, genetic modifications, and many more, which will be further explored in the later paragraphs. This body section will dive deep into

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the current and developing treatments for celiac disease and assess their effectiveness in treating patients. (Figure 1)

One method to deal with celiac disease is by using genetically modified grains that lack the immunogenic epitopes that make people sick. However, this method is very challenging as gluten contains many of these said epitopes, and they are spread across the wheat genome in the genetic loci, making it impossible to perform deletion or silencing. Gluten is a complex group of proteins found in certain grains, not a gene. It is associated with various wheat genomes, including the 42-chromosome hexaploid genomes and the 28-chromosome tetraploid genomes. All these complex genomes evolved from simpler genomes, such as the 14-chromosome diploid genomes. A majority of these complex genomes, including the tetraploids and hexaploids, contain a peptide known as the 33-mer peptide, which is the most potent trigger of the HLA-DQ molecules and plays a key role in celiac disease pathogenesis.[15] While most wheat genomes contain this peptide or those similar to it, studies show that wheat with the simple AA and BB genomes lack this type of peptide and is, consequently, much less immunogenic.[15] This research has led to exploring mRNA interference technology to reduce the immunogenic gliadin peptides in patients.[16] While all of this seems promising, this solution's challenges and side effects make it seem far-fetched. A potential risk with this treatment would be the cross-contamination of this genetically modified wheat with normal wheat, undermining the effects of the solution. Another issue with this genetically modified gluten is that the modifications cause gluten to lose its baking capabilities. These issues, however, are not very severe, and so the real problem lies in the fact that scientists do not know what the immunogenic peptides in wheat are. So, it would be nearly impossible to take out all the immunogenic components of the grain without knowing all of these elements.

Glucocorticoids, also known as corticosteroids, are a type of steroid hormone that is used mostly for severe celiac patients who suffer from refractory celiac disease or EATL.[17] These hormones bind to glucocorticoid receptors within cells in the cytoplasm, which changes the structure of these receptors, causing them to activate.[18] The activated complexes then move to the cell nucleus, where they bind to glucocorticoid response elements (GREs) DNA sequences.[19] This binding then leads to decreased inflammation in a patient's body by slowing down the production of harmful lymphokines and reducing the proliferation of T and B cells. There are many different types of glucocorticoids, such as prednisone or budesonide, which all have their own advantages. Multiple studies show that this drug when combined with a gluten-free diet, can decrease the harmful effects on the body, but at a cost. While this drug has been proven to reduce the overall apoptosis rates in cells, it has also shown very harmful side effects, such as damaging the intestinal barrier through decreased epithelial cell regeneration.[15] This damage done to the epithelial barrier severely impacts the patient's body, rendering this treatment unproductive by scientists as the side effects are as bad as the actual disease.

Interleukin-15 (IL-15) is a cytokine that promotes the activation of IELs and plays a crucial role in the immune response during celiac disease. IELs, when activated by IL-15, cause intense damage to the intestinal barrier, so blocking IL-15 is hoped to prevent the inflammatory effects from taking place.[15] Some antibodies such as AMG 714 and NZV930 that target IL-15 are being tested to block this cytokine and are proving to work at doing this but are showing some severe negative possible side effects.[20] This is because IL-15 does not only cause intestinal damage but plays a crucial role in the function of Natural Killer cells and CD8+ T cells, which help maintain immune homeostasis and fight infections. So, by blocking the IL-15, we risk ruining the cytokine's positive functions.[21] Another issue is that celiac disease is a very complicated disease that involves multiple pathways, so just blocking one cytokine is not likely to be enough to help with this disease.

Interleukin-10 (IL-10) is an immunoregulatory cytokine that helps maintain the immune response to dangerous substances and helps sustain homeostasis in the body of ordinary people. However, this cytokine is deficient in those with celiac disease, which contributes to the inflammatory immune response to gluten. Increasing the amount of IL-10 is an idea being explored by scientists, and one way they plan to do it is through recombinant human IL-10 gene therapy.[22] This boost in the anti-inflammatory effects of IL-10 seems like the perfect solution, but the side effects are very severe for this type of treatment. Some consequences of this disease include flu-like symptoms, blood pressure changes, and possible damage to the immune system.[23] With these harsh symptoms, many scientists argue that the side effects outweigh the effectiveness of the treatment.

Transglutaminase 2 (TG2) is an enzyme that catalyzes the forming of many intermolecular isopeptide bonds, such as the one between glutamine and lysine.[24] It is also responsible for the deamination of gliadin peptides, which helps generate complexes that lead to an immune response to gluten. Studies reveal that T cells are more likely to recognize TG2-treated (deaminated) gliadin over non-TG2-treated (undeaminated) gliadin. Further studies also proved that when TG2 was blocked by cystamine, the T cells were a lot less likely to cause an immune response.[15] Overall, these studies confirm that by inhibiting TG2, it is possible to prevent the deamination of gluten peptides, greatly decreasing the immune response, but the problem arrives because TG2 is not only used for deamination. TG2 performs many functions, such as wound healing, cell signaling, cell differentiation, apoptosis regulation, angiogenesis regulation, helping with vascular function, and many more.[25] The risk of damage to any of these can lead to severe effects. For example, since TG2 enzymes impact apoptosis, this blockage could cause unintended cell death due to dysregulated apoptosis. TG2 enzymes also control vascular function, and the disruption of that system could damage tissue perfusion and oxygenation, which could lead to intense or even fatal harm to a person's body. After consideration of all of these consequences, this method is not very effective.

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`	Properties					
Treatments	**Treatment type**	**Approach**	**Method**	**Consquences**	**Practicality**	**Target**
Genetically Modified Grains	Gluten Modification	To use Ancient Wheat as a replacement for modern gluten, since it is less immunogenic than the evolved, complex wheat used now. (16)	The usage of mRNA interference technology to reduce the immunogenic gliadin peptides. (17)	There is a risk of cross contamination, a loss of the baking capabilities of gluten, and a lack of knowledge of what the immunogenic peptides in wheat are. (16)	This is not very likely to be used in the near future due to lack of knowledge on what the target peptides actually are.	Gluten peptides
Glucocorticoids	Anti-Inflammatory Drug	This drug binds to receptors in the body to decrease the inflammatory effect of celiac disease. (19)	The usage of this drug combined with a gluten-free diet provides decreased inflammation in a patient's body.	This causes a decrease epithelial cell generation along with serious damage to the epithelial barrier. Also, this method only works for refractory celiac disease patients. (16)	Considering that a main goal of celiac disease treatments is to decrease the epithelial barrier damage, this method is not very practical.	Glucocorticoid receptors
IL-15 Blocking	Cytokine Blocking	To block the cytokine IL-15 since IL-15 plays a big role in the inflammatory immune response of celiac disease. (14)	The usage of antibodies such as AMG 714 and NZV930 to block the cytokine IL-15. (21)	IL-15 has other helpful functions such as its big role in the maintenance of Natural Killer cells. By blocking this cytokine, we risk harming the beneficial functions it performs through Natural Killer cells such as maintaining homeostasis and helping fight infections. (22)	Since IL-15 is just one of the many pathways impacted by this disease, the blockage of this cytokine is not likely to make a difference. Also, the damage to such crucial functions make it an ineffective solution.	IL-15 cytokine
IL-10 Increase	Cytokine Increase	To increase the amount of the immunoregulatory cytokine IL-10, which is significantly less prevalent in those with celiac disease. (16)	The usage of recombinant human IL-10 gene therapy to increase IL-10 cytokines. (23)	Flu-like symptoms, blood pressure changes, and possible damage to the overall immune system. (24)	The risk of damage to the already inpaired immune system of patients makes this treatment pointless.	IL-10 cytokines
Transglutaminase 2 Blocking	Enzyme Blockage	To block the enzyme Tranglutaminase 2 since it is a main factor in the dysregulated immune response of this disease. (25)	The usage of the complex Cytamine to block the enzyme Tranglutaminase 2. (16)	Transglutaminase 2 also plays a huge role in many positive body functions such as wound healing, cell signaling, vascular function, apoptosis regulation, and many more. By blocking this cytokine we risk damaging any of these crucial body	The serious risk of severe side effects and even fatality makes this treatment completely irrational.	Trangluta minase 2 enzymes
				functiona which could lead to severe and even fatal effects. (26)		

Figure 1: ***Overview of Current and Traditional Treatments of Celiac Disease***
This table summarizes current and emerging treatments for celiac disease, highlighting their mechanisms, limitations, and practicality. While each approach targets different aspects of the disease, all face significant challenges that limit their effectiveness or feasibility for widespread clinical use.

Overall, while traditional and developing therapies for celiac disease show some promise in helping heal patients, in most cases, the side effects greatly outweigh the benefits. These treatments are only valuable for a worst-case scenario in which a patient needs to be treated to survive. In other cases, these therapies just prove to be ineffective in treating this disease because of how complex it is, as it is with the IL-15 therapy and probably many others. However, scientists have been developing a new method that tackles the complexities of celiac disease through a precise treatment known as stem cell therapy. This therapy is currently being developed, and it utilizes the stem cells in a person's body to treat the damage done to the patient's body.

Hematopoietic Stem Cells and Induced Pluripotent Stem Cells in Celiac Disease:

Stem Cells are an emerging therapy for celiac disease and are constantly proving to give positive results with minimal side effects. There are many different kinds of stem cells, and in this section, we will go over two of the three main types of stem cells that are being used to treat this disease. These stem cells are HSCs and iPSCs, and while they are both types of stem cells, they are very different in many ways, and both have unique properties. Both stem cells can help with celiac disease in many different ways, and in this section, we will go over how each stem cell impacts those with celiac disease and determine how effective each treatment is.

HSCs are a population of multipotent stem cells that reside primarily in the bone marrow and have the ability to differentiate into all the different blood cells in the body.[26] Three main types of HSCs are classified by their differentiation and self-renewal abilities. At the top of the HSC hierarchy are the long-term HSCs (LT-HSCs). These cells have a lifelong self-renewal ability and can repopulate the entire blood system.[27] Then are the short-term HSCs (ST-HSCs), which have a limited capacity to self-renew and cannot uphold blood cell production, or hematopoiesis, for long periods of time.[28] At the end of this hierarchy are multipotent progenitors (MPPs). These HSCs can differentiate into multiple blood cells but cannot self-renew.[29] HSCs have numerous qualities that make them an effective treatment for celiac disease. For example, HSCs can activate pericryptal myofibroblasts, vascular cells, and epithelial cells, all of which help maintain the intestinal mucosa. Additionally, tests done with HSCs on different kinds of diseases reveal that it is likely to work for celiac disease, and there is a possibility that it can induce immune tolerance. Furthermore, studies show that HSCs can not only help heal the intestinal mucosa but can also help recover the immune system by making it tolerant to antigens that would usually cause an immune response in celiac disease.[30] However, HSC treatment poses a risk of death, making it only a tool to save those from refractory celiac disease.

Refractory celiac disease is confirmed by continued malabsorption syndrome and intestinal villous atrophy while on a gluten-free diet for around half a year. Two types of RCDs have been identified: type one and type two. Type one refractory celiac disease, there is an increase in IELs, but none of these IELs display any signs of abnormalities.[31] In contrast, type two refractory celiac disease does include genetic abnormalities along with changes to the T-cell receptor and the aberrant phenotype on T-cells.[32] These aberrant T-cells are very dangerous and have a high chance of helping a patient develop EATL. To counteract the damaging effects of celiac disease, regulatory T-cells are increased, but IL-15 restrains their healing abilities.[33] While the traditional treatment for refractory celiac disease includes immunosuppressive therapies and nutritional support, these treatments have shown minimal impact in type two refractory disease, which has led to the consideration of HSC treatment. There were 2 studies done with 10 patients each on HSC autologous therapy, and both studies have shown promising results.[34] In these studies, the patients had significantly fewer aberrant T cells, partial healing to the intestinal mucosa, and recovery to a normal body weight, which immensely helped with the patient's health. However, some patients still developed EATL, so it can be determined that the aberrant IELs are greatly resistant to the HSC treatment. Also, most of the patients who developed EATL died, so while this strategy is mostly effective in those with type 2 refractory disease, it is not a solution for EATL patients. Furthermore, a couple more tests were run with auto-HSCT

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on thirteen patients between 2004 and 2010. One patient died due to complications in the transplantation of the auto-HSCT, but the majority of them received some improvement in their condition; 5 of them got their immune system "reset," and their epithelial barrier showed no complications. Another one of the patients did develop EATL but survived for much longer than usual EATL patients at around 7 years.[35] Overall, this treatment can help those with stage two refractory celiac disease and can delay the fatality of EATL, but the risk of death makes it only a solution in case of extreme need. On the other hand, an (HLA)-identical matched-sibling HSC allogenic treatment was run between two patients for celiac disease, and these patients experienced a cure for their celiac disease.[34] This meant that these patient's symptoms, serological markers, and all other effects of celiac disease were gone. In one of these patients, the abnormalities in their epithelial barrier also completely disappeared, freeing them of their condition. This proves that the allogenic HSC treatment completely resets the immune system and removes the damaged cells, replacing them with the ones from the donor. Furthermore, two other celiac disease patients also went through an allogenic HSC treatment to treat their Thalassemia major disease and were completely treated for their celiac disease.[34] They were reintroduced to a gluten-containing diet and showed no side effects or signs of celiac disease after 7 years. Another child who also received this HSC treatment received similar results, and after five years, they were still on a non-gluten-free diet.[34] However, with allogenic HSC treatment, there is a risk of developing Graft-versus-host disease (GVHD), in which the patient's body thinks the donor's cells are a dangerous substance and attacks them.[36] GVHD is a serious, life-threatening disease that causes symptoms all over the body, and in HSC transplantation, patients have around 30-70 percent of getting this disease.[37] Overall, while allogenic HSC transplantation seems to work, it should only be used to treat those in life-threatening conditions due to its intense risks. However, iPSCs may not be as bad as other emerging stem cell therapies.

Another emerging stem-cell therapy is iPSCs. iPSCs are pluripotent stem cells that can be made by reprogramming somatic cells within a person's body. These stem cells have the ability to differentiate into almost every cell in the body, including the three embryonic germ layers: the endoderm, mesoderm, or ectoderm.[38] In this therapy, the somatic cells are induced by transcription factors such as Oct4, Sox2, Klf4, and c-Myc, which causes them to become pluripotent.[39] Previously, pluripotent stem cells could only be derived from human embryos, raising some ethical concerns, so changing to somatic cells is a great breakthrough in this research. With this technology, we can produce almost any cell that a patient may need, eliminating the need for immunosuppressive therapies for celiac disease.

This therapy can help with celiac disease by decreasing the damage done to the T-regulatory cells (Tregs) in this disease. Tregs help to maintain a state of equilibrium in which the immune system does not mount a damaging response to a person's body. Tregs such as CD4, CD25, and Foxp3 positive, help regulate the T cell-mediated immune response; ensuring it does not go overboard can cause autoimmunity.[39] The Tregs also help to maintain peripheral tolerance by suppressing the overactivation of T-cells. These Tregs perform these functions by secreting a variety of inhibitory cytokines, such as interleukin-10 (IL-10), transforming growth factor-beta (TGF-β), and interleukin-35 (IL-35).[40] These cytokines help suppress the proliferation of various immune cells, including T effector cells, B cells, and natural killer cells, helping maintain homeostasis and preventing a damaging immune response.[40] The Tregs are damaged in celiac disease and have fewer immunosuppressive abilities. So far, studies have shown that using autologous Tregs to suppress immune responses in multiple autoimmune diseases has proven successful. The problem arises when acquiring these Tregs since they are only visible in inflamed parts of the body.[41] Obtaining these Tregs from the inflamed site can cause unwanted inflammation in other body parts. Another issue is that it is difficult to cause the Tregs to proliferate, making it hard to gather enough Tregs to inhibit the immune response. On the other hand, it is possible to induce functional Tregs with iPSCs rather than having to collect them from the patient. Using this method, people can avoid all the complications of extracting Tregs from the patient while having the same effect. The immunoregulatory cytokines TGF-β and IL-10 were also shown in the Tregs developed by iPSCs, proving that they work the same as those accumulated from the body.[42] So far, animal studies with other autoimmune diseases have shown promising results in both autologous and allogeneic iPSC transfers.[42] This method of using iPSCs to utilize Tregs can help suppress the intense immune response in celiac disease patients. However, even with the use of iPSCs, there is a risk of tumor formation from small parts of undifferentiated cells.[43] While some scientists are trying to find ways to eliminate these undifferentiated cells, no method has shown great results for autoimmune conditions. Another risk with this method is that the reprogramming process may create abnormal Tregs, causing them to not work properly. Additionally, no tests have been done yet on celiac disease with this treatment, so while this may be a great tool in the future, it is too risky to use for a very long time, and considering its extreme side effects, it will take a while until scientists can come up with a safe way to use this so it can be implemented.[42] On the other hand, there has been much research on a new type of stem cell for celiac disease, known as MSCs.

Overall, the side effects of both of these treatments make them useless until all these consequences can be addressed, which would be nowhere in the near future. The lack of testing in iPSCs and the extreme consequences to the body in both of these treatments make them too risky to utilize. On the other hand, a new emerging stem cell therapy known as MSCs has shown promising test results with minimal side effects, making them a better option for this condition.

