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RESEARCH ARTICLE

Enhanced Neuroprotective Delivery of Ashwagandha via Chitosan Nanoparticles: Improving Blood-Brain Barrier Permeability for Accessible Mental Health Therapeutics

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ABSTRACT: Tailored approaches to mental health care are crucial in enhancing its accessibility and efficacy. Focusing on cultural sensitivity, Ashwagandha (ASH), a serum cortisol-reducing evergreen shrub, is one of many herbal remedies in non-Western traditional medicine. Since existing off-the-counter ASH supplements lack efficiency in permeating the blood-brain-barrier (BBB), this study aims to investigate methods to increase the efficient delivery of ASH to brain cells and simultaneously enhance protective effects on neuron cells. We used a transwell model to mimic the BBB and tested the protective effects of the ASH-chitosan complex on brain cells subjected to H_2O_2-induced oxidative stress. Using a transwell BBB model with human endothelial and neuronal (SH-SY5Y) cells, we evaluated neuroprotection against H_2O_2-induced oxidative stress (7% concentration, inducing ~65% cell death). ASH-CNP complexes demonstrated significantly enhanced cell viability (83.0 ± 2.1%) compared to H_2O_2 controls (63.0 ± 1.8%, $p = 0.0016$), while individual ASH or CNP treatments showed no significant improvement. This higher cell viability was more prominent in the ASH-chitosan complex as compared to ASH or chitosan, individually. Through this study, a low-cost and applicable method of creating a complex that has protective effects and permeates the BBB more effectively, demonstrating possible improvements that can be made to existing ASH supplements. These findings demonstrate at least approximately 32% improvement in neuroprotection, supporting the development of enhanced over-the-counter ASH supplements for underserved populations with limited access to conventional mental health care.

KEYWORDS: Neurobiology, Ashwagandha (ASH), Blood-Brain-Barrier (BBB), Cultural Competence, Accessible Mental Health Care.

Introduction

Withania somnifera, an evergreen shrub commonly known as Indian ginseng or Ashwagandha (ASH), is a medicinal herb usually found in South Asia, Northern Africa, and the Middle East.[1] It is known for its effects on lowering stress and anxiety, which can be attributed to its ability to reduce serum cortisol levels. In addition, studies have shown that it can heighten sleep quality and improve cognitive function. The use of this Indian traditional medicine can be traced back approximately 3000 years, demonstrating the prominence of the shrub within the Indian culture of Ayurveda medicine.[2]

Similarly, traditional medicine such as Chinese Traditional Medicine (TCM), Unani Medicine, and Siddha Medicine are widely practiced in various regions of the world with their roots deeply ingrained within the respective cultures.[3] Involving acupuncture, herbal medicine, and spiritual therapies, traditional practices are preferred for some due to their perceived capabilities, affordability, and cultural significance. Globally, the TCM market size in 2024 was approximated to be 247.22 billion United States dollars, with projections for it to reach around 450 billion United States dollars by 2033.

Still, Modern Western Medicine (MWM) remains the dominant medical system globally. In contrast to traditional medicine, it employs a method in which medical conditions are treated through evidence-based approaches involving pharmaceutical drugs, radiation, and surgery.[4] In the United States alone, from the years 2000 to 2018, the pharmaceutical industry invested an estimated 879.3 million dollars into novel drug development, including failure and capital costs.[5]

Despite the proven effectiveness of MWM, the incorporation of traditional methods, also known as Complementary and Alternative Medicine (CAM), can increase the care accessibility of marginalized communities. This is executed by addressing cultural needs, reducing health gaps related to race and ethnicity, and providing a more comprehensive approach to health.[6] The World Health Organization encourages the evidence-based integration of CAM into conventional medical care to increase the availability of healthcare.

Specifically within the domain of mental health and psychiatric care, consideration of one's culture is especially important.[7] Studies have found that racial minorities have a greater skepticism toward prescription drugs compared to their white counterparts. This calls for more research to deepen the understanding of cultural attitudes toward MWM and strengthen the effectiveness in the integration of CAM and culturally competent practices that respond to the needs and limitations of various groups.[4]

In the case of Korean Traditional Medicine, acupuncture is often used to alleviate symptoms of depression and anxiety. Other methods, such as Uwhangchungsimwon, a herbal med-

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icine, are also used to treat mood disorders.[8] Alternatively, in Unani Medicine, which originates from Greece, hydrotherapy in the form of baths and compresses, along with yoga, is used to manage mental health.[9] Currently, despite their long-standing histories in the respective cultures, there is still a need for further research to support their treatment efficacy and cultural significance. Therefore, culturally competent integrative practices of medicine within the realm of mental health and psychiatric care can allow for decreased stigma and a greater receptivity to treatment for non-Western communities.[10]

This study aims to address the current limitations of CAM, specifically over-the-counter ASH supplements, by investigating the method of infusion in chitosan nanoparticles (CNPs). Though ASH has been extensively studied, it still has a restricted delivery capacity to human brain cells. Previous studies have shown that ASH is unable to effectively penetrate the blood-brain barrier (BBB).