Mesenchymal Stem Cells in Celiac Disease:

MSCs are multipotent stromal cells with many properties and are currently being tested for celiac disease treatments.[44] These stem cells can differentiate into numerous cell types within multiple lineages, including the mesoderm, ectoderm, and endoderm lineage. MSCs are also plastic-adherent under

standard culture conditions. MSCs express CD105, CD73, and CD90 molecules but do not express CD45, CD34, CD14, CD11b, or CD19 and HLA-DR molecules.[45] These cells have a wide range of capabilities, from repairing tissues to decreasing inflammation, along with their ability to transform into multiple cell lineages, making them a valuable tool in future therapeutic research. These cells have been proven to have the same impact on the epithelial barrier as HSCs but have an advantage over them due to their greater immune abilities. MSCs lack many HLA molecules, such as CD40, CD80, and CD86, that can trigger a cytotoxic T-cell attack or cause the CD4 + T cells to activate. This makes them able to impact the immune system without the risk of a rejection response.[46] MSCs also help maintain immune tolerance through their anti-inflammatory and immunomodulatory properties. A mouse model done for colitis proved that MSCs were able to create Tregs to suppress the pro-inflammatory T-helper 1 cell (Th1).[47] This evidence proves that MSCs may influence the immune cells to make them more tolerogenic by creating a microenvironment known as a "quasiniche" through the secretion of various cells.[48] MSCs can induce immune tolerance through the paracrine release of protective substances such as indoleamine 2,3-dioxygenase (IDO), prostaglandin E2 (PGE2), nitric oxide (NO), and many more.[49] HLA-G cells are another MSC substance that helps with immune system regulation through the apoptosis of CD8+ T cells, suppressing damaging NK cell activity, increasing the amount of Tregs, and many more functions. Overall, all of these functions of MSCs make it a useful solution for the treatment of celiac disease in many ways. (Figure 2)

The epithelial barrier involves a complex interplay of many substances to maintain selective permeability within the system, but, as stated before, celiac disease damages the tight and adherent junctions of this system, decreasing its selective permeability. To find a solution for this, some traditional therapeutic methods were tested, such as larozotide acetate, but after extensive tests, these did not show significant results.[50] In a study done on a mouse model of colitis, MSCs helped heal the epithelial barrier by reassembling claudins, which are one of the most important proteins of the tight and adherens junctions.[51] Certain MSCs also proved to help reduce the increased enterocyte apoptosis rate by protecting all the stem cells from radiation. Additionally, MSCs secreting Interleukin-16 (IL-6), hepatocyte growth factor (HGF), and vascular endothelial growth factor (VEGF) caused the Fas receptor to be intercepted from reaching its ligand.[34] This is essential since when Fas connects to its ligand, it can activate the mediators caspase-3 and -8, which cause the apoptosis of enterocytes. Since the Fas receptor does not reach the ligand, the apoptotic effect is not reached. Overall, MSCs play a huge role in maintaining the function of the epithelial barrier and preventing damage to it.

Intraepithelial lymphocytes and NKs play a crucial role in the maintenance of the intestinal barrier, but the overactivation of these cells in celiac disease causes a damaging immune response. This issue causes scientists to turn to MSCs as a possible solution for this destruction. MSCs can reduce the expression of key activation receptors of natural killer cells NKp30, NKp44, and NKG2D and suppress IFN-y production, hindering NK cell cytotoxicity.[52] This inhibition of the NK cells impairs their ability to recognize and attack target cells so that the MSCs can do their job without getting killed by the NK cells. Also, although MSCs are recognized and destroyed frequently by IL-2-activated NK cells, the increased amount of IFN-y in the CD mucosa interferes with the destructive ability of these NKs. This means that the MSCs could properly function in the patient's body.[53] The MSC's impact on intraepithelial lymphocytes and NKs helps their ability to function and make an impact on the epithelial barrier.

Antigen-presenting cells play a huge role in celiac disease pathogenesis, making their regulation a significant concern for scientists. The strong binding of the HLA-DQ molecules on the dendritic cells to the deamidated gliadin peptides promotes the presentation of these peptides to CD4+ T cells.[54] MSCs can harm the monocyte differentiation into dendritic cells by a blockage in the G0 or G1 phase of the cell cycle or by the secretion of suppressive paracrine factors such as PGE2, IL-6, and monocyte-colony stimulating factor.[55] Also, the exposure of the MSCs to the fully mature dendritic cells can cause the dendritic cells to become less mature. Some ways this is confirmed is by the dendritic cells expressing less HLA class II, CD80, CD86, CD40, and CD83 molecules along with demonstrating an increase in their endocytic action.[34] This shift to more tolerogenic dendritic cells can decrease the proliferation of allogeneic T cells, which would, consequently, reduce inflammation in this disease. MSCs can immensely impact antigen-presenting cells, which helps decrease the inflammatory response in celiac disease.

T-cells are the main cells that drive the whole immune system response in celiac disease, so utilizing MSCs for this cell would greatly impact its whole function. Studies have shown that MSCs support the suppression of pro-inflammatory T-helper 1 response while skewing the T-helper 1 to T-helper 2 ratio more towards the T-helper two side.[56] Since T-helper 2 cells have anti-inflammatory effects, this would greatly help decrease the inflammation caused by the T-cells. MSCs can also inhibit the proliferation of gliadin-specific T-cells and increase the apoptosis rate for these cells. MSCs also have the ability to hinder pro-inflammatory cytokines that are directly involved in tissue injury, such as IFN-y and Interleukin-21 (IL-21).[57] All of these effects on T-cells from MSCs are done due to an enzyme known as IDO, which causes a lack of the amino acid tryptophan which is crucial for T-cell growth and activation.[58] Two other complexes that help with this T-cell MSC response are the PGE2 and NO mediators.[59] PGE2 helps with the skewing of the Th1 and Th2 ratio, while the NO mediator helps with the overall immune response of T cells. HLA-G molecules also help in the skewing of Th1 and Th2 cells and support the expansion of CD4+CD25highFoxP3+ regulatory T cells.2 Lastly, the reduction of tumor necrosing factor (TNF)-a, caused by MSCs, can prevent patients from getting a more severe form of celiac disease as there is an excess of (TNF)-a in those with refractory celiac disease.[60] Overall,

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MSCs' impact on the T-cell response may be the driving factor pushing this treatment to be further tested.

Regulatory T cells are a key asset in the modulation and maintenance of the immune response in celiac disease. The CD4+CD25highFoxP3+ T regulatory cells help achieve peripheral tolerance, helping preserve the harmless antigens outside the thymus and modulating the immune response in this area.[42] One experiment run on Crohn's disease or autoimmune enteropathy showed that a patient was successfully treated using an autologous MSC treatment. Since patients with Crohn's disease and autoimmune enteropathies experience an increase in regulatory FoxP3+ T cells in the intestinal mucosa and peripheral blood, just as do those with celiac disease, it is safe to assume that this could also work on celiac disease patients.[34] When the gliadin-specific T-cells were cultured together with MSCs, the levels of the immunomodulatory cytokine TGF-β increased, and so did the inhibition of IL-15.[61] The increase in TGF-β created modulating effects for the immune system, and the inhibition, not blockage, of IL-15, decreases the pro-inflammatory effects in this disease, showing that MSCs can use Tregs as a tool for inflammation in celiac disease.

B cells are white blood cells vital in the humoral immune response to celiac disease.[62] Intestinal plasma cells produce increased serum class A immunoglobulins (IgA) specific for gliadin and tissue transglutaminase, which plays a big factor in CD pathogenesis.[4] Studies have shown that the development of B-cells partially relies on MSCs and that these MSCs can impact the B-cell's activation and differentiation into plasma cells.[63] Also, in MSCs, IFN-γ stimulated IDOs cause the lack of the amino acid tryptophan, which is necessary for B-cell expansion.[2] The chemokines CCL2 and CCL7 secreted by MSCs bind to receptors on plasma cells, triggering a cellular signaling pathway that inhibits the STAT3 pathway.[64] By inhibiting STAT3 activity, MSCs can suppress plasma cell differentiation and immunoglobulin secretion. Overall, by decreasing B-cells' impact on the body, the MSCs help reduce the humoral immune response in CD.

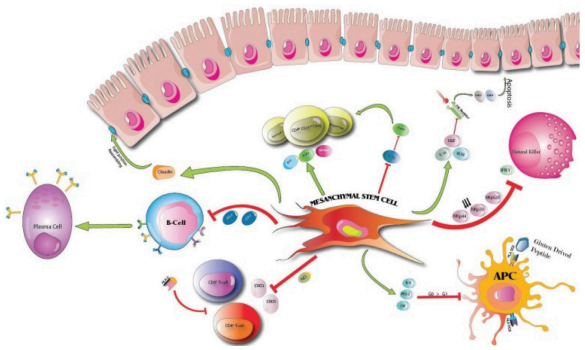

Figure 2: ***MSCs Modulation of Immune Response[2]***
This figure explains how MSCs (mesenchymal stem cells) regulate the immune response in celiac disease by interacting with immune cells like B cells and T lymphocytes Additionally, MSCs modulate claudin to maintain intestinal barrier integrity. Reproduced with permission from Moheb-Alian A *et al.* Gastroenterol Hepatol Bed Bench. 2016;9(Suppl1):S1-S7. Licensed under CC BY 4.0

Overall, MSCs have the most influence on celiac disease's immune response with minimal side effects. Unlike other stem cell therapies, MSCs impact many parts of the immune system, and they are easily accessible as they can be isolated from various adult tissues, which is a lot harder to do in other stem cell therapies. Furthermore, their exceptional differentiation and paracrine effects make them a more effective therapy than the others. Although MSCs have shown some potential side effects, such as consequences to the immune system and dysregulated differentiation, the risk of most of the side effects for MSCs is incredibly low and can be almost entirely eliminated through safety procedures. While it is true that further research and testing needs to be done before this can be implemented, considering the risk factors and the lack of success in other therapies, MSCs seem to be the most effective in curing this disease, and their incredibly high impact greatly outweighs its minimal risks.

Conclusion

As shown in this review, MSCs have incredible immune regulation abilities. They can target almost every part of the body affected by celiac disease, proving that they are the most effective treatment for this disease. While HSC transplantation poses a high risk of developing GVHD, in MSCs, this is, in fact, quite rare and can be prevented through necessary precautions. Furthermore, unlike iPSCs, MSCs have a shallow risk of tumor formation since they are not being reprogrammed like iPSCs are. Additionally, they do not pose any of the risks that traditional therapies do since they are not blocking any part of the immune system but are rather inhibiting it, eliminating the possibility of immune system dysfunction due to the blockage of particular systems. Furthermore, the low risks of MSCs and the high impact it has on the body make it a potential therapy for those with normal celiac disease. There has also been a test done on a 51-year-old woman with refractory celiac disease with MSCs, which yielded positive results as her symptoms disappeared and her intestinal mucosa was healing.[65] Also, there has been evidence that MSCs work on patients with diseases highly similar to celiac disease. For example, a 61-year-old woman with steroid-refractory adult autoimmune enteropathy and severe malabsorption syndrome was treated through an autologous MSC therapy.[66] Within a month, she lost all traces of celiac disease and also developed an increase in FoxP3+ Tregs. Furthermore, celiac disease also shares many properties with Type 1 diabetes, and MSCs have shown auspicious results in treating Type 1 diabetes, which implies that this treatment has a high chance of working in celiac disease, too.[67] However, before MSCs can be put to use, some important questions need to be addressed, such as how long the effectiveness of MSCs lasts. Due to the MSC's extensive range of immunomodulatory complexes and their ability to form a "quasiniche," they do not need to remain in the patient's body for long. Also, it is beneficial to mention that there have been no biological differences concerning which tissue the MSCs have been derived from. However, the invasiveness of this method and the potential constraints of gathering it from the bone marrow have led to the idea of gathering

placenta, and many more. These treatments will likely be the most impactful due to their increased proliferative capacities and high tolerogenic properties. Furthermore, before MSCs can be truly put to use, many things need to be worked out, like the standardization of the procedure and the dosage at which this will work.

Provide a summary of the results of your review concisely. Wrap up your review by drawing everything together and making sure it is clear what conclusions you draw about your topic or field of study based on the research studies you read and analyzed. This can include making suggestions for future research on the topic as part of your conclusion.

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Suhani is a young author interested in possibly pursuing biotechnology in the future and was inspired to write about celiac disease due to her mother's complications with this condition.

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REVIEW ARTICLE

Are Sports Endorsements Beneficial to the Sponsoring Firm?

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ABSTRACT: This review paper aims to determine whether sports endorsements are truly beneficial to the sponsoring firm by comparing and contrasting various prior studies. Relevant literature was identified to examine sponsorships at three levels—athlete, team, and event—and the findings are consolidated. The results show that the value of sports endorsements is based on multiple factors, including the athlete's reputation, how connected the sponsor is to the particular sport (congruence), the characteristics of the sponsoring firm, and the expectations of potential winners. Congruence is determined to be the most important factor, with congruent firms facing more extreme effects. Winning is proven to positively affect the returns for the sponsor, while the size and type of sporting event seem to differ significantly in sponsorship value. This synthesis highlights that the return on investment on these sponsorships can be high, but they are case-specific.

KEYWORDS: Behavioral and Social Sciences, Economics, Sports Economics, Marketing, Endorsements.

Introduction

As of 2023, the value of the sports sponsorship market worldwide exceeded $100 billion, and this figure is projected to double by 2030, making it a highly significant aspect of marketing. These sponsorships have become a pervasive strategy for firms that aim to use them to improve their brand image, credibility, and visibility.[1] This recent gain in popularity can be attributed to sports having a captive audience and needing to be consumed live, meaning that viewers cannot skip advertisements.[2] Moreover, the emotional investment in sports is high, and athletes are influential, making the audience more receptive to on-screen endorsements.[3] Hence, the volume of sports sponsorships is increasing, causing economists to question their true value to endorsers.

This question resonates with me, and I have chosen to explore it in this paper. My motivation to write on this topic stems from my personal on-court experiences in sports. I've been a competitive tennis player in South India for the last 5 years, and I am a multiple-time awardee at All India Tennis Association (AITA) tournaments, which improved my national ranking. While I had continued exposure to viewing it from an athlete's perspective, I had never considered sponsorships from a firm's point of view. As a result, I became curious about these effects as I traveled for tournaments. This curiosity, coupled with my ever-growing interest in economics, led me toward the aim of determining whether endorsements in sports are truly beneficial to the sponsor.

To determine this, I review and summarize various papers, answering similar questions and consolidating my findings. Through this, it explores varying levels of sports sponsorships - including athlete, team, and event- to better understand the impacts at each stage. These results are then tied together to form a holistic view, helping emphasize the similarities and differences across the three levels. Results indicate that many factors affect the value of sports endorsements. Of these, the one that makes the most significant difference is the connectedness (congruence) between the sponsor and the event, with more congruent firms tending to receive greater returns on their sponsorships. Another important factor is winning, with successful athletes and teams being more valued by the market than unsuccessful ones. Meanwhile, an element of contrast highlighted across the papers is the impact on the market of the expected winner winning an event differing based on the sport. Thus, we can conclude that determining the value of sports endorsement is complex.

Discussion

The paper's organization is as follows: This section contains the literature review, which describes six papers studying the impacts on various sponsorship levels, followed by the conclusion.

Athlete Endorsements:

This subsection explores the financial partnership, not just ambassadorship, between an athlete and a brand by examining three cases focused on Tiger Woods.

A study by Farrell *et al*. uses analysis of athlete performance and the value of endorsing firms following the endorsement to find the economic impact of sports sponsorships, focusing on Tiger Woods.[4] Firms often regard celebrity endorsements as efficient marketing methods due to celebrities having high influence, credibility, and attention. The celebrity's association with the firm leads to the hypothesis that it will positively impact its value. This then ties into the idea of exploring how these celebrity endorsements affect consumer behavior.

Tiger Woods was an immediate sensation in the world of golf, creating a legacy that set him apart. He became an endorser of major firms, including Nike, Titleist, American Express, etc. Hence, he became the prime candidate for performing this analysis. Golf is also an individual sport, minimizing the effect of any confounding variables. Moreover, the nature of the sport is such that if the athlete is in contention to win, they receive

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increased media coverage. This helped establish a clear relationship (or lack thereof) between athlete performance and the endorsing firms' value. This study primarily focuses on Nike's endorsement of Woods, aiming to analyze whether there is a direct correlation between Woods' performance and Nike golf ball sales and to determine whether the sponsorship is economically beneficial to Nike.

An event study method is employed to show the effect of certain events, in this case, Woods' tournament performance and sponsorship announcements, on stock prices. This is done by measuring the excess/abnormal returns, which is the deviation from the expected returns that a firm would receive based on prior stock price trends. These abnormal returns serve as the metric to quantify the net benefit for endorsing firms, varying based on how an event is perceived by the market. That is, if the market views Woods' tournament performance favorably, the abnormal returns will be positive, and vice versa.

Meanwhile, the impact of Woods' tournament performance is also estimated by regressing excess returns against it. Data on the excess returns was collected for the three main sponsoring firms — Nike, American Express, and Fortune Brands (parent of Titleist), as well as for the main TV networks that broadcast golf tournaments. The excess returns of the latter were used to analyze whether they had any positive economic effects during tournaments in which Woods participated. The regression also takes into account other variables that might affect the sponsors' sales, including performance measured by a dummy variable based on whether Woods finished in the top 10 at the tournament or the negative of his end-of-day finish. The other variable in the regression is the industry average excess returns, which accounts for any abnormal effects in the market on that particular day. It must also be noted that on weekdays, excess returns are calculated on the same day as tournament play, but there is a one-day lag after Sunday's results.

Firstly, there seems to have been a positive reception by the market on the date of announcing each of the endorsements, with the cumulative excess returns of Nike and American Express crossing 2% on the day of and the day after the announcement. This event study method shows that celebrity endorsements are often looked upon positively by the market. Meanwhile, three of the four golf majors' broadcasting firms showed small positive excess returns after the major's conclusion, which could potentially be attributed to the increased viewership due to Tiger Woods.

Running the regression for the three main sponsors yields very similar results for American Express and Fortune Brands in comparison to Nike. For Fortune Brands, the p-values of the performance variables were found to be statistically insignificant, taking values below 0.05. This result has been attributed to the fact that Fortune Brands' golf division made up only 15% of its total revenue, and hence, the performance of other divisions likely superseded Tiger Woods' impact. American Express also has insignificant results for the performance variables, with the correlation coefficient for a top-10 finish being 0.04, which could have occurred for two reasons. Firstly, in 1997-98, when American Express signed Tiger Woods, his performance had dropped considerably. Hence, this may have led shareholders to believe that the endorsement was a mistake. Second, it has previously been proven that the market prefers endorsements by experts in that field.[5] American Express services have no relation to golf, therefore making the market skeptical of whether Woods is an appropriate choice. This suggests that news about Tiger Woods' tournament finishes does not affect their excess returns for both firms, causing them to show no relationship.

However, Nike's results show that they gained from choosing to endorse Woods. The performance variables - "finish" and "top ten" are both statistically significant (coefficients 0.0001 and 0.0065, respectively), indicating that the market views Woods' performance favorably if he is in the top 10 and in contention to win the tournament. This is because the publicity for Nike is greater if Woods is in contention to win, as there is more media exposure for their products. The result that is obtained from regressing Woods' performance against the standard excess returns in Nike's industry further proves the hypothesis that only Nike reaps the benefits of sponsoring Tiger Woods.

In conclusion, the study provides convincing evidence on the nature of celebrity endorsements and their value through the case study of Tiger Woods. It indicates that only the most relevant firms to the industry the celebrity belongs to will face positive economic benefits, but that these benefits are significant and worth the sponsorship deal.

Chung *et al*. continue to explore the case of Tiger Woods, this time using his deal with Nike to quantify the worth of celebrity endorsements for the endorsing firm and to determine whether these endorsements lead to increased product sales.[6] A significant increase in the number of athlete endorsements over the last 30 years inspired this research. It is often suggested that celebrity sponsorships benefit firms, but this study uses empirical proof to indicate the extent to which this is true.[7] This is done by looking at how much a product's sales and other financial outcomes, like profitability and market share, are influenced by a single athlete's endorsement deal.

Unlike prior studies in this field, this paper analyzes the product, accounting for the quality and credibility of Tiger Woods, the athlete. By adopting a long-term analysis approach with data on Nike golf ball sales from 1997 to 2010, there is more precision in the analysis of one specific product. This shows the sustainability of these returns for Nike, years after the date of the agreement. The paper further explores how the public perception of Woods affected Nike's sales following his marital scandal to test the hypothesis that the endorsed athlete's personal life directly plays a role in the utility of a consumer.

Initially, background research on celebrity endorsements in the golf industry and an overall analysis of golf ball sales are presented. It is noted that the two major factors in selecting golf endorsers are the influence the celebrity commands and their credibility in endorsing the product. The structures of golf balls are also deemed important as they are the differentiating factor among brands. Meanwhile, the sales of golf balls are established to be seasonal, consistently peaking during the summer and hitting a trough during the winter months.

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Hence, in constructing a model to estimate golf ball sales, a seasonal indicator is used. A comparison is also made between the market share of 15 golf ball manufacturing firms in 1997 and that in 2010, with Nike showing a very significant increase from 1.59% to 10% beginning immediately after Woods' endorsement announcement in June 2000. The firm also benefited from a clear jump in golf ball sales at the same time.

This study makes use of a dual-methodological approach to analysis, making use of both "reduced form analysis" and a "structural model". The reduced form analysis uses a model that includes five golfers - Tiger Woods, Phil Mickelson, Ernie Els, Vijay Singh, and David Duval. Each golfer is assigned a variable attributed to their skill at a particular point in time, corresponding to their PGA ranking. To improve the statistical power of the test, rankings are instead grouped into three bins, which are then used in the model. A variable that is identified to affect the estimation of the endorsement is the exposure that the endorsing brand receives in the media. This exposure can be divided into two types: planned, which accounts for spending from firms' marketing budgets, and unplanned, which refers to the TV exposure the golfer receives due to his performance in a particular tournament. To check whether there is a difference in spending on marketing based on the endorser's ranking, the planned advertising on the endorsement variable (ranking) is regressed against the endorsement variable and the unplanned advertisement variable. The endorsement variable was found to be insignificant, suggesting that planned advertising wouldn't be likely to alter the estimation. A dummy variable was defined for unplanned exposure, taking different values based on whether the golfer won the tournament and the significance of the tournament. In the case of Tiger Woods, this unplanned exposure is concluded to have made a significant impact on Nike sales due to the frequency of his victories during the timeframe.

The sales of the three major golf ball manufacturers — Nike, Callaway, and Titleist are then regressed against the endorsement variables (bins). This yields results that indicate that the model fits the data well and shows that 3 out of 5 endorsers have significant estimates for ranking variables. For Tiger Woods and David Duval in particular, there is a downward trend in estimated effect on sales as world ranking falls, indicating decreasing effects through the rankings. Meanwhile, for Vijay Singh, there is a statistically insignificant inverse relationship, while the other two golfers have significant inverse relationships among the variables. To check whether the loss of statistical power in the bin method was too great, another regression was taken without grouping of rankings. Very similar results are attained, indicating that the marginal effect for Tiger Woods was the greatest of the three golfers with statistically significant results.

Specifically for Woods, another regression is taken that estimates the additional sales of Nike golf balls as a result of the endorsement deal. It indicates a significant effect on sales from the ranking variable, suggesting that for every month Woods is ranked world number 1, he contributes an additional 1,416,000 golf ball sales per month. This analysis provides near-accurate estimations of the effect of golfers' rankings on golf ball sales across firms, not specific to Nike.

In addition to the reduced form analysis, the structural model is used to show the deeper effects of Woods' endorsement on Nike sales by analyzing how it affects other sections of the market, including consumer preferences, market share, profits, etc. It considers factors like how much more consumers were willing to pay for Nike golf balls because they were endorsed by Tiger Woods and how this endorsement impacted Nike's market share and profitability. The model helps to figure out if the increase in sales was mainly due to the endorsement or other factors like price changes or competitors' actions. Further steps were also taken to establish a total market size of 40 million, and rough calculations were used to determine the average rate of consumption of golf balls to better understand human preferences in choosing to buy/not buy the product. Similarly, price analysis is also done to conclude how much customers would be willing to pay for a dozen Nike golf balls. In creating a variable for the time trends as a control and for the scandal, which captures the impact of sales from the scandal while taking into account seasonality, the model can estimate the effects on Nike following Tiger Woods' publicly accepted misdemeanors.

The results show statistically significant results for Woods' endorsement effects on both Nike and Titleist products, suggesting that he contributed to the satisfaction associated with consuming both of those products. The quantitative results to prove these conclusions are the following. Nike gained over $103 million in profits from Woods' endorsement effect while getting a price premium of around 2.5%. This reiterates the conclusions from the reduced form analysis. Woods' competitors, though, had slightly differing results, with Duval's marginal impact falling steeply once he switched to Nike, but both he and Ernie Els contributed a greater marginal impact for Titleist than Tiger Woods. Meanwhile, analysis of the effects of Woods' marital scandal shows delays in negatively affecting Nike, happening only three months after the lead-up to April 2010. While the variable attributed to time trends accounts for this, it is reasoned that the delay is due to the time taken for all details of the accident and related infidelities to get released to the public, happening only in December 2009. As a result of this scandal, Tiger Woods seems to have lost approximately $1.4 million in profit for Nike. As a consolidation of the findings illustrated above, Table 1 below (taken from Chung *et al.*) provides the correlation coefficients for the major factors analysed

Table 1: Endorsement Effects

Random Coefficient Model				
	(Supply and Demand)		(Demand)	
Linear Parameters	**Estimate**	**SE**	**Estimate**	**SE**
Winning of Tournaments (unplanned exposure)	0.023	0.015	0.021	0.014
Woods Scandal on Nike products (12.2009-01.2010)	-0.037	0.090	-0.044	0.089
Woods Scandal on Nike products (02.2010-04.2010)	-0.169 **	0.094	-0.180 **	0.093
Woods Nike Endorsement	0.301 ***	0.079	0.302 ***	0.079
Duval Nike Endorsement	-0.364	0.247	-0.387	0.244
Mickelson Callaway Endorsement	1.284 ***	0.207	1.299 ***	0.206
Els Callaway Endorsement	1.289 ***	0.311	1.357 ***	0.311
Woods Titleist Endorsement	0.271 ***	0.076	0.291 ***	0.074
Mickelson Titleist Endorsement	-0.329 **	0.194	-0.340 **	0.192
Duval Titleist Endorsement	0.484 ***	0.108	0.488 ***	0.106

Singh Titleist Endorsement	-0.117	0.094	-0.121	0.093
Els Titleist Endorse	0.664 ***	0.138	0.668 ***	0.136
Non Linear Parameters	**Estimate**	**SD**	**Estimate**	**SD**
Price	-0.088 ***	0.021 ***	-0.089 ***	1.71E-08 ***
	(0.003)	(0.008)	(0.008)	(9.984)
Layers		-4.362e-08		-2.17E-07
		(547.839)		(1438.543)

Note: Signif. codes: 0 < *** < 0.05 < ** <0.1

All in all, research by Chung *et al*. provides a thorough picture of the true value of celebrity endorsements for a firm by making use of a dual-methodological style of analysis. It can be concluded that, especially when the congruence between the endorser and the product is high, it is extremely beneficial for the firm to endorse the athlete to reap long-term benefits. The paper further suggests that a fall in an athlete's reputation, like Woods' scandal, has severe effects on the endorsing firm. This very situation is explored further below by Knittel *et al*.[8]

That paper analyzes the potential negative effects that endorsements could have on corporate sponsors as a result of negative events concerning the athlete by examining Tiger Woods' marital scandal in 2009. It explores the reputation risk that firms take when choosing to attach themselves to an athlete while also examining the effect of the negative perception on competing firms. As a result, the paper aims to conclude whether endorsements are worth the potential damages, particularly in a risk-laden scenario.

In 2009, Tiger Woods was at the height of his popularity and was deemed one of the most influential people in the world. However, late in the year, several pieces of information regarding Woods' car accident and marital infidelities came to public light, resulting in the athlete taking an indefinite break from competitive golf on December 10. The data focuses on the 10-15 following the scandal's emergence, aiming to conclude how much celebrity endorsements affect the value of the firms, which in this case are primarily Accenture, Gillette, EA (Electronic Arts), Nike, and PepsiCo/Gatorade. This situation is deemed a good case study due to the surprise effect related to it, meaning that the market could not preempt the scandal and adjust accordingly before the event, thus giving more accurate conclusions.

An event study methodology is employed, along with an examination of Google Search intensity analytics to research the claim. The 5 major endorsing firms listed above are first divided into two categories: those who have developed product lines based on Tiger Woods and hence belong under the "Tiger Brand"- like Gatorade, EA, and Nike- and those who have not. Each of these 5 companies reacted differently to Woods' scandal, with some dropping him altogether and others limiting his role as a brand ambassador. For each of these firms, upon looking into the average Google Search intensity in 2009, the highest interest was recorded to be during the week of the scandal. Meanwhile, the metric used to measure the impact on stock prices is abnormal returns, the deviations from the expected returns. A regression model is formed to estimate these abnormal returns, taking into account the return on shares, the return on the total market index, the return on competitor firms, and the number of days after November 30th, when the data is being measured. Similarly, cumulative abnormal returns are also calculated to indicate the rate of change in these abnormal returns in the days following the scandal.

The results of the cumulative abnormal returns show substantial negative impacts for all endorsing firms in the market, particularly 8 days after the accident. The eighth day refers to the first day a sponsor took action against Woods, with Gatorade dropping him, proving that the market reacts to endorsement-related news. These return estimates are larger for the "Tiger Brand" groups and are statistically significant. The average daily abnormal returns for each group of firms also provide statistically significant results that help provide strong evidence that abnormal returns are consistently negative.

To link the findings from the abnormal returns to the pattern of search intensity, the two are plotted against each other, showing a high correlation between the two variables. To further confirm these results, abnormal returns are regressed against a measure of news intensity, which themselves have been divided into three different measures. The results prove the hypothesis true, with every day having a higher search intensity on days when there are more negative abnormal returns shown. It is also vital to note that these effects are significantly greater for firms under the "Tiger Brand" than for the overall sample. Upon testing the regression for non-endorsement-related but scandal-related search data, we establish that the above findings are specific to endorsement deals.

Competitors of sponsoring firms are also carefully examined, with each of the 7 sponsors compared with 10 corresponding competitors in their respective industry. Two potential results are theorized - either the competitors are successful in stealing business after a fall in the endorser's abnormal returns, or they are unaffected by the change. Another question that's addressed is whether endorsement-intensive (those who have at least one endorsement) competitors showed different trends from those who do not endorse. A regression model is then developed, consisting of the return on portfolio and the abnormal returns of the competitor along with time after the accident to yield the return on shares for the competing firm.

The results of the initial competitor model yield that their Cumulative Abnormal Returns (CARs) are positive while having an inverse relationship with the abnormal returns of sponsor firms. This confirms the hypothesis that the competitor firms received the business lost by the sponsors. However, there was a difference detected between endorsement-intensive and non-intensive competitors, with the returns of the latter turning negative from day 2 onwards. Relative to non-endorsement-intensive competitors, endorsement-intensive competitors lost 2 to 3% more value. This difference was statistically significant, indicating that across the market, the scandal sent a signal that doubted the decision to engage in celebrity endorsements. That is to say, the estimated 2% lost by sponsoring firms 10 days and onwards after the event was gained by their non-endorsement-intensive competitors on the same day.

Another regression is run, where a dummy variable that represents whether a firm is endorsement intensive and a measure of news intensity is added to the original equation. These results support the prior ones, showing a strong positive re-

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lationship between search intensity and abnormal returns for non-endorsement-intensive competitors and a less positive relationship with endorsement-intensive ones.

Overall, the findings of this study are such that endorsing firms are quite vulnerable to reputation risk due to the negative publicity of the endorsed athlete, supporting the surface analysis conducted by Chung *et al*. Furthermore, it concludes that the benefit of this negative publicity is significant for competitor firms, particularly those that are not endorsement-intensive. Besides providing the quantitative values for this relationship, the paper questions the reasoning behind certain trends, making it extremely helpful in researching the shortcomings of celebrity endorsements.

This section explored the case study of Tiger Woods, with particular emphasis on his relationship with Nike and on the outcomes of his marital scandal for sponsor shareholders. It is concluded that performance is highly beneficial to sponsors and that the expectation of victory, based on PGA rankings, improved sponsor returns. The importance of congruence is also emphasized, with sponsors like Gatorade and Titleist feeling a greater impact than non-Tiger Brand firms. The analysis of the scandal introduced a new angle to the question, where despite positive returns from the endorsement being common, there would always be a high level of risk associated with it.

Team Endorsements:

Moving away from Tiger Woods' case, Cornwell *et al*. choose to find out whether sponsoring a winning team is more beneficial than sponsoring a participating one.[9] This is done by assessing the true value of winning a motorsports event for the endorsing firm. The sport itself is quite conducive to advertising, with sponsor banners very easily visible to viewers, increasing the exposure for sponsoring firms. The Lone-standing Indianapolis 500 is selected as the primary event for its international presence, viewership, and ease of data collection.

The study looked at all 268 sponsoring firms (including winners) for all cars between 1962 and 1997, ignoring the firms that did not have stock price data available. Stock prices are the best metric to take qualitative changes, like winning, into account. Hence, similar to prior papers, an event study method with abnormal returns is employed. The method includes evaluating the time series for each of the sponsoring firms to measure the impact of the events 20 days before and after the event.

Next, cross-sectional regressions were taken. While regressing the sponsorship winners against abnormal returns, it is noted that motorsports are designed in a way that, more often than not, the expected winner ends up winning the race. Therefore, to be precise in estimating the regression, the following parameters are included - the relative value of the sponsor, the ratio of the eventual winner's qualifying speed to that of the fastest qualifier, the winner's margin of victory, and dummy variables to establish congruence between the sponsor and the sport, and to identify whether the athlete is a first-time race winner. The margin of victory is specifically included to account for the fact that sponsors with wins at a greater margin of victory have more exposure. Meanwhile, as mentioned in an earlier paper, congruence establishes how closely linked the effect of the race would be on the sponsor. For example, Valvoline would be impacted far more significantly than Domino's Pizza.

The initial results show that there is no reason to suggest that winning the Indy 500 resulted in statistically significant abnormal returns for sponsoring firms, with no notable change in the abnormal returns in the days immediately following the race. Even over a month of analysis, the cumulative abnormal returns were deemed to be negative and insignificant. Similarly, for analysis on the other 232 non-winning sponsors, barring two days post the race, there are no notable changes in the abnormal returns, and they remain statistically insignificant for the most part. The positive returns two days after the event (1.04%) indicate that the firms do receive some, however small, benefit from sponsoring the race.

The results of the regression show that of the 6 major variables, 3 are recorded to be statistically significant. With the "new winner" variable showing positive returns, the paper concludes that the surprise aspect attributed to a new winner results in gained returns for the sponsors. For qualifying speed, the negative returns show that when a car is considered less likely to win, there is a fall in the abnormal returns of their main sponsors. As for congruence, the results are similar to the study on title sponsorships, with the positive and significant relationship between the variables indicating that firms with greater congruence to the sport yield greater abnormal returns. The regression line itself is deemed significant as it passes at the 10% significance level. Meanwhile, upon regressing the non-winning sponsors, it is made clear that winning is important to shareholder wealth, as none of the seven variables are deemed significant. The qualifying speed and "new winner" variables are positive and negative, respectively, opposite to the results of the winning title sponsors. Table 2 below summarises the regression analysis by providing the coefficient of correlation for each of the primary variables

Table 2:

multiple regression analysis of the mean abnormal returns (registered over event days t = -2....2) by winning sponsors of the indianapolis 500

Variable	Variable Coefficient	Variable t-statistic	t-statistic p-value
INTERCEPT	1.0343	1.3728	0.1843
NEW WINNER	0.0422*	2.3474	0.0144
MATCH	0.0281*	1.4226	0.0848
QUALIFYING SPEED	-1.1553*	-1.4395	0.0824
MARGIN	-4.85E05	-0.4905	0.6288
TELEVISION	0.0059	0.2549	0.4006
FIRM SIZE	-0.1528	-0.2866	0.3886
Multiple R	0.6118		
R^2	0.3742		
Adjusted R^2	0.1955		
df/Regression	6		
df/Residual	21		
df/Total	27		
F-value	2.0932		
F-probability	0.0975		

* The indicated variable coefficient t-statistic is significant at the 10% level, one-tailed test.