To minimize this limitation, our study aims to develop a new delivery system using nanoparticles, specifically CNP. This type of nanoparticle is known to be effective in delivering specific particles through the BBB while maintaining low costs and being biodegradable. By examining how to improve the efficacy of over-the-counter ASH supplements, understanding appropriate dosage, and maintaining an appropriate price range, this study targets those with limited access to quality mental health care. Including cultural and ethnic minorities, communities of a lower socioeconomic status, and those in rural areas, we have aimed to address gaps in vital care.

Methods

Cell culture and materials:

RPMI1640 cell culture media was used to culture the human neuronal SH-SY5Y cell line (initially, the cell passage number was 10). The cell culture media were exchanged every three days to maintain the cells' health with nutrition. The cells were incubated in the CO2 incubator at 37 degrees Celsius. The cells were detached using Trypsin-EDTA dissociation buffer. Then, the experimental cell groups were prepared in 24 24-well cell culture plate.

Preparation of Chitosan Nanoparticles (CNPs) from Chitin:

The chitosan nanoparticles were synthesized from chitin. The molecular weight and degree of deacetylation were not measured in this process. The chitin was incubated with 1M NaOH for 8 hours to remove all proteins. The resulting materials were incubated with HCl to remove excess alkali. Then, the purified chitosan was used to synthesize the nanoparticles with sodium tripolyphosphate with 1% (v/v) acetic acid solution.

Ashwagandha (ASH) infusion into CNPs:

The ASH extract solution was prepared, and only solubilized fractions were isolated to remove the insoluble debris. The ASH extract was added dropwise to the chitosan nanoparticle solution to allow the complex to be formulated.

Cell Viability Test Using Hydrogen Peroxide on Human Brain Cells:

To stimulate the high-stress environment in neurodegenerative conditions, SH-SY5Y cells were exposed to hydrogen peroxide concentrations (0%, 0.5%, 1%, 3%, 7%, 10%) for 24 hours. Then, a cell viability assay was conducted using an automated cell counter machine (LUNA-FL). The topical concentration that induced around 50-70% cell death (7% H_2O_2) was selected for the next experiments.

Designing the Blood-Brain Barrier (BBB) System with Transwell:

A two-chamber transwell (0.4 μm) was used to model the BBB. Human endothelial cells were added to the upper chamber, and the neuronal cells were cultured in the lower chamber. The transwell was maintained for seven days to ensure the barrier formation.

Brain Cell Viability test of ASH-infused CNPs:

Following barrier formation, the upper chamber was treated with the following groups: Untreated control, H_2O_2 only (7%) – positive control for oxidative stress, H_2O_2 + ASH extract, H_2O_2 + CNP, and H_2O_2 + ASH–CNP complex. Each treatment was applied to the upper chamber while hydrogen peroxide was added to the lower chamber. This setup allowed for the analysis of the nanoparticle complex transport through the simulated BBB and its protective effects on neuron cells.

Results and Discussion

Optimizing hydrogen peroxide concentration for stress test on brain cells:

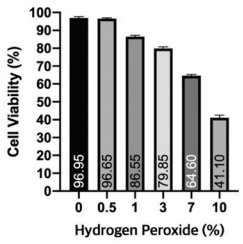

Figure 1: Increasing the hydrogen peroxide concentration decreased the viability of brain cells. The bar graph represents the mean and standard deviation of cell viability for each hydrogen peroxide concentration. This stress test provided more information that aided in the selection of hydrogen peroxide concentration based on its effect on cell viability in brain cell samples.

Table 1: Statistical analysis of cell viability under varying hydrogen peroxide concentrations. One-way ANOVA followed by Tukey's multiple comparisons test was performed to determine statistically significant differences in cell viability between untreated cells (0% H_2O_2) and each treatment group. Significant reductions in viability were observed from 1% H_2O_2 and higher concentrations compared to the control. The 7% H2O2 concentration sample was selected because it had a sufficient level of damage, allowing for neuroprotective effects to be observed most effectively.

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
0 vs. 0.5	0.3	-3.139 to 3.739	No	ns	0.9989
0 vs. 1	10.4	6.961 to 13.84	Yes	***	0.0002
0 vs. 3	17.1	13.66 to 20.54	Yes	****	<0.0001
0 vs. 7	32.35	28.91 to 35.79	Yes	****	<0.0001
0 vs. 10	55.85	52.41 to 59.29	Yes	****	<0.0001

The purpose of testing the cell viability levels of brain cell samples with various hydrogen peroxide concentrations was to simulate a high-stress environment. Therefore, this experiment aimed to find the optimal concentration of hydrogen peroxide for inducing brain cell stress. This optimal concentration of hydrogen peroxide was then planned for the downstream ex-

periment to investigate the impact of ASH-infused CNPs on brain cell protection.