One particular sponsor, Scientifically Tested Products (STP), had unique results for two major reasons. One was that the firm had been the winning sponsor 4 times in the period, and the other was that STP was especially congruent with the motorsports industry, being the only one of the winning sponsors that produced a product that would improve race performance. Hence, the abnormal returns of every winning title sponsor are regressed and compared to STP's, resulting in the conclusion that the results attained by STP were far greater in magnitude than any of the other firms due to its extremely high congruence levels. The mean CAR of STP was 8.2439%,

roughly translating to a $134 million increase in market valuation purely due to race results.

This goes in tandem with results from previous papers that emphasize the importance of congruence, stating that if there's alignment between the sponsor and the sport, the returns are very high. Hence, for the right firm (like STP), there is an extremely high value to motorsports sponsorships. But even otherwise, no harm is done to sponsoring firms if the sponsored team wins, as they receive no explicit benefit or loss. In motorsports, this lack of benefit is usually attributed to the winner often being expected, contradicting the results from the athlete-specific level.

Event Endorsements:

Now, Clark *et al.* shift the focus of this literature review from the impact of sponsoring individual athletes to that of sponsoring events.[10] They conducted a study that analyzed the worth of title sponsorships by measuring their impact on the stock prices of sponsoring firms across industries and sports. While there have been qualitative arguments for and against endorsements, this paper aimed to use abnormal returns to identify whether these sponsorships create value for the endorsers. The primary hypothesis formed was that announcements of title sponsorships have a positive relationship with stock returns across sports, regardless of whether the announcement is a renewal agreement or a new deal. It is also predicted that if a sponsor is congruent with the sport, meaning that their products align with the sport, they will receive healthier abnormal returns.

This is done by selecting 114 title sponsorship announcements between January 1990 and January 2005 from the following sports - golf, tennis, college football, and motor racing- and using an event study methodology to observe the impacts of these announcements on the respective endorsers' share prices. The method relies on the idea that the market reacts to newly disclosed information on the firm or economy and hence varies the stock price accordingly. As for the regression model, a precision-oriented Scholes-Williams market model is used.

First, the mean CARs for the 114 events are calculated for certain time intervals, 20 days before to 20 days after the event, with data from the CRSP (Center for Research in Security Prices). The 114 sports events are then broken down sport-wise, and the same calculations are done with them. The abnormal returns are also studied after splitting the endorsements into "new" and "renewing" categories to test the hypothesis that there would be no difference. Then, cross-sectional regression was conducted, which investigated each of the above variables' correlation with the abnormal returns of the endorsing firms. These included the market value of the firm's equity, the firm's cash flow (movement of money), as well as dummy variables denoting congruence, whether the endorser belongs to an industry that's considered "high-tech", and which of the 4 sports it sponsors.

Each of the hypotheses listed above generates unique results. Firstly, from the broad standpoint of all 114 events, there seems to be no net value generated from title sponsorships. That is to say, what the endorsing firm gains from title sponsorships is merely worth the same as what they invested in it. However, while splitting the 114 events by sport, it can be concluded that title sponsorships do not have an equal effect across sports. NASCAR race sponsorships yield positive abnormal returns of roughly 2% in the 10 days following the announcement of the deal, while the returns of NCAA title sponsorships are negative 2% in the same duration. With golf, across both the men's and women's tours, there seems to be no consistent evidence to suggest that the returns from title sponsorships are not zero. Tennis follows a similar trend to the NCAA, with abnormal returns being significantly negative. Hence, there's a clear difference in the market's reactions to sponsorships in different sports. For the study on "new" vs "renewing" sponsorships, there are differences between the two in the case of golf and NCAA bowl games. For the former, renewals were looked upon negatively by investors (-3%), whereas for the bowl games, they were looked upon favorably. This difference is attributed to the fact that PGA sponsorship fees continued to rise, while NCAA sponsorship fees remained more or less the same.

The regression shows three of the included 8 variables to be significant - congruence, "high-tech", and market value. The significance of market value indicates that a larger firm, with more market value, is more likely to reap benefits from title sponsorships than a small firm. For congruence, the significance suggests that a firm more closely tied to the sport it's sponsoring is, on average, likely to receive 3.4% greater abnormal returns than a firm that isn't. This supports evidence from previous research, which shows that for congruent firms (like Nike to Woods' golf), the impact on abnormal returns is greater. As for the relationship of the sponsoring firm to technology, the regression suggests that high-tech firms receive 2.6% more shareholder wealth from other firms. Cash flow is the only non-dummy variable to show no significant result. The four sports follow similar trends as when they were analyzed individually, with NCAA bowl events and PGA Tour events negative and of similar magnitude to the earlier analysis.

Overall, this study provides convincing evidence as to the worth of title sponsorships for firms, taking into account a wide sample of sporting events across four sports. Overall, the results show that title sponsorships can be viewed as indifferent to firms that are willing to invest, as abnormal returns tend to have little to no value. However, the paper breaks this analysis down into sports and groups of sponsoring firms to conclude that under apt conditions (like congruence), and for the right firm (high-tech or not), title sponsorships can be extremely useful as a market strategy. By isolating these factors, the paper can generalize its findings for the sporting industry as a whole.

Farrell *et al*. also aim to find the value of corporate sponsorships in sporting events, by focusing on just one event- the 1996 Summer Olympic Games in Atlanta.11 In the lead-up to the Olympics, the costs of sponsorships rose significantly from previous editions, causing firms to question their true financial value. This paper aims to answer that question, determining whether or not Olympic sponsors receive net benefits. Other considered aspects include whether these sponsorships

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are due to agency costs (choices made by firm managers for personal gain) and whether their values vary based on the type of sponsor. Further, the paper aims to examine other variables that influence the market perception of Olympic sponsorships to show the benefits of such an investment.

Data is collected on the stock returns for all firms that announced sponsorship agreements before the Atlanta Games. Those that were not publicly traded were excluded from the analysis, resulting in a sample size of 26 firms. Analysis was done by measuring the impact of sponsorships on shareholder wealth through an event study methodology. To test the hypothesis that Olympic sponsorship announcements do not affect shareholder returns, mean abnormal returns for the sample were taken within the interval of announcement day 5 days. The CAR is then calculated for a 3-day interval, the day of the announcement, and the two subsequent days. To explore differences across sponsor groups, the firms are divided into two categories—"new sponsors" and "repeat sponsors".

Next, a cross-sectional regression of abnormal returns was conducted. The independent determinants included the total value of the sponsors' assets, whether the firm is a repeat sponsor, the percentage of its insider and outsider ownership, and a dummy variable for the sponsorship category. The sponsorship categories are based on the value-adding potential of the sponsors, with Group 1 containing the most lucrative sponsors and Group 3 containing the least. It's hypothesized that there will be positive relationships for both the asset value and "repeat" variables. It's also suggested that the lower the value-enhancing potential of the sponsorship group, the more negative the abnormal returns would be. Hence, Group 1 is predicted to have a positive correlation, but Group 2 is said to have a negative one.

Meanwhile, the reason for including insider and outsider ownership is to examine the role that agency costs play in Olympic sponsorships. In more insider-owned firms, managers can make decisions for personal gain easily, as they are not bound by the wishes of shareholders who want to maximize value. The opposite is true for primarily outsider-owned firms. The level of insider ownership is then split into three categories based on percentage ownership.

Primary results showed that 4 days before the announcement, returns were positive and significant, with an associated coefficient of 0.425. Whereas, two days following the announcements, the market viewed the event as unfavorable, leaving significant negative returns of approximately the same value. Conducting a t-test also produces the same results, supporting the notion that a lack of specific information to the market about the endorsements is the reason for the negative returns. As for the 3-day CAR, the sample mean showed a 4.3% decrease in returns, making for statistically significant results. Meanwhile, the categorical analysis showed negative mean abnormal returns for both groups, but there was a statistically proven difference.

The coefficients of the cross-sectional regressions support the results from the 3-day CAR calculation, showing a negative and statistically significant constant coefficient despite accounting for various endorser-related variables. The model itself has a low coefficient of determination that isn't statistically significant. However, of the eight other variables included in the analysis, six have positive relationships with abnormal returns (per the hypothesis), but only one is deemed significant - the one on outsider ownership. This shows that for outsider firms, the effect of agency costs is less as they are prone to greater shareholder pressure. Hence, they follow the expected result of having a positive relationship with abnormal returns.

Overall, the findings suggest that Olympic sponsorships as a marketing strategy are not value-adding to the endorser. The study finds that around the time of announcing the sponsorships, the abnormal returns for firms turn negative, implying the negative perception of these sponsorships by the market. The impact of agency costs is also prevalent, corroborated by the cross-sectional regression showing a positive relationship between outside ownership and abnormal returns.

At first glance, endorsing sporting events, whether mega-events or smaller ones, does not provide value to firms. However, upon closer examination, it is suggested that the returns for a sponsor depend heavily on the type of event, dependent on various factors, including the sport, the type of firm sponsoring it, and whether it was a renewed sponsorship. Meanwhile, the Olympics are proven not to be value-adding for the sponsor, but agency costs are the reason attributed to sustained high investment in them, proven by analysis of insider vs outsider ownership. It can therefore be concluded that sponsoring sporting events is only beneficial to a firm if the conditions are tailor-made for them.

The results of each of the three levels provide significant insight into the impact of sports sponsorships on endorsing firms. Across these levels, several similarities and differences can be identified. Congruence is established to be a key factor that affects the endorsers' returns, with more congruent firms facing more significant impacts, whether the endorsed team or athlete wins or suffers damages to their reputation. Some level-specific factors, like the type of endorsing firm, its ownership, and other characteristics, are also proven to alter their returns. A factor that differed between the team and athlete endorsements was the "expected winner". In golf, it was determined that sponsors of higher-ranked players, who had a higher expected likelihood of winning, received higher returns, while in motorsport, there is no apparent benefit to sponsoring a winner who is expected to win. Hence, this literature review concludes that sports endorsements can have high benefits associated with them, but these are claimed only by the right firm at the right time.

Conclusion

In recent years, sports endorsements' popularity has been on a steep rise, with the market projected to grow by over 8.39% every year. Therefore, questions have arisen as to whether these high levels of investment are worth it. Through a holistic literature review, this paper has aimed to answer these questions by determining whether sports sponsorships are economically beneficial for the sponsoring firm. Papers on various aspects of sports sponsorships have been analyzed, of which most employed an event study methodology to conclude that multiple factors, such as congruence, the type of endorsing firm,

and athlete performance, help determine the impact of sports sponsorships.

The most important factor was congruence, the level of connection between the sponsor and the sport, which can be seen when examining the case of reputation risk, with more congruent firms facing greater negative effects when the reputation of the athlete they sponsor falls. Another common factor was that better performance by the sponsored athlete or team significantly improves the returns for the sponsor, proving that winning is quite financially beneficial. Major differences are found across results at the event endorsement level, such as the difference in the expected winner metric and events having inconsistent returns based on their type. Besides this, other factors' relationship with shareholder returns are examined, showing that characteristics of the sponsoring firm- including ownership, industry, and size- also play a role in determining returns. Hence, these consolidated results go to show that for a firm to receive positive benefits, it must satisfy certain conditions. First, it must be in the right industry, typically one that is connected to the sport. It must also sponsor an event that is lucrative but also unpredictable, unlike the motorsports example described above. Lastly, it should sponsor the right teams and athletes–those with healthy reputations and worldwide influence. These three factors, when taken together, make for healthy returns for a firm investing in sports sponsorships.

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REVIEW ARTICLE

Regulation of Gut Microbiota a Potential Therapeutic Option for Insulin Resistance - A Review

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ABSTRACT: There has been an exponential rise in the incidence of metabolic diseases globally in recent years. In the metabolic disease spectrum, insulin resistance is considered to be a precursor of Type 2 Diabetes Mellitus. If insulin resistance is left untreated, it leads to hyperglycemia, hyperuricemia, dyslipidemia, and eventually, frank diabetes. While genetic susceptibility plays a role in disease development, non-genetic factors such as diet and lifestyle have been instrumental in disease progression. One such factor that has been increasingly linked to insulin resistance and type-2 diabetes is gut dysbiosis. A healthy gut microbiome regulates metabolism and endocrine signaling. An imbalance of the gut microbiome has been shown to increase gut permeability, cause low-grade inflammation and immune dysfunction, and lead to insulin resistance. This review focuses on the role of gut dysbiosis in metabolic disease and explores interventions like diet, prebiotics, probiotics, and fecal microbiota transplantation as potential strategies to regulate gut microbiota to ameliorate insulin resistance. By providing an up-to-date analysis of the therapeutic options, this review underscores the potential of targeting gut microbiota as a promising approach to prevent the progression of insulin resistance to type 2 diabetes mellitus, suggesting the need for personalized gut microbiota-based therapies in the future.

KEYWORDS: Translational Medical Sciences, Disease Treatment and Therapies, Gut Microbiota, Gut Dysbiosis, Insulin Resistance.

Introduction

Insulin resistance (IR) is an impaired biological response to insulin stimulation of target tissues like the liver, muscle, and adipose tissue, which eventually results in hyperglycemia, hyperuricemia, dyslipidemia, and ultimately full-blown type 2 diabetes mellitus (T2DM) if left untreated. Insulin resistance, therefore, is considered as a precursor to T2DM.[1]

In IR, there is inadequate disposal of glucose from the bloodstream into the skeletal muscle. The circulating glucose then requires more insulin to facilitate its uptake into the insulin-resistant tissues resulting in hyperinsulinemia. Hyperglycemia occurs over a period of time as the beta cells in the pancreas are unable to meet the insulin demand. This vicious cycle that continues between insulin demand and supply over the years leads to blood glucose levels consistent with T2DM. Excess circulating glucose enters the hepatocytes, creating excess fatty acid production in the liver, which is not only deposited in the liver but also throughout other organs. Similarly, when adipose tissue becomes insulin-resistant, there is insufficient lipolysis, causing an increase in the circulating free fatty acids (FFA). Higher levels of FFA lead to lipotoxicity-induced beta-cell dysfunction, contributing to the development of T2DM.[2]

A recent analysis of the National Health and Nutrition Examination Survey (NHANES) data from 2021 found that 40.3% of US adults aged 18-44 are resistant based on Homeostatic Model Assessment for Insulin Resistance (HOMA-IR) measurements.[3] Although IR is known to affect all ethnicities and races, comparable data between them is limited. The global diabetes prevalence in 2019 is estimated to be 9.3% (463 million people), rising to 10.2% (578 million) by 2030 and 10.9% (700 million) by 2045.[4] Although genetic susceptibility plays a role in T2DM development, non-genetic factors such as diet and physical activity cause insulin resistance eventually leading to T2DM.

An association between gut dysbiosis (alteration of healthy microbiota), insulin resistance, low-grade inflammation, T2DM, and obesity has been reported over the past decade **(Figure 1)**.[5] Gut microbiota (GM) is the microbial population in the gut and is the largest microbial community in the human body. A healthy gut microbiome regulates metabolism, endocrine signaling, and brain function (brain-gut axis).[6] Diet and lifestyle modification are the mainstay of treating insulin resistance. The relationship between gut microbiota and IR suggests the role of regulating gut microbiota as a potential therapeutic target for the treatment of IR and prevention of its progression to T2DM. In this article, we summarise the role of GM in insulin resistance and provide an up-to-date review of diet, prebiotics, probiotics, and treatments like Fecal microbiota transplantation (FMT) as probable interventions for amelioration of IR and prevention of its development into DM.

Discussion

Gut microbiota (GM) and Insulin resistance (IR):

Human GM is primarily anaerobic, belonging to phyla *Firmicutes* (~60%), *Bacteroides* (15%), *Actinobacteria* (~15%), *Verrucomicrobia* (2%), *Proteobacteria* (~1%), and *Methanobacteriales* (~1%).[7] These are distributed throughout the gastrointestinal tract, and balancing the composition of GM is critical to maintaining gut permeability, metabolism, immune function, and prevention of metabolic disease. Several studies have shown that gut dysbiosis (alteration in the GM) can lead to disease. Dysbiosis can be of three different kinds- over-

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microbiota, and loss of overall microorganism diversity.[8] These can occur in isolation or, more often, simultaneously. For example, dysbiosis in the form of low microbiota diversity has been shown to increase the risk of weight gain, insulin resistance, low-grade inflammation, and T2DM in human studies.[9] Similarly, patients with T2DM compared to healthy individuals have a decreased ratio of *Firmicutes* to *Bacteroidetes* in the majority of studies.[10] Specific bacterial species have a definite role in maintaining gut health and preventing disease.

A decrease in *Prevotella* species, a bacteria that helps in glucose homeostasis, was observed in 50 Japanese T2DM patients compared to healthy subjects,[19] but studies in 291 Nigerians and 171 Chinese with T2DM showed an increase in Prevotella.[20] This contrasting result may be due to inter-ethnic variation with genetics, diet, medication use, and sequencing technique, necessitating the use of new technologies to establish firm associations between GM in healthy and diseased individuals among different ethnicities. Similarly, data on *lactobacillus* is dependent on species. While *L. gasseri*, *acidophilus*, and *salivarius* are positively correlated with T2DM, *L. amylovorus* demonstrates a reverse correlation.[17]

Table 1: Role of various intestinal bacterial species in maintenance of gut health and gut dysbiosis in the form of increase in harmful bacteria or decrease in beneficial GM in IR/T2DM patients compared to healthy controls in human studies.

Gut bacteria	Changes in IR/T2DM	Role in glucose metabolism	Reference studies
Akkermansia muciniphila	↓	SCFA-producing bacteria strengthen the gut barrier against pathogenic bacteria	11
Roseburia intestinalis *Roseburia feces*	↓	SCFA producing bacteria	12
Faecalibacterium prausnitzii	↓	Inhibits pro-inflammatory cytokine secretion	13
Bacteroides caccae *Bacteroides vulgatus*	↑	Opportunistic bacteria	14
Bacteroides intestinalis	↓	Preserves intestinal wall integrity; reduces LPS production	15
Firmicutes	↓	Mucin-producing bacteria help with the gut barrier	15
Clostridium clostridioforme	↑ in DM ↓ in prediabetes	Opportunistic bacteria: increase in plasma glucose levels	16
Lactobacillus gasseri *Lactobacillus acidophilus* *Lactobacillus salivarius*	↑	Maintain mucosal barrier function by increasing the mucin levels in the gut	17
Streptococcus mutans	↑	Opportunistic bacteria	18
Prevotella capri	↓ in Japanese ↑ in Nigerians	Increases inflammation and risk of obesity	19,20
Clostridia sp.	↑	Opportunistic bacteria	21
Bifidobacterium	↓	Maintains glucose homeostasis	22
Ruminococcus gnavus	↑	Pro-inflammatory bacteria	23

A decrease in mucin-producing bacteria increases intestinal permeability, allowing pathogenic bacteria to enter the bloodstream and causing 'metabolic endotoxemia,' triggering low-grade inflammation involved in the pathogenesis of IR and the development of T2DM.[24] *Akkermansia*, *Roseburia*, *lactobacillus*, and Bacteroides can decrease pro-inflammatory cytokines (IL-6, IL-8, IL-7, and TNF-alpha) and are thought to be protective against IR and T2DM by restoring insulin sensitivity and improving glucose homeostasis, while *Fusobacterium nucleatum* and *Ruminococcus gnavus* can increase cytokine production.[25] Another study from Denmark with 123 non-obese and 169 obese individuals showed that individuals with high gene count microbiome had reduced susceptibility to metabolic disease and individuals with low gene count were more prone to harboring pro-inflammatory bacteria such as *Ruminococcus gnavus*.[26] Thus, depending on the GM composition, the microbiota can increase or decrease inflammation and metabolic dysregulation impacting IR **(Table 1)**.

Role of GM and Their Metabolites in Metabolic Pathways:

Metabolites are molecules derived from gut microbiota, and their role is to mediate a response between the host and the intestinal bacteria. Gut microbiota is responsible for the fermentation of dietary plant fibers in the intestine, producing short-chain fatty acids (SCFA) such as butyrate, propionate, and acetate. SCFA is instrumental in the regulation of appetite, insulin response, and inflammatory processes. Propionate and butyrate have anti-obesogenic action, while acetate promotes fat storage.[27] Propionate improves the function of beta-cells in the pancreas, promotes insulin secretion and glucose uptake in muscles, and decreases glucagon production in the pancreas. Butyrate is also responsible for maintaining the integrity of the intestinal barrier. Various bacterial components such as lipopolysaccharides (LPS), flagellin, and peptidoglycans can enter the bloodstream if the intestinal barrier is compromised, triggering a chronic inflammatory response that, over time, contributes to metabolic dysregulation and insulin resistance **(Table 2)**.[28] Bacteroidetes produce acetate and propionate, and Firmicutes produce butyrate by fermenting dietary fibers.[29] Reduction in butyrate-producing bacteria such as *Faecalibacterium* and *Roseburia* can, therefore, potentiate insulin resistance and T2DM, as evidenced in human studies.[16] Obese subjects treated with the antibiotic Vancomycin steadily developed insulin resistance due to inhibition of the growth of butyrate-producing bacteria in the gut.[30]

Table 2: Role of gut microbiota dysbiosis in the pathogenesis of insulin resistance. The table shows the cascade of events triggered by an imbalance in the gut microbiota leading to impaired glucose metabolism, setting the stage for insulin resistance and eventually diabetes mellitus over time.

1. Increased gut permeability
2. Lipopolysaccharide leakage into the bloodstream (metabolic endotoxemia)
3. Increase in pro-inflammatory cytokines
4. Increased oxidative stress
5. Increased chronic low-grade inflammation
6. Altered glucose homeostasis

While the association between gut microbiota, glucose metabolism, and insulin resistance in diabetic patients has been relatively well-established through human studies, similar data in patients with pre-diabetes (characterized by abnormal blood glucose levels below the T2DM threshold) is limited. In a recent study from Asia, 57 pre-diabetic patients were compared to 60 healthy individuals in the age group of 18-65, and the pre-diabetics showed a lower GM diversity with respect to the healthy cohorts.[31] More such studies are needed with larger cohorts and across different population groups to substantiate the GM variability in insulin resistance. **Figure 1**

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shows how gut dysbiosis leads to metabolic dysregulation and insulin resistance.

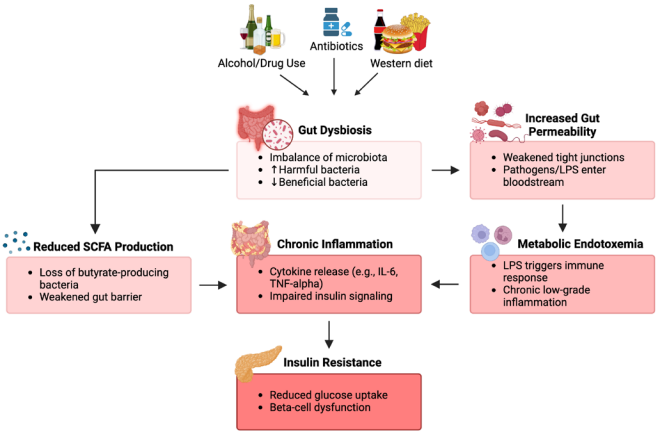

Figure 1: Impact of gut dysbiosis on the pathogenesis of insulin resistance and T2DM through various mechanisms eventually leading to altered glucose metabolism. Abbreviations: LPS: lipopolysaccharide, SCFA: short-chain fatty acids, IL-6: interleukin 6.

Modification of Gut Microbiota to ameliorate Insulin Resistance:

In recent years, there has been an increase in the incidence and prevalence of metabolic disorders like obesity, insulin resistance, type 2 diabetes mellitus, and non-alcoholic liver disease, underscoring the need for therapeutic options to prevent disease onset and progression. With increasing evidence supporting the link between gut dysbiosis and metabolic disorders in animal and human studies, the use of microbiome-based therapies through the manipulation of intestinal bacteria and their metabolites to restore metabolic health is promising.

Gut dysbiosis can be induced by certain medical treatments such as the administration of broad-spectrum antibiotics and chemotherapy, a diet characterized by intake of highly processed foods and foods with low-fibre content, sedentary lifestyle, poor sleep, excessive alcohol consumption, chronic stress, and exposure to environmental pollutants.[32] This section will review the current literature on the interventions aimed at regulating GM to prevent insulin resistance and its eventual progression into T2DM.

Diet and Gut Microbiota:

The major predictor of GM composition is diet.[33] Long-term consumption of the Western diet increases gut permeability, lipopolysaccharide leakage, oxidative stress, and the release of proinflammatory cytokines, leading to IR and T2DM over time.[34] Studies have reported that a Mediterranean diet can impact intestinal bacteria positively. It increases the *Firmicutes* to *Bacteroidetes* ratio and reduces the abundance of *Ruminococcus* and *Prevotella* while increasing the presence of *Faecalibacterium prausnitzii*, as shown in a randomized control trial with 20 obese men following a Mediterranean diet for 1 year.[35] Another study showed an improvement in HOMA-IR compared to the control group after 6 months.[36] However, changes in HOMA-IR were dependent on the baseline gut microbiota composition. Those with increased levels of Bacteroidetes and decreased levels of Prevotella showed a reduction in insulin resistance, providing protection from T2DM and metabolic syndrome.[36] Similarly, lower saturated fatty acid and high-fibre-containing plant-based diets have shown an increase in *Bacteroidetes* and a decrease in *Ruminococcus* compared to omnivorous diets,[37] though studies are limited. Patients with prediabetes who maintain a high-fiber and low carbohydrate intake have increased intestinal barrier integrity and reduced inflammation as the gut bacteria are able to utilize the dietary fiber to produce short-chain fatty acids (specifically butyrate) effectively.[38]

Natural bioactive compounds found in certain food sources can also positively influence gut microbiota (Table 3). Anthocyanidins contained in strawberries, blueberries, and cherries have been shown to increase lactobacillus and bifidobacterium, thereby improving glucose and lipid metabolism and, consequently, IR by improving gut barrier function.[39] The action of GM modulation by bioactive compounds is attributed to increases in the levels of *Bifidobacterium spp. Lactobacillus* spp. *Akkermansia spp*., as well as to the reduction in the *Firmicutes* to *Bacteroidetes* ratio.[47]

Table 3: Effect of foods containing bioactive compounds on the gut bacteria composition and their mechanism of action in reducing insulin resistance.

Bioactive compounds and foods containing them	Gut microbes impacted	Effect on insulin resistance	Reference papers
Anthocyanidin (Berries)	↑ *Bifidobacterium* ↑ *Lactobacillus*	Improved gut barrier function and IR	39
Hesperidin, Naringin (citrus fruits)	↑ *Bifidobacterium* ↑ *Lactobacillus*	Increased production of SCFA and improved insulin sensitivity	40
Berberine	↑ *Bifidobacterium*	Reduced endotoxemia and inflammation	41
Alkaloids and polyphenols (oat bran)	↑ *Lactobacillus* ↓ *Ruminococcus* ↓ *Prevotella*	Reduced inflammation through SCFA production	42
Polyphenols (Green tea, black tea, oolong tea)	↑ *Akkermansia* ↑ *Bifidobacterium* ↑ *Lactobacillus*	Increased production of SCFA Improved insulin secretion	43
Resveratol (Red wine)	↑ *Bifidobacterium* ↑ *Lactobacillus*	Improved glucose tolerance	44
Lycopene (tomato, moringa)	↑ *Bifidobacterium* ↓ *Proteobacteria*	Better gut barrier function Reduced LPS-induced insulin dysfunction	45
Beta-glucans (mushrooms, yeast)		Reduces blood glucose levels by retarding sugar absorption	46

Prebiotics:

Prebiotics are non-digestible food ingredients (fibers) that affect the host positively by stimulating the growth or activity of one or more species of gut microbiota. There is growing evidence to suggest that prebiotics can improve glucose homeostasis in insulin resistance.[48]

One such prebiotic is Inulin, a fructose polymer that cannot be digested by humans, but the gut microbes break it down into short-chain fatty acids. Inulin increases *Bifidobacterium* and *Faecalibacterium*, resulting in increased butyrate levels.[49] A systematic review summarised clinical trials evaluating the effect of inulin on *Akkermansia muciniphila* in control versus T2DM patients and found increased abundance in the treated group compared to the control.[50] A meta-analysis of randomized controlled trials to assess the impact of prebiotics in general on IR reported a decrease in serum insulin and blood glucose levels.[51] Keeping the high-fiber content as a basis, a

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study using 5 raw materials acorn, quinoa, sago, sunflower, and pumpkin seeds, assessed the effect of these prebiotics in healthy and diseased individuals. The study established that prebiotics not only produce beneficial metabolites like SCFAs but also improve gut dysbiosis by promoting the growth of favorable gut bacterial species.[52] *Lactobacillus* and *Bifidobacterium* are the usual targets for prebiotics and diet, as shown in **Table 3**.

The ability of prebiotics to produce a positive impact on glucose metabolism makes it a good adjunct to traditional anti-diabetic drugs. However, their benefit, if started early in the process of metabolic dysregulation, still needs to be established to truly evaluate their role in disease prevention.

Probiotics:

Probiotics are live bacteria that are beneficial to human health in ways such as improving gut health, inhibiting the growth of pathogenic bacteria in the gut, producing SCFAs, and stimulating the immune system.[53] There is enough data to support that alteration of the GM through administration of probiotics improves T2DM by reducing intestinal permeability and pro-inflammatory cytokines.[54]

A randomized, double-blind, placebo-controlled trial of administration of *Akkermansia muciniphila* in IR volunteers improved insulin sensitivity, decreased plasma insulin levels, and reduced body fat mass due to its anti-inflammatory effects.[55] Recent meta-analysis of studies showed that probiotic supplementation improved fasting blood sugar, HbA1C, and HOMA-IR in T2DM.[56]

Probiotics influence GM composition at multiple levels, like strengthening the intestinal barrier, production of SCFAs, and immune modulation, especially specific strains of *Lactobacillus* and *Bifidobacterium*. Numerous studies have shown the beneficial effects of individual bacterial strains, but there are more than enough studies to also show that when a combination of probiotics using more than one bacterial strain is used, it is more effective in improving glucose metabolism.[57] Supplementation with a mix of probiotics containing *Lactobacillus acidophilus*, *Lactobacillus casei*, and *Bifidobacterium bifidum* in DM patients showed a decrease in insulin levels, HOMA-IR, and improvement in insulin sensitivity compared to controls after 6 months in a study.[58]

Several butyrate-producing bacterial species have shown improvement in insulin sensitivity in both animal and human studies. One such study showed that supplementation with a capsule containing a butyrate-producing species, *Anaerobutyricum soehngenii*, improved glycemic controls in patients with metabolic syndrome.[59]

The positive effects of probiotic supplementation have also been studied in pre-diabetics. A double-blind, randomized controlled trial in which patients were randomly supplemented with a probiotic (*L. acidophilus, B.lactis, B.bifidum*, and *B.longum*) or synbiotic (inulin and probiotic) over 24 weeks showed a significant decrease in fasting insulin, HOMA-IR, and HbA1c levels.[60] This suggested that probiotics and synbiotics can potentially reduce the risk of developing metabolic disease in patients with IR. Similar results were shown in a study by Kassian *et al.*, which found a higher concentration of bacteria in the probiotic supplement.[61]

Studies in patients with T2DM have shown less impactful results on glycemic control when probiotics have been compared with antidiabetic drugs, emphasizing their role more as an adjunctive treatment and not a replacement to mainstay therapy. However, the same may not apply to patients of IR or pre-diabetics who are not on any pharmacological anti-diabetic drugs and rely primarily on diet and lifestyle changes to improve their metabolic dysregulation. As previously elucidated, patients with IR differ in the composition of their GM compared to healthy controls. In such cases, the administration of prebiotics, probiotics, or a combination of these can be efficacious supplements to ameliorate the disease or prevent its further progression by improving glucose metabolism. Though most of these studies have been randomized, double-blinded, and placebo-controlled, sample size, inconsistency in measurement, and short duration of the study period warrant the need for more well-designed, longitudinal studies to establish a clear impact of probiotic supplementation as an adjunctive treatment to diet and lifestyle modification for insulin resistance reversal.

Synbiotics:

Synbiotics are dietary supplements that use a combination of both pre and probiotics. Studies have reported a decrease in HbA1c levels when diabetic patients on hemodialysis were administered a probiotic containing different bacteria species together with inulin. Meta-analysis of randomized controlled trials in T2DM patients treated with synbiotics has shown similar results with a reduction in fasting blood sugar levels in addition to HbA1c.[62] A double-blind, randomized control trial from China showed that berberine with probiotics improved HbA1c levels better than berberine alone.[63] These results highlight the synergistic effects of pre and probiotic co-administration. There have been some studies, however, comparing synbiotics with control supplements, which did not report any changes in insulin or glucose response and no alteration of glucose metabolism.[64] Inconsistency in the results of studies has prevented widespread use of synbiotics currently, necessitating larger studies across varied population groups.

The available evidence suggesting the use of probiotics, prebiotics, or synbiotics is not strong enough and, therefore, the therapeutic use of these supplements for metabolic disorders has not been recommended yet. Also, there are only a small number of studies designed to analyze the effects of probiotic and/or synbiotic administration in the prediabetes population who are at risk of developing diabetes and cardiovascular diseases.[61] Inconsistent use of microbial strains and formulas, heterogeneity of target population, and variation in the bioavailability of synbiotics may limit their use in patients with metabolic dysfunction.[63] Additionally, uncertainty in the duration of supplementation of synbiotics or pre- and probiotics may also pose a challenge when used in the clinical scenario. Furthermore, synbiotics are almost always used in conjunction with lifestyle modifications as part of the treatment plan making it difficult to quantify their impact in reducing insulin resistance.

Fecal Microbiota Transplantation (FMT):

Fecal Microbiota Transplantation (FMT) is an intervention wherein fecal material from a healthy donor is transferred to a recipient to improve their gut microbiota composition. The process involves meticulous screening of the healthy donor and processing the microbiota from their fecal material. This is then administered to the recipient through the upper gastrointestinal route (nasogastric tube), lower gastrointestinal route (colonoscopy), or oral route (capsule form). Fecal Microbiota Transplantation has been successfully used for recurrent Clostridium difficile infection secondary to antibiotic-induced gut dysbiosis and is currently being evaluated for GM modulation in IR/T2DM patients.[65]

A study published by Mocanu CV *et al*. suggested that FMT, especially with low fermentable fiber supplementation, can improve insulin sensitivity by increasing the microbial diversity within the gut microbiota, proposing it as a potential therapy for metabolic syndrome.[66] In one study, obese patients with IR given frozen FMT capsules had significantly improved HbA1c levels after 12 weeks with an abundance of *Prevotella* in the recipient.[67] Other studies have also shown improvement in gut barrier function, an increase in GM diversity, and an increase in butyrate-producing bacteria such as *Roseburia intestinalis* and *Bifidobacterium pseudopodium*.[68] Allegreti JR *et al*. studied the effect of FMT in obese but metabolically healthy individuals and found improvement in both glucose and insulin levels after 6 and 12 weeks of treatment compared to a placebo.[69] Administration of FMT capsules led to improvements in total cholesterol, fasting glucose, and HbA1C levels compared to placebo in subjects who had low microbiome diversity to start with. The authors, however, state that the changes in microbial composition were not specifically correlated to the metabolic outcomes suggesting FMT as an adjunct to dietary intervention and exercise to achieve statistically significant changes in insulin sensitivity.[69]

All these studies provide promising evidence; however, other studies showed no significant change in insulin sensitivity in patients with mild to moderate insulin resistance.[70] The difference in the outcome of the studies could be due to multiple factors like the degree of GM dysbiosis in recipients, FMT preparation, and route of administration. This warrants the need for standardization of the procedure to tap the true potential of this innovative therapy to stop the progression of early IR into frank T2DM. Additionally, while FMT is considered relatively safe, transmission of infectious agents does pose a potential risk preventing FMT from becoming a widely accepted form of treatment.[71] One way to offset this risk would be to manufacture synthetic bacterial communities resembling eubiotic gut microbiota for administration to patients with metabolic dysregulation.[72]

Drugs and GM:

Studies assessing interactions of anti-diabetic drugs and GM composition are emerging. GLP-1 receptor agonists, a class of anti-diabetic drugs can change the *Firmicutes* to *Bacteroides* ratio modifying the GM composition. However, most studies are animal-based.[73] Metformin, on the other hand, has a well-established therapeutic effect mediated through GM. It increases bacteria such as *Enterobacteriales* and *Akkermansia muciniphila*.[76] Additionally, metformin use has been associated with a higher production of SCFA's and is known to strengthen the intestinal barrier. Through the modulation of GM, increasing the SCFA levels and enhancing the intestinal barrier integrity, metformin prevents metabolic endotoxemia and thereby reduces insulin resistance.[75]

Conclusion and Future Direction

Insulin resistance is considered a precursor of T2DM in the spectrum of metabolic disorders. Recent data has shown the role of gut microbiota dysbiosis in both IR and T2DM. An increase in pro-inflammatory microbes and a decrease in anti-inflammatory GM causes low-grade chronic inflammation and metabolic dysregulation through an increase in gut permeability and immune dysfunction, leading to IR and, eventually, T2DM. This gut dysbiosis is further exacerbated by a Western diet and poor lifestyle, accelerating the progression of the disease. Modulation of GM through bioactive compounds in diet, prebiotics, probiotics, synbiotics, and FMT to restore gut eubiosis can improve insulin sensitivity and slow the progression of IR along the metabolic disease spectrum, as demonstrated by various animal and human studies. There is a definite and increasing need for large-scale, longitudinal studies across population groups to establish a role for gut-modulating therapies for the prevention of metabolic dysregulation, which may need to be tailored to each patient considering inter-individual variations in GM and different responses to nutritional strategies, further emphasizing the need for individualized treatment strategies.