Figure 1 shows six samples of brain cells with the respective hydrogen peroxide concentration (%) of 0, 0.5, 1, 3, 7, and 10, and their cell viability. The sample with 0% hydrogen peroxide yielded the highest cell viability of 96.95%; the 0.5% hydrogen peroxide sample yielded cell viability of 96.65%; the 1% hydrogen peroxide sample yielded cell viability of 86.55%; the 3% hydrogen peroxide sample yielded cell viability of 79.85%; the 7% hydrogen peroxide sample yielded cell viability of 64.60%; and the 10% hydrogen peroxide sample yielded the cell viability of 41.10%.

Compared to the 0% sample, the 0.5% sample showed no significant difference in cell viability ($p = 0.9989$) (Table 1). However, compared to the 0% sample, the 1% sample indicated a significant difference in cell viability ($p = 0.0002$). Between the 0% sample and the 3% sample, results showed a significant difference in cell viability ($p < 0.0001$). Similarly, compared to the 0% sample, both the 7% and 10% samples showed a significant difference in cell viability ($p < 0.0001$).

We aimed to use a sample with cell viability within the 50% range. This range was most desirable as cell viability of less than 50% would be too damaged to test the impacts of ASH-infused CNPs. On the other extreme, samples with greater than 70% cell viability would not be the most desirable for observing the protection effects of the ASH-infused CNPs, as they are already in relatively healthy condition compared to the 50% cell viability sample. As the 7% H2O2 concentration sample was within this range with a cell viability of 64.60%, it was selected for the following experiments.

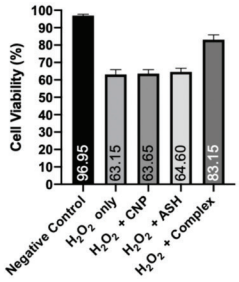

Figure 2: Ashgawanda with chitosan nanoparticle complex showed efficient transport through human blood vessel cells to human brain cells and showed a protective effect against hydrogen peroxide cellular stress. CNP, ASH, and CNP + ASH (Complex) were treated on top of the transwell for seven days. This result demonstrated a synergistic positive relationship between the CNP-ASH complex and cell viability.

Table 2: Statistical comparison of treatment groups under hydrogen peroxide-induced stress. One-way ANOVA followed by Tukey's multiple comparisons test was used to assess the significance of each treatment's effect on neuronal viability. The ASH–CNP Complex significantly improved cell viability compared to H_2O_2-only treatment, while CNP or ASH alone showed no statistically significant effect. The combination of CNP and ASH showed enhanced neuroprotective effects ($p=0.0016$) as compared to CNP and ASH, respectively ($p>0.05$).

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant	Summary	Adjusted P Value
H2O2 only vs. H2O2 + CNP	-0.5	-9.491 to 8.491	No	ns	0.9992
H2O2 only vs. H2O2 + ASH	-1.45	-10.44 to 7.541	No	ns	0.96
H2O2 only vs. H2O2 + Complex	-20	-28.99 to -11.01	Yes	**	0.0016

The experiment shown in Figure 2 was performed to test the impact of CNP, ASH, and the Complex on the cell viability of neurons treated with hydrogen peroxide. This was conducted by inducing hydrogen peroxide stress to the bottom chamber of the transwell, where the neurons lie, and by treating CNP, ASH, or the Complex on the upper chamber of the transwell, where the endothelial blood vessel cells lie. We hypothesized that the combination of CNP and ASH, the Complex, would increase the BBB permeability of ASH. Simultaneously, we tested the protective effects of ASH on H_2O_2 stress-induced environments.

The first sample was a negative control sample with no H_2O_2, CNP, or ASH. Such samples without any treatment maintained a high viability of approximately 97%. It serves as the reference for normal, healthy cell conditions. The second sample was exposed to hydrogen peroxide, inducing a stress environment. Treating cells with H_2O_2 alone significantly reduced cell viability to approximately 63%, confirming the known cytotoxic effect of H_2O_2 via oxidative stress. The third sample was exposed to H_2O_2 and CNP. This did not yield a statistically significant improvement in cell viability compared to sample 2, as adding CNP resulted in a viability of about 63% (Table 2). This result suggests that CNP by itself, under these conditions, provides minimal to no protective effects. The fourth sample was the addition of H_2O_2 and ASH. Treating ASH alone showed a slight improvement in viability of around 64%, but it was not statistically significant (Table 2). This indicates some protective trend, but not enough to fully counteract oxidative stress. Finally, the fifth sample of H_2O_2 and the ASH–Chitosan Complex resulted in a marked increase in cell viability of around 83% (Table 2). This improvement, compared with sample 2 of only H_2O_2, was statistically significant ($p = 0.0016$), indicating a synergistic or enhanced protective effect against H_2O_2-induced damage.