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REVIEW ARTICLE

K-POP Composition with Generative AI

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ABSTRACT: This research aimed to create a K-POP music composition model with Long Short-Term Memory and analyze K-POP datafication by integrating technology and other fields. K-POP was converted with a graph by Librosa, and it differed from people listening to music with their ears and looking at the data. It shows more relationships between pitch, time, and frequency. The LSTM model trains three data sets: all K-POP genre songs, BLACKPINK's songs, and BTS's songs. The output has a repeated melody when a model trains songs on all K-POP genre songs. It was a repetition of high notes. Conversely, when it focuses on learning one specific group, the output of the model has a wide range of notes. The model trained on a specific group's song generates a song that is closer to K-POP.

KEYWORDS: Robotics and Intelligent Machines, Machine Learning, K-POP Composition, LSTM, Music Composition.

Introduction

Artificial Intelligence, one of the most ideal technologies ever invented, has changed human lives. It brought convenience to individuals and the world. AI is inseparable from people's daily lives through Siri by iPhone[1] and Bixby by Samsung.[2] YouTube, an online video platform, uses AI for its video recommendation algorithm and distinguishes malicious comments to ban.[3] Instagram used AI to analyze and collect data about user preferences. They analyzed user preferences and exposed feeds related to user preferences on the user's screen.[4] AI is no longer separate from humans. For example, generative AI penetrates our lives.

The generative AI resolves diverse tasks. For instance, an AI drawing generator, Midjourney, creates an image or drawing depending on the user's prompt in a few minutes.[5] Large Language model, ChatGPT, is helpful in writing a novel or letter and speech outline. The sentences made by ChatGPT are very natural. It has proficiency in generating sentences.[6] Alexa, made by Amazon, is an AI personal secretary for people. It serves to enhance human comfort. It answers the questions humans ask.[7] As generative AI is in the spotlight, it keeps developing. AI generative models in the music field have also advanced. AI voice cover videos are especially favored on social media. On YouTube, a song cover video using AI got millions of views. People are using AI voice covers to make their favorite song cover videos. Through this example, I noticed the possibility of using AI to compose music. Thus, I attempt to create a K-POP music composition model.

K-POP is a category of Hallyu (Korean wave). After 2015, K-POP was moving forward to the global market. Then, in 2023, K-POP was fashionable worldwide. Its fame is centered around teenagers and those in their twenties. It won the Billboard Award, which is globally recognized and well-regarded. K-POP stands for Korean Pop. In a broader context, it means all Korean songs, but from a narrow perspective, it represents the end of 20th-century music's dance, hip-hop, R&B, and electronic music. K-POP shows breakneck development. Originally, K-POP was renowned in Asia. However, due to the commercialization of the internet, other countries can access K-POP through social media. This has resulted in K-POP becoming famous globally and promoting Korea. K-Pop has increased the number of tourists in Korea. Because of this, K-POP is also called South Korea's greatest export. Before the existence of K-POP, there was Hallyu (Korean Wave). There has been an increase in international interest in South Korean popular culture, especially music, film, fashion, and food. Hallyu has started getting attention at the end of the 20th century. Beginning with the export of Korean drama, exporting K-Drama expanded to songs and became famous. In the 21st century, a phenomenon favored Korean cultures, such as kimchi, electronics, and food. As K-POP became famous, as a new context of the Hallyu, K-POP was established.

The most prominent feature of K-POP is the ocular effect. It is considered a factor of K-POP fame. Usually, other singers and bands didn't sing a song while dancing. Conversely, almost all K-POP idols dance while they sing a song. Most K-POP songs are dance-pop. Not only these, but K-POP also has attractive melodies and lyrics. K-POP idols work in bands and both girls' and boys' groups. This paper divided K-POP into two categories: Group A is a boys' group, and Group B is a girls' group.[25]

This proves that K-POP has the value of research about the incredible speed of spreading its influence worldwide. To study K-POP, it requires a unique tool, MIDI. I used a MIDI file to construct a training dataset. Similar to how other songs are composed, and similarly to other songs, K-POP is also composed with verse and hook. It has repeated parts. Since K-POP's time series data is repetitive, I used LSTM to create a model. This research shows the positive side of AI and various possibilities for improvement and development. It is not only

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used in computer science; it can also connect with other fields. Thus, the whole society can improve together at the same time. I wish other education fields were developed through generative AI, not only art and writing. The development of technology has brought a lot of comfort to citizens. After the invention of generative AI, such as Midjourney, the quality of the student's project work has rapidly increased. For example, by developing drawing books for children, using my methods, students could submit high-quality work with the help of AI.

Methods

K-POP Data:

To analyze the characteristics of K-POP more clearly, I chose songs and K-POP groups that are publicly well-known. The data was collected in 2016 when K-POP started to attain sudden acclaim. The criteria for data (K-POP idol group) are the groups that at least won the music TV show and have a music video with over 100 million views on YouTube. I collected data from Twice, BLACKPINK, BTS, and NewJeans.

Table 1: K-POP MIDI File Data. It shows how many MIDI files were collected from the K-POP IDOL group. All the songs are after 2015. Some of the K-POP IDOL groups in the list debuted around 2021.

Group	Artist	Number of Songs
A	BTS, Tomorrow by Together, Stray Kids, NCT, EXO, ENHYPEN, Seventeen	79
B	BLACKPINK, Twice, NewJeans, IVE, NMIXX, Asepa, (G)-IDLE	75

I made up seven groups per group A and B. Group A had 79 songs, and Group B had 75 songs. In total, it consists of 154 songs. I collected at least five songs per group (Table 1).

MIDI file:

MIDI stands for Musical Instrument Digital Interface. It is a digital music sheet for playing digital instruments. It shows when and what pitch has to be played. Moreover, using MIDI files, requires special tools, such as PrettyMIdi and Py Fluidsynth.[28]

The song should be in a MIDI file format to model and learn the music. The MIDI file data used in this research paper are from the Musescore website. The MIDI file data is piano MIDI data for the consistency of the collected data. Musescore's website shares a music sheet or MIDI file with everything needed to write a song.

Table 2: "Still With You" MIDI note. A new note is played in less than one second.

Pitch	Start	end	Step	duration
73	0.685714	1.1010714	0.000000	0.325000
71	1.028571	1.353571	0.342857	0.325000
73	1.371428	1.859285	0.342857	0.487857
80	1.885713	2.373570	0.514285	0.487857
73	2.399999	2.724999	0.514285	0.325000

MIDI NOTE is a converted version of MIDI. I changed the MIDI file into a note using three variables: pitch, step, and duration. Start and end also represent duration. To train for the model effectively, I changed MIDI to note. If it was converted, the results are in Table 2. The model will be trained by this MIDI NOTE and converted to MIDI NOTE again so humans can listen to the generation output.

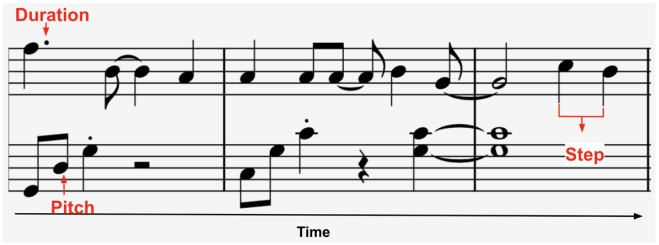

Figure 1: JungKook's "Still With You" music sheet is labeled with duration, pitch, and step. Each note represents duration, pitch, and step; the rest represents only duration.

Pitch:

Like in Figure 6 above, the pitch in MIDI NOTE is pitch. In the table, the pitch is expressed as an integer. It can be converted as 'A4' or 'G4'. In Figure 1 (music sheet), the position of the note represents the pitch.

Duration:

The duration of a MIDI note shows the duration of the pitch. A note is a whole note with four counts of duration; if it is a quarter note, it has one of the four counts as duration. In Figure 1, the type of note represents the duration.

Step:

The Steps represent the length of the previous note and the following note. It can calculate using the table by the previous starting point and subtract the start point.

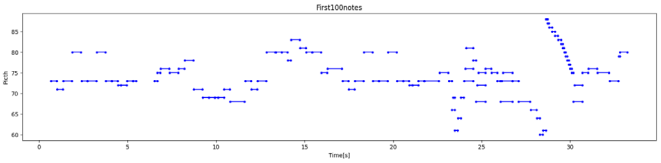

Figure 2: It is JungKook's "Still With You" first 100 notes floating graph. It had a wide range of pitch classes.

Each point on the graph represents each note in the music sheet. The y-axis shows the pitch, and the x-axis shows the times. The length of the line is the duration of the note. As the line is short, it means it has a short duration, and as the line is long, it means it has a long duration (Figure 2).

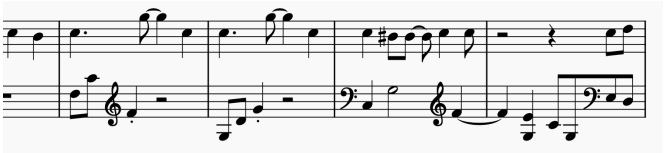

Figure 3: JungKook's music sheet for "Still With You" music sheet shows the song's first few seconds. In the bottom part of the sheet, there are several changes between G glef and F clef.

Figure 3 shows that staccato is represented as a concise line and two points on the graph. The floating graph makes it easier to see how a song will play than people who don't know how to read a music sheet. It is because, as one picture, it tells everything, such as when this pitch starts and where it ends. The floating graph provides a more expeditious comprehension.

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Deep Learning Model:

K-POP train data:

The train progressed throughout MIDI NOTE. After centralization, like Table 1, convert MIDI to MIDI NOTE with three columns using step, duration, and pitches. It has three columns. Figure 4 is one example of a sequence. The model will train 154 K-POP songs.

```
[[0.5703125  0.         0.32499986]
 [0.5546875  0.342857   0.32499986]
 [0.5703125  0.342857   0.48785694]
 [0.625      0.5142855  0.48785694]
 [0.5703125  0.5142855  0.32499986]
 [0.5703125  0.342857   0.48785694]
 [0.625      0.5142855  0.48785694]
 [0.5703125  0.5142855  0.32499986]
 [0.5703125  0.342857   0.32499986]
 [0.5625     0.342857   0.16214279]]
```

Figure 4: Example of a sequence of "Still With You" by JungKook MIDI NOTE format. All values are between 0 and 1.

LSTM:

LSTM was invented by Sepp Hochreiter in 1997. It stands for Long Short-Term Memory. It is invented to solve vanishing gradient problems. Construction of the original LSTM is input gate, output gate, forget gate and cell. The forget gate intends to teach to remove resources by resetting.[29] The architecture of LSTM is composed of sub-network (memory blocks). Three gates (input, output, and forget) adjust the flow of information related to the cell while the model remembers values over time intervals.[30]

It is a neural network architecture. This deep learning model is used in many fields. It is useful for training time series data. It is an advanced step of RNN. It prevents forgetting previous data.[31] The author uses LSTM. Most of the References and related work use LSTM to train MIDI files in composition, and LSTM is valuable data for time series.

Model:

```
Model: "model"
_________________________________________________________________________________________
 Layer (type)                Output Shape              Param #   Connected to
=========================================================================================
 input_1 (InputLayer)        [(None, 25, 3)]           0         []

 lstm (LSTM)                 (None, 128)               67584     ['input_1[0][0]']

 duration (Dense)            (None, 1)                 129       ['lstm[0][0]']

 pitch (Dense)               (None, 128)               16512     ['lstm[0][0]']

 step (Dense)                (None, 1)                 129       ['lstm[0][0]']

=========================================================================================
Total params: 84354 (329.51 KB)
Trainable params: 84354 (329.51 KB)
Non-trainable params: 0 (0.00 Byte)
```

Figure 5: The model summary of learning MIDI file. Every dense is connected to LSTM.

It also helps the model remember previous data to learn MIDI NOTE more productively. Three variables are added as inputs: step, pitch, and duration (Figure 5). I gave temperature parameters 1 and 2 to demonstrate the effectiveness of temperature on the music composition. The temperature parameter tells the model when it composed the music using only model knowledge or credibility.

Results and Discussion

K-POP data organize:

Usually, a song is about 3 minutes long. However, since each song has a different length, I cut it into 60 seconds to make it the same length for productive training. The model will learn every song for 60 seconds. Sixty seconds is the minimum time that includes the song's verse and chorus. In addition, 60 seconds also consists of the hook.

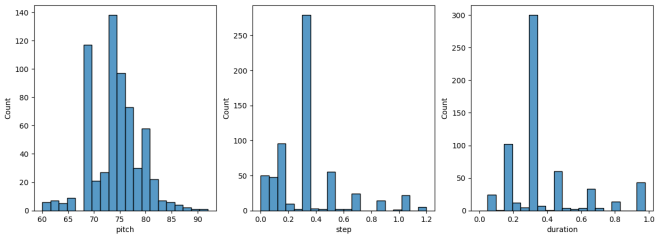

Figure 6: It is "Still With You" by JungKook's plot distribution. It has three graphs: pitch, step, and duration. Step and duration have the same highest count, at 0.3.

The Figure 6 graph shows the song "Still With You" as a composition of pitch, step, and duration. The major pitch is between 70 to 80. The average step is about 0.3. The average duration is also about 0.4. The song features the same step and duration length.

Temperature Parameter and Train Model:

The temperature parameter is used to manage the shape of the probability distribution. The low temperature intends to advance the quality of generation results. On the other hand, it creates repeating issues.[32]

With 50 epochs, the model did train. While model training MIDI notes, to prevent overfitting, it is coded to stop learning if a certain amount of loss continues to rise (Figure 7).

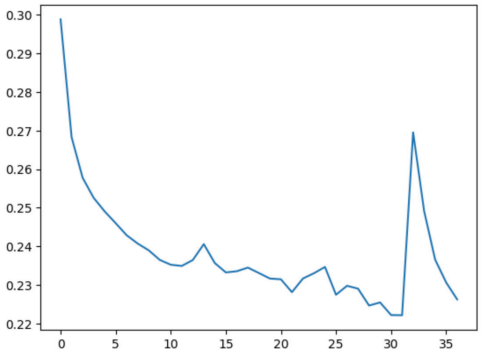

Figure 7: Train the loss history of the model that stops learning. Due to the loss increase at epoch 30, it stops learning at epoch 35.

Result of composed song:

The model's output is MIDI NOTE. The generated song should be converted to MIDI to hear it. Compared to the original song, the melody became simple. Before training the model, when MIDI files were converted to Note and changed into MIDI, there was a similar problem. The sound was more direct. Moreover, even though all songs didn't have a high pitch, the resultant music had a high pitch. Like Figures 8 and 9, most notes had a high pitch and kept repeating.

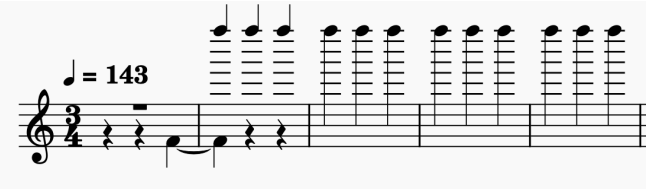

Figure 8: MIDI output music sheet when temperature is 1. It has high pitches.

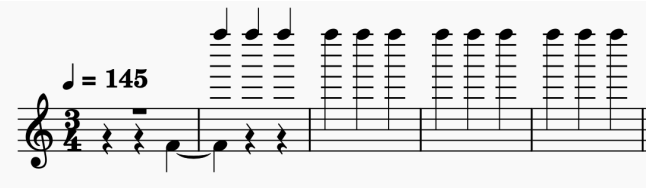

Figure 9: MIDI output when temperature is 2. It shows notes similar to those in Figure 8 but has two more notes in the sheet.

Figures 8 and 9 are music sheets of output MIDI files from the Model. Those didn't make a big difference in the beginning part.

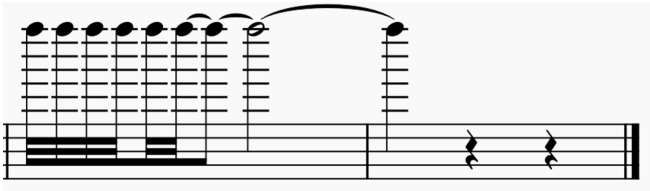

Figure 10: End of MIDI output's music sheet when temperature is 1. The notes are repetitive.

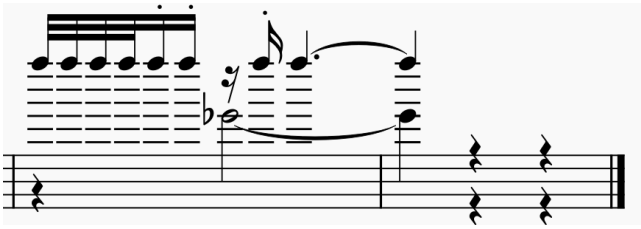

Figure 11: The end of the MIDI output's music sheet when the temperature is 2. It has an extra two rests and notes.

However, the bottom of the music sheet and the end of the music remained the same. In temperature 2, music has modulation (Figure 10, 11).

One K-POP Idol group music composition:

When the model learned 154 K-POP songs, the melody was too simple, and it didn't feel like catching the features of K-POP. There is a hypothesis because it learned too many different genres in K-POP, such as dance-pop or R&B. Thus, the hypothesis is that if only one group is trained, the song will show its own group's characteristics and K-POP characteristics more. Therefore, the model was learned using Black Pink and BTS group songs.

BLACKPINK:

The color of the group's music is evident; most of BLACKPINK's songs were written by the same writer, TEDDY, so the similarity of the songs is higher than that of other groups. In the case of (G)-IDLE, similar to BLACKPINK, Jeon So-Yeon, leader of (G)-IDEAL, wrote and composed all the title songs, so each music has a similar color, but there is a limit to the data that can be collected, so BLACKPINK was chosen. Nineteen songs were learned in the same condition and method as all K-POP songs.

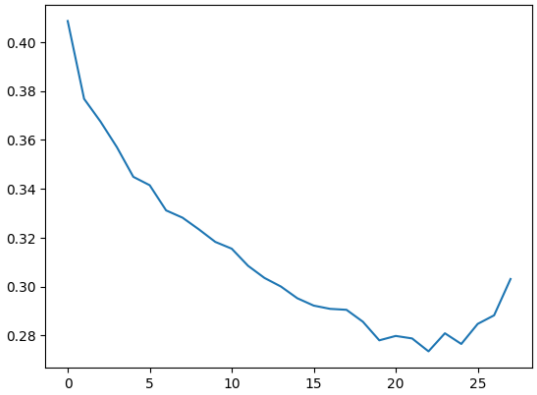

Figure 12: Model train loss history when train BLACKPINK data. After about 22 epochs, the loss increased, and when the epoch is over 27, it stops learning.

While training the songs, when epochs are about 23, it shows the loss is rapidly rising. As the code is set, it stops learning due to the rising of the loss (Figure 12).

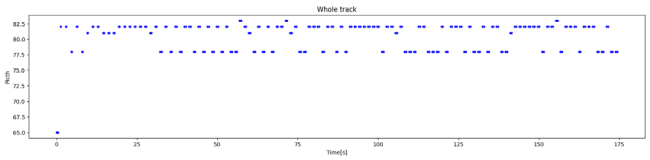

Figure 13: BLACKPINK model output plotting graph when the temperature is 1. It has high-pitch classes.

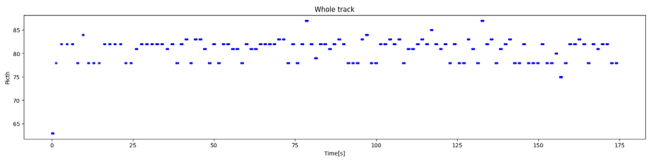

Figure 14: BLACKPINK model output plotting graph when the temperature is 2. It has different pitch classes compared with Figure 21.

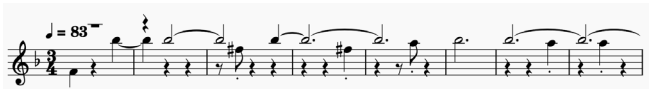

Figure 15: The BLACKPINK model outputs a music sheet when the temperature is 1. It has more rest than when the temperature is 2.

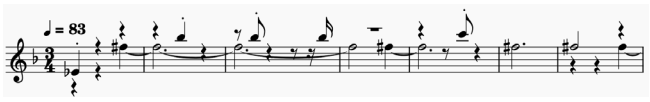

Figure 16: BLACKPINK model output music sheet when the temperature is 2. It has the same number of notes as when the model temperature is 1.

Figures 13 and 14 are MIDI files that the model composed. Unlike when training 154 K-POP songs, there is a difference between temperatures of 1 and 2, and the melody is more abundant. In Figure 14, the note has a higher pitch and less

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notes than in Figure 13. Figure 14 has the highest pitch, which is about 84. The song's beginning melodies resemble BLACKPINK's "How You Like That." Figures 15 and 16 are music sheets of Figures 13 and 14. They have similar melodies and the same number of notes in the sheets.

BTS:

BTS has a clear music color. Their recent music genre is dance pop. I collected more MIDI files than other groups. BTS was a group that had enough data to train. It has 15 songs. The method and condition of learning are the same as training all 154 K-POP songs.

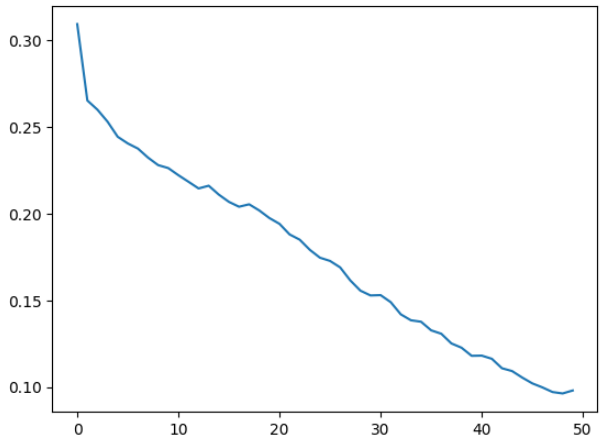

Figure 17: Model train loss history when training BTS data. It is an ideal train loss history. When the epoch is 2, the loss dramatically drops.

While training for a BTS song, there was no stopping. The loss kept decreasing (Figure 17).

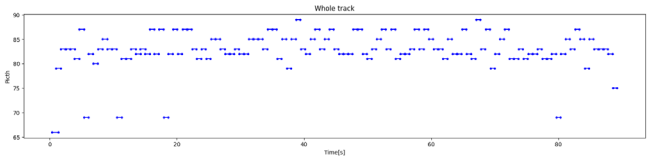

Figure 18: BTS model output plotting graph when the temperature is 1. It is a similar melody when the temperature is 2.

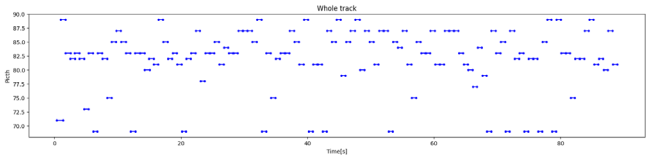

Figure 19: BTS model output plotting graph when the temperature is 2. It shows the whole track of model output. It has a smaller pitch range than when the temperature is 1. The pitch class range is 70 to 90 compared to 65 to 90 when the temperature is 1.

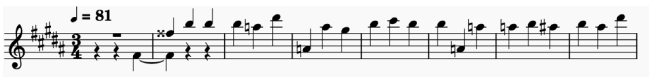

Figure 20: BTS model output music sheet when temperature is 1. It is four-thirds beat.

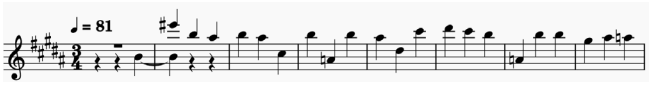

Figure 21: The BTS model outputs a music sheet when the temperature is 2. It has a similar melody to when the temperature is 1. They have the same number of rests and notes.

Figures 18 to 21 show a music model generated depending on the temperature. Figure 19 has more notes than Figure 18. The main melodies and duration of the notes are mostly similar, but the range of pitches and number of notes were different. Figure 18 has a wide range of pitches from 65 to 90.

When the temperature parameter is high, the melody and notes are more plentiful and feel like real music. Rather than training too many diverse genres simultaneously, training the same or similar genres simultaneously is more effective in writing music than humans.

Like collaborative generative AI technology and K-POP music, it is expected that other fields can be incorporated. With these technologies, society can expect to develop by combining each field.

However, commercializing this technology would take much work due to data limitations. While converting the MIDI file to NOTE and converting MIDI again, the song became simple.

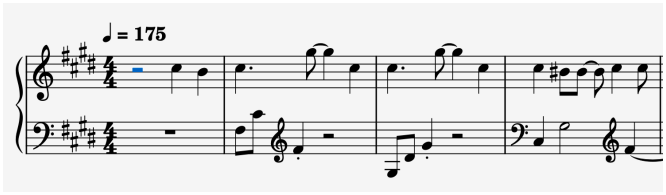

Figure 22: Music sheet of the original MIDI ("Still With You" by JungKook). It is double-handed.

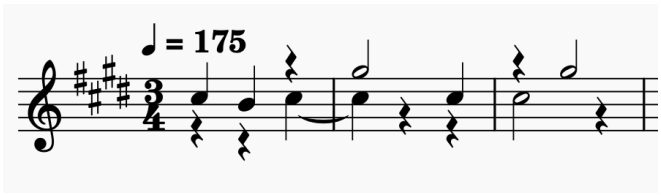

Figure 23: Music sheet after MIDI NOTE to MIDI ("Still With You" by JungKook). It has the same number of notes after it changed into single-hand sheet music.

Like Figure 22, the original MIDI file has two hand sheets of Music, but after converting the MIDI note and changing it into MIDI format, it became single-hand sheet music (Figure 23). Only the main melody of the song was extracted. Moreover, even though the model studies songs for 60 seconds, sometimes, the result is much shorter than that.

■ Conclusion

This paper created a generative K-POP music composition model by LSTM. However, when converting to a MIDI file, music becomes simplified. The MIDI file that the model generates comprises only one instrument, the Piano. Thus, if a song has more instruments, the song becomes more complete. Due to the limited availability of MIDI files for K-pop songs, I was only able to train the model on 154 songs. However, in the future, I plan to increase the size of the dataset by convert-

ing commercially available K-pop songs into MIDI format. In addition, I will improve the learning algorithm to maximize training efficiency even with a relatively small number of songs.

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REVIEW ARTICLE

Monitoring Heavy Metals by Microbial Fuel Cell: A Review

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ABSTRACT: Heavy metals, often introduced into surface waters through anthropogenic activities, pose significant environmental and human health risks. The urgent need for their rapid, simple, and portable detection has intensified due to the increased leaching of these metals into aquatic systems. Microbial fuel cells (MFCs) have emerged as a promising and cost-effective bioelectrochemical technology for sustained, long-term operation *in situ* application. This paper reviews the progress in MFC-based biosensors for detecting heavy metals, which can be categorized into two types: biofilm-based MFC biosensors and those utilizing redox reaction at the cathode. In biofilm-based MFC biosensors, the presence of heavy metals inhibits the metabolism of exoelectrogenic bacteria, leading to a proportional reduction in electrical signals (voltage or current), which can be exploited for metal detection. Conversely, the redox reaction of heavy metals at the cathode proportionally increases electrical signals, providing another detection method. Future research should focus on the enrichment of functional bacteria, including novel exoelectrogenic strains and microbial consortia that work synergistically, as well as the development of innovative materials that offer cost-effective alternatives to expensive noble metal catalysts.

KEYWORDS: Environmental Monitoring, Pollution Control, Biosensors, Heavy Metals, Electrode.

Introduction

Heavy metal pollution in surface water is a significant global environmental issue, stemming from both natural sources, such as rock weathering, and anthropogenic activities, including industrial production and sewage discharge. Zhou and colleagues analyzed historical data on total concentrations of 12 heavy metals in global surface water from 1972 to 2017, revealing a shift in the primary sources of metal pollution from mining and manufacturing to rock weathering and waste discharge.[1] Industries such as mining, electroplating, and smelting release large volumes of heavy metal-laden wastewater, raising widespread concerns about water contamination.[2] For instance, Li and colleagues reported that in 2012, approximately 221.6 × 10^8 tons of industrial wastewater were discharged, containing around 388.4 tons of heavy metals, including Pb, Hg, Cd, Cr, and T-Cr.[3] In typical electroplating wastewater, Cu concentration can reach up to 1500 mg/L.[4] When such wastewater is discharged without adequate treatment, it contaminates surface water, posing significant threats to human health. In response, many countries have enacted stringent regulations to limit the discharge of heavy metals in wastewater systems. The detection and monitoring of these heavy metals has become a critical area of focus due to their excessive leaching into the environment.

Conventional testing methods and modern innovative sensors can be used to detect heavy metals. Traditional testing techniques for heavy metals, such as flame or furnace spectroscopy and atomic absorption spectroscopy, are highly selective and sensitive, making them the gold standard for quantifying and monitoring heavy metal ions. However, these methods are costly, lacking field portability, requiring skilled operators, and thus not suited for rapid detection.[5, 6] With the rise in heavy metal pollution due to industrial development, there is increasing interest in developing fast, portable, and automated detection systems.[7] This leads to advancement in sensors, including Enzyme-immobilized biosensors, Aptamers-based biosensors, Ion imprinted sensors, and electrical sensors.

Microbial fuel cells (MFCs) have emerged as a cost-effective and promising bio-based electrochemical technology for long-term running and *in situ* monitoring. Researchers have explored MFC-based technologies for detecting environmental contaminants and degrading contaminants while simultaneously generating power.[8–12] MFCs function by harnessing microbial metabolism to convert chemical energy directly into electrical energy via microbial metabolic processes. In most MFC biosensors, exoelectrogenic bacteria are inoculated in the anode chamber to sense contaminants. When the metabolic activity of these bacteria is inhibited by a contaminant, the value of voltage or current is weakened, forming the basic principle of biofilm-based MFC biosensors.[13] Recently, novel MFC-based sensors have been developed that utilize the cathode through redox reaction, instead of exoelectrogenic bacteria, to sense heavy metals.[14, 15] These sensors can monitor repeated pollution events, as the sediment adsorbs heavy metals, thereby protecting exoelectrogenic bacteria from being harmful by heavy metals.

Several recent review articles have explored MFC-based biosensors for detecting heavy metals.[16, 17] Kumar and colleagues provided an overview of the mechanisms behind MFC-based biosensors for on-site toxicity compounds' detection, such as BOD, COD, heavy metals, organic toxicants, antibiotics, and acidic toxicity.[17] They emphasized the critical roles of biofilm and external resistance in these processes. On the other hand, Noori and colleagues focused on microbe-electrode interacti-

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ons within MFC for heavy metal detection, highlighting how the electrode's microenvironment influences these interactions, which in turn leads to a decrease in current due to the intrinsic toxicity of heavy metals.[16]

This review addresses recent advancements in MFC-based heavy metals detection, categorizing the technologies into biofilm-based MFC biosensors and cathode-based MFC biosensors driven by redox reactions. It further discusses the monitoring mechanisms and limitations of each approach while providing a forward-looking perspective on the development of advanced MFC biosensors to overcome existing challenges.

Discussion

MFCs Technologies and Their Functions:

A classical MFC is a bio-electrochemical system that transforms chemical energy into bioelectric energy using microbes as biocatalysts. It consists of an anaerobic chamber and an aerobic chamber, separated by a proton exchange membrane, with an anode and a cathode electrode placed in their respective chambers.[18] These electrodes link through an external circuit, allowing energy to be harvested through an electric load.

The source of organic matter in the MFC system varies widely, ranging from pure compounds to complex mixtures, including organic acids (e.g., acetate, glucose, and sodium formate), sugars (e.g., glucose and mannitol), alcohol, phenolic compounds, polysaccharides, disaccharides, biomass, and synthetic wastewater,[18] with acetate and glucose being the most commonly utilized substrates for electricity generation.[19] Recent advancements have led to the development of novel MFC technologies, such as plant-MFC (P-MFC), sediment-MFC (S-MFC), algae-MFC (A-MFC), and constructed wetland integrated MFC (CW-MFC).[18, 20] These advanced MFCs utilize different fuel substrates; for instance, P-MFCs harness plant roots, S-MFCs rely on organic-rich sediment, and A-MFCs use algae biomass as fuel.

The primary function of MFC is electricity generation through the oxidation of organic matter, a key feature of this technology.[21] Beyond generating bioelectricity, MFCs offer significant benefits in wastewater treatment by utilizing the organic matter in wastewater as a fuel source. This dual capability positions MFCs as a highly balanced and environmentally harmonious form of energy production, directly converting waste into electric power. Recently, MFCs have gained attention for their innovative application as biosensors for quick and online monitoring of wastewater, including chemical oxygen demand (COD), toxic compounds, volatile fatty acids, and microbial activity.[22] MFCs can also detect heavy metals using biofilm-based MFC biosensors, where the inhibition of exoelectrogenic bacteria's metabolic activity results in weakened electrical signals (voltage or current),[16] including anodic biofilm-based (Figure 1) and cathodic biofilm-based (Figure 2) biosensors. Alternatively, heavy metals can be detected at the cathode-based biosensors through redox reactions, as shown in Figure 3. Table 1 provides a summary of MFC-based sensors for monitoring heavy metals in aqueous solution, which will be discussed in the following sections.

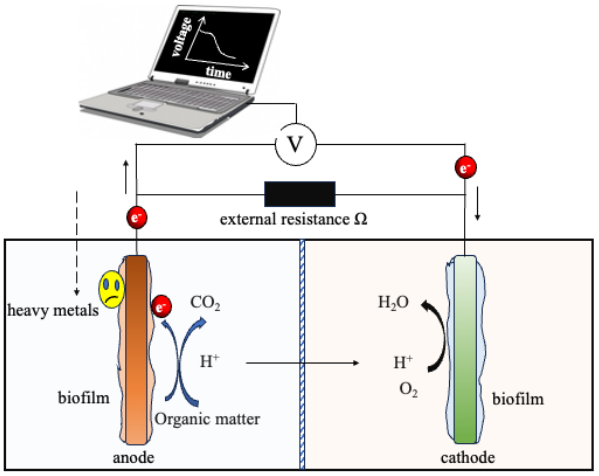

Figure 1: Schematic illustration of the anodic biofilm-based MFC biosensors setup for heavy metal detection. Exoelectrogenic bacteria within the anode chamber generate electrical signals, which serve as indicators of metabolic activity. This kind of biosensor operates by measuring the inhibition ratio of electrical signals, as increasing heavy metal concentrations suppress exoelectrogenic bacterial metabolism on the anode. The inhibition ratio is defined as the relative decrease in output current (or voltage) caused by the toxicant, compared to the baseline current (or voltage) before exposure.

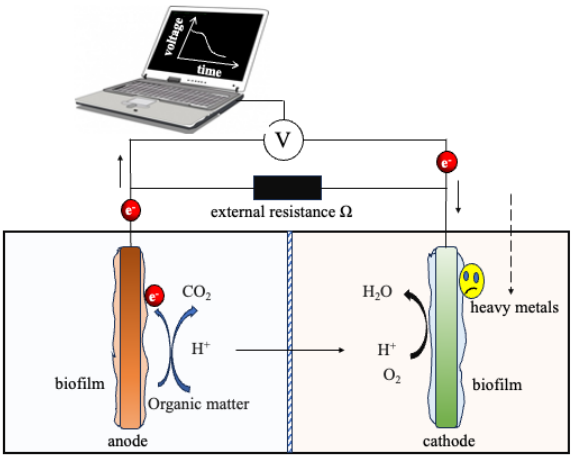

Figure 2: Schematic illustration of cathodic biofilm-based MFC biosensors setup. The formation of electroactive biofilm on the cathode enables certain microbes to utilize electrons for respiration through reversible metabolic pathways. The kind of biosensor operates by measuring the inhibition ratio of electrical signals, as elevated heavy metal concentrations suppress electroactive bacterial metabolism on the cathode. An electroactive biofilm is a layer of microorganisms that grow and attach to the electrode surface of an MFC.

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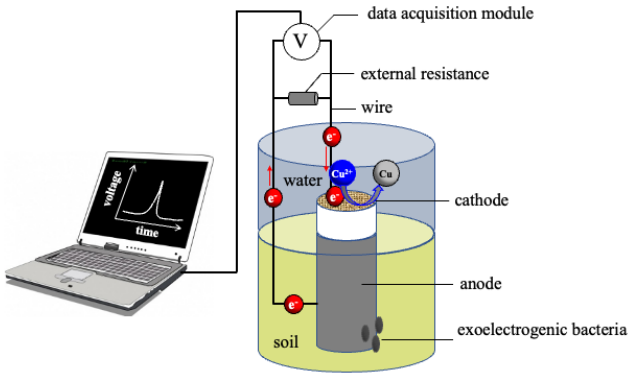

Figure 3: Schematic illustration of a cathode-based MFC biosensor utilizing redox reaction. The cathode-based MFC sensor detects heavy metals through cathodic reactions, generating a voltage signal that peaks as heavy metal concentrations increase on the cathode. The kind of biosensor operates by measuring the increase ratio of electrical signals, as elevated heavy metals produce more electrons on the cathode.

Table 1: Types of MFC-based biosensors for heavy metal detections using various cathodes, anodes, and microorganisms. This table summarizes the types of heavy metals detected, the corresponding MFC biosensor types, the anode and cathode materials used, and the main microorganisms involved in MFC systems.

Heavy metals	Biosensor types	Anode	Cathode	Microorganisms	Detection range and response time	Reference
Cu^{2+}	anodic biofilm	Carbon Cloth	Carbon cloth/Pt	Mixed microbial consortium	–	23
Cu^{2+}	anodic biofilm	Stainless steel	Platinum	Mixed microbial consortium abundance with Geobacter and Clostridium	5.0-320 mg/L, 30 s	14
Cu^{2+}	anodic biofilm	Stainless steel	Platinum	Paddy sediments	12.5-400 mg/L, 20 s	15
Cu^{2+}	Cathodic biofilm	MWCNT/ activated carbon sheet	MWCNT /activated carbon sheet	Soil	100-500 mg/L, 24 min	24
Cu^{2+}	anodic biofilm	Carbon felt	Carbon felt	Activated sludge	1.0-50 mg/L, 1.0 mg/L (45 min), 50 mg/L (10 min)	25
Pb^{2+}	anodic biofilm	Graphite felt	Graphite felt	Mixed microbial consortium	1.0-5.0 mg/L, 10 min	26
Pb^{2+}	anodic biofilm	Graphite plate	PVDF/activated carbon	Mixed microbial consortium	0.1-5.0 mg/L	27
Pb^{2+}	anodic biofilm	Graphite plate	PVDF/activated carbon	Sewage sludge	1.0-2.0 mg/L, 3 min	28
Pb^{2+}	anodic biofilm	Graphite plate	PVDF/activated carbon	Sewage effluent	1.0-3.0 mg/L, 60 min	9
Cd^{2+}	anodic biofilm	Carbon Cloth	Carbon Cloth	Enriched electroactive bacteria	1.0-50 µg/L, 4 min	29
Cd^{2+}	anodic biofilm	Carbon paper	Carbon paper/Pt	Mixed microbial consortium	1.5 mg/L	30
Ni^{2+}	anodic biofilm	Graphite plate	Graphite plate	Mixed microbial consortium	20-180 mg/L	31
Cr^{6+}	anodic biofilm	Carbon Cloth	Carbon cloth/Pt	Mixed microbial consortium	1.0-8.0 mg/L	32
Cr^{6+}	anodic biofilm	Carbon Cloth	Carbon cloth/Pt	Anaerobic sludge	1.0-100 mg/L	33
Cr^{6+}	Cathode-based with redox reaction	Stainless-steel tube	Platinum mesh	Soil	10-120 mg/L	34
Hg^{2+}	anodic biofilm	Graphite felt	Graphite felt	Mixed microbial consortium	1.0-5.0 mg/L	26
Cu^{2+}, Zn^{2+}, Ni^{2+}, Cr^{6+}	anodic biofilm	Activated carbon/carbon felt/stainless	Activated carbon/carbon felt/stainless	Aerobic sludge and anaerobic sludge	0.0.4-82.71 mg/L	35
Cu^{2+}, As^{5+}	anodic biofilm	Carbon felt	Carbon fiber brush	Anaerobic sludge	1.0-10 mg/L for Cu^{2+}, 0.5-5.0 mg/L for As^{5+}	36
Cu^{2+}, Cr^{6+}, Zn^{2+}, Ni^{2+},	anodic biofilm	Graphite sheet	Graphite sheet	Anaerobic sludge	5-20 mg/L	37
Hg^{2+}, Cr^{6+}, Pb^{2+}	Cathodic biofilm	Titanium-coated TiO_2/IrO_2 mesh	Graphite rods	Soil	Hg^{2+}: 0.5-2.5 mg/L; Cr^{6+}: 1-19 mg/L; Pb^{2+}: 10-48 mg/L; 1 min	38

Biofilm-Based MFC Biosensors for Monitoring Heavy Metals:

In most MFC biosensors, exoelectrogenic bacteria are employed to detect heavy metals. The monitoring mechanisms of these biosensors rely on measuring the inhibition ratio of the generated electrical signals. As heavy metal concentrations increase, they inhibit the metabolic activity of the bacteria, resulting in decreased electrical signals. Commonly used exoelectrogenic bacteria are acclimated within the anode chamber of the MFC. For example, a dual-chamber MFC-based biosensor has been developed and optimized for monitoring Cu in wastewater. Under conditions of 1000 Ω external resistance and 50 mM $K_3Fe(CN)_6$ as catholyte, a linear relationship was observed between maximum voltage output and the concentration of heavy metals.[36] The proportional inhibition ratio for both metals suggests that the electrical changes were primarily due to electrogenic bacteria's activity on the surface of the anode. Researchers have also isolated electroactive microbial consortia from industrial wastewater to assess their bio-sensing capabilities against four heavy metal contaminants: Cr^{6+}, Cu^{2+}, Ni^{2+}, and Zn^{2+}.[37] The inhibition ratios for these metals increased with their concentrations, and Sodium acetate was used as a major organic substance to enhance biosensors' reusability. These electrochemically active biofilms, typically formed under anaerobic conditions, require a lengthy formation period.

Compared with the extensive research on exoelectrogenic bacteria in the anode chamber, there is a limited investigation into the role of these bacteria in cathodic biofilm-based MFC biosensors.[16] Developing electroactive biofilm on the cathode

leverages the reversible metabolic pathway of certain microbes, allowing them to uptake electrons for respiration. This setup enables biological reactions at the cathode, with reaction kinetics potentially influenced by the adverse effects of incoming pollutants. Cathode-based biosensors have been proposed to enhance the detection range of MFCs in autotrophic microbial systems. For instance, Prévoteau and colleagues constructed an electroautotrophic biofilm on a carbon electrode at +0.2 V vs. Ag/AgCl, successfully detecting heavy metals such as Hg^{2+}, Cr^{6+}, and Pb^{2+}.[38] The observed current decline and subsequent recovery demonstrate the self-regenerative capabilities of cathode-based microbial sensors. To further advance sustainable, low-cost, and responsive technology, photo-microbial fuel cell sensors for detecting heavy metals in wastewater have been explored. These sensors offer a broader detection range compared to traditional MFC biosensors.[39]

Key factors influencing the performance of MFC biosensors include the types of microorganisms used, concentration and type of heavy metal, environmental conditions, electrode materials, design, substrate availability, and the presence of interfering substances. For instance, Nong and colleagues investigated two types of MFC biosensors for Pb^{2+} detection, operating at temperatures of 10 ˚C and 25 ˚C. Their study revealed that the inhibition rate of voltage was 22.81% at 10 ˚C, compared to only 5.9% at 25 ˚C.[9] Electrochemically active biofilms, typically formed under anaerobic conditions, often require prolonged periods to develop. However, Wang and colleagues demonstrated that using aerobic sludge as an inoculation source enabled the rapid formation of stable electrochemical biofilms within 35 hours.[25] To enhance MFC performance, various anode materials have been explored. For example, Xu and colleagues modified a 3D porous nitrogen nanotube sponge anode with chitosan and Polyaniline, improving microbial enrichment, adhesion, and power density.[40] Additionally, the combination of a photocathode with a microbial anode enhanced sensitivity for Cu^{2+}due to the P-N heterojunctions in CuO/ZnO that improved electron transport.[39] Furthermore, incorporating biochar as a filler in CW-MFC biosensors increased output voltage and detection range due to oxidative phosphorylation promoted by biochar.[35] Biofilm-based MFC biosensors face limitations in differentiating individual metals in mixed contamination, as their signals reflect cumulative metabolic inhibition. Future designs could integrate synthetic microbial consortia with metal-specific genetic reporters to enhance selectivity.

Cathode-based MFC biosensors utilizing redox reaction:

Biofilm-based MFC sensors that rely on exoelectrogenic bacteria for heavy metals detection typically struggle with repeated contaminations, as the bacteria must be recovered or reinoculated after each event. To address this limitation, Wu and colleagues introduced a sediment MFC (SMFC) sensor that uses the cathode to detect heavy metals, thereby protecting the exoelectrogenic bacteria from inhibition and eliminating the need for reinoculation.[14] When Cu^{2+} solutions were added to the overlaying water, the sensor produced a voltage signal that peaked, with the increase from baseline to peak voltage linearly correlated with Cu^{2+} concentrations up to 160 mg/L. Liu and colleagues further explored this method to monitor Cu^{2+},[15] demonstrating that a cathode-based SMFC sensor could detect Cu^{2+} with minimal stress on exoelectrogenic bacteria, as Cu^{2+} was inactivated by the flooded soil. Additionally, a carbon-felt-based cathodic SMFC biosensor was developed for long-term monitoring of heavy metal ions in soil, generating a stable output voltage of about 400 mV within 2-5 minutes of metal ions injection and effectively detecting Cd^{2+}, Zn^{2+}, Pb^{2+}, and Hg^{2+} over four months without significant performance degradation.[41] These SMFC biosensors, utilizing a floating cathode for detection of heavy metal shocks, showed the ability to keep long-term stability in the field, producing voltage peaks in response to repeated heavy metal pollutant events. The voltage peaks refer to the maximum voltage output observed during a certain period of operation. The oxygen reduction reaction (ORR), which typically occurs on the cathode in the MFC system, plays a crucial role in power generation. When heavy metals are introduced, they are reduced to the lower valence state on the cathode by consuming electrons generated by bacteria, as confirmed by the detection of $Cr3^{+}$ on the cathodic surface after the introduction of $Cr6^{+}$.[34] Similarly, the reduction of Cu^{2+} to Cu^{+} on the SMFC sensor's cathode triggered a voltage peak, further confirming this reduction process.[14] This reduction reaction accelerates electron consumption on the cathode, instantly increasing the current and generating a voltage peak.[34] The key redox reactions involved are as follows:

$$Cu^{2+} + e^{-} \rightarrow Cu^{+} \quad (1)$$

$$Cr_2O_7^{2-} + 14H^{+} + 6e^{-} \rightarrow 2Cr^{3+} + 7H_2O \quad (2)$$

While redox-based cathode biosensors excel in detecting specific metals (Cu^{2+}, Cr^{6+}), their performance in multi-pollutant systems requires further validation.

Biofilm-based and redox-based MFC biosensors each offer distinct advantages and limitations for heavy metal detection. Biofilm-based sensors rely on the metabolic activity of exoelectrogenic bacteria, where the presence of heavy metals inhibits electron transfer, leading to a measurable decline in electrical output. This approach is often limited by slow response times and reduced reusability due to biofilm damage from repeated metal exposure. In contrast, redox-based biosensors detect heavy metals through direct electrochemical reactions at the cathode, producing rapid voltage peaks at metals are reduced. These biosensors exhibited faster response times, greater reusability, and enhanced stability. However, redox-based systems are particularly effective for detecting redox-active metals.

Challenge and Future Perspective:

Despite significant progress in the MFC biosensors for heavy metals detection, several challenges and opportunities for future research remain:

Enriching functional bacteria:

Exoelectrogenic bacteria, the biocatalytic core of MFCs, are vital for converting chemical energy into electrical energy through extracellular electron transfer. However, microbial activity can decline over time due to factors like nutrient depletion, toxicity of metals, or microbial aging. Long-term stability is essential for practical monitoring, but microbial performance can degrade, affecting the lifespan and reliability

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of the biosensor. What's more, only a limited number of exoelectrogenic species that significantly enhance power output have been identified to date.[42] Discovering new exoelectrogens and gaining a deeper understanding of their electron transfer mechanisms are essential for further advancement. While research has predominantly focused on exoelectrogens at the anode, there is a pressing need to explore those at the cathode more extensively. Additionally, microbial consortia that cooperate synergistically could further enhance power generation.[43] Understanding individual microbial behaviors and community interactions in natural habitats is crucial for developing effective synthetic consortia. Future research should focus on exploring cooperative microbial communities and applying metabolic engineering techniques to optimize electricity generation. Genetic modifications to enhance specific metal ion interactions or boost electron transfer capabilities are promising.

Materials innovations:

Advancements in materials science are poised to significantly enhance the efficiency of MFC. The development of advanced materials with improved catalytic activities can facilitate more effective interactions between microbes and electrodes, promoting biofilm growth and enabling more efficient electron transfer. This, in turn, can increase the sensitivity and selectivity of MFCs.[44] Conductive materials that bridge biological connections for electron transport and those that enhance electron transfer rates are particularly crucial for the advancement of MFC technology. Cost-efficient materials, such as metal oxides and conductive polymers, are especially promising as they can replace expensive metals while improving overall MFC efficiency. The properties of anodic materials, including biocompatibility, microbial adhesion, electrochemical efficiency, and effective electron transfer mechanisms, are vital for biocurrent generation and the practical application of MFCs.[45, 46] Additionally, the development of efficient and low-cost catalysts, such as $CoMn_2O_4^-$ doped graphene oxide, can accelerate electron transfer and enhance electrochemical activity at the cathode, offering a viable alternative to expensive noble metal catalysts.[47] Further research into such efficient and cost-effective catalysts is needed.

Moreover, introducing sunlight into MFC to construct advanced photo-MFC technology offers numerous benefits and can be pursued.[48] Theoretical guidance for selecting electron shuttles to optimize sustainable energy production is also crucial. The performance of MFC heavily depends on the properties of the cathode, with redox potential being a crucial factor. Cathode materials should possess high redox potential to efficiently capture protons, thereby enhancing MFCs' performance.[49] In an MFC sensor utilizing redox reactions at the cathode, heavy metals can act as electron acceptors. To maintain high reaction rates, platinum (Pt)-based catalysts are often employed due to their effectiveness in reducing the activation energy of cathode reactions. Thus, exploring alternative cathode materials, like single-atom-doped carbons and transition metal complexes, could provide a cost-effective substitute for expensive noble metal catalysts.

Wireless and remote sensing for MFC biosensors:

Wireless and remote sensing capabilities are key to the advancement and practical deployment of MFC biosensors. These capabilities enable real-time, continuous, and long-distance monitoring of water quality without the need for on-site intervention, making it particularly suitable for large-scale or remote areas that are otherwise difficult to monitor. However, wireless MFC biosensors typically require power for data transmission and processing, which can be a challenge when deployed in remote or off-grid locations. While MFCs themselves can generate electricity, the additional power requirements for wireless communication and data storage might exceed what the MFC can produce, particularly in continuous operation scenarios. To address the energy demand, there is a need to optimize MFCs for better energy harvesting, potentially incorporating energy storage systems such as capacitors or rechargeable batteries. Alternatively, integrating energy harvesting technologies (e.g., solar panels) alongside the MFC system could help ensure a long-term, uninterrupted power supply. However, balancing the energy needs for MFC functionality and wireless data transmission while maintaining system longevity is still a challenge.

Conclusion

This paper reviewed recent advancements in MFC biosensors for the detection of heavy metals, focusing on biofilm-based MFC biosensors and those employing redox reactions at the cathode. It was observed that MFC-based biosensors can detect a variety of heavy metals across a wide range of concentrations through appropriate techniques. However, further research is needed to explore MFC biosensors that utilize redox reactions at the cathode, particularly regarding detection mechanisms, influencing factors, and reaction pathways. Enhancing the effectiveness of MFC biosensors for heavy metals monitoring will likely depend on advancements in chemical processes, molecular biology, and innovative materials. Additionally, integrating wireless and remote sensing capabilities will be crucial for the broader deployment and practical application of MFC biosensors.

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