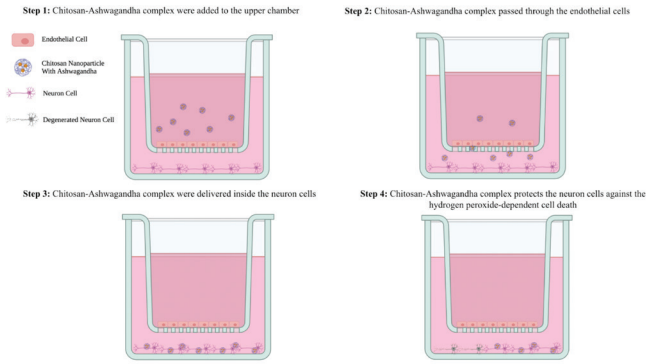

Figure 3: The overall summary of how the chitosan-ASH complex protects human neuron cells against hydrogen peroxide-dependent cell death. The results highlight the complex's potential to improve BBB-like delivery and offer a more accessible, safe alternative to conventional neuroprotective treatments. While limitations exist in model complexity, this study supports further optimization of CNP+ASH formulations and positions them as promising candidates for over-the-counter mental wellness support, particularly in underserved populations.

The transwell method was adopted, in which an upper and lower chamber are present with endothelial cells in the upper chamber and neuron cells in the lower chamber (Figure 3). This method was employed to study the particle migration of

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the Chitosan-ASH complex through the endothelial cells to the neuron cells in the lower chamber. The transwell with a pore size of 0.4μm was selected as it was most suitable for the relatively small size of the nanoparticles as compared to cells. This process can be divided into four distinct steps. First, the Chitosan-Ashwangandha complex was added to the upper chamber. Second, the Chitosan-ASH complex passed through the endothelial cells and then through the pores of the transwell. Third, the complex was delivered inside the neuron cells after entering the lower chamber. Fourth, the Chitosan-ASH complex was observed to protect neuronal cells against hydrogen peroxide-induced cell death, as cell viability was higher than that of the sample with H_2O_2 but without the Chitosan-ASH complex.

ASH is known for its antioxidant and adaptogenic properties. ASH alone showed a slight improvement in viability. Though not statistically significant, it suggests some protective potential, but insufficient improvement to counteract the oxidative stress induced in the setup.

The Chitosan-ASH complex, however, demonstrated a significant increase in cell viability. This indicates the potential enhanced delivery of ASH's bioactive compounds via CNP. As such, conjugation with chitosan can improve cellular uptake, stability, and sustained release of active molecules, enhancing the protective effect of ASH. These results demonstrate the synergistic protective effects of combining CNP and ASH for relieving H_2O_2 stress-induced cellular environments (Figure 3).

This enhanced neuroprotective effect of the ASH + CNP complex can be explained by the intracellular antioxidant defense mechanisms and improved BBB permeability. Due to their cationic nature, CNP interacts with endothelial cell membranes (negatively charged), to move across the BBB and thereby allowing a greater amount of ASH to reach the neurons. Then, the compound exerts adaptogenic and antioxidant effects by finding reactive oxygen species, reducing lipid peroxidation, and regulating stress-responsive signaling pathways. These include the hypothalamic-pituitary-adrenal (HPA) stress pathway. With the demonstrated enhanced neuronal survival, it is suggested that the CNP-mediated delivery simultaneously improved bioavailability while prolonging the retention and stability of ASH in neuronal environments.

The limitations of this study are that the representative models used, such as the transwell system and cell type, do not perfectly represent the characteristics of the BBB and the complexity of neuronal cells. The BBB is composed of multiple cell types, while there is only one cell type (SH-SY5Y) represented in the experiments. To better mimic the BBB, more cell types could be included in future studies to increase similarity to the multicellular nature of the barrier. Alternatively, mouse animal models can be used to test the efficacy of CNP + ASH (Complex). This mouse model may represent a structure more similar to the human BBB.

Though this study supports the idea that the infusion of ASH in CNP enhances its delivery and protection qualities, the optimization of the ratio between ASH and CNP is yet to be investigated. By studying this further, the overall stability of the ASH + CNP complex may reveal the understanding of its real-life applications. To further determine the approximate increase in cost-efficiency, additional research without the presence of the H_2O-induced environment must be conducted.

One of the most common ways in which the public consumes ASH is through over-the-counter supplements. Taking this into account, analyzing its overall stability and optimal ratio is crucial in understanding the viability of over-the-counter ASH-infused CNP supplements. The main factors in determining viability include shelf life, efficient mass production, and human metabolism rate.

This study increases the accessibility of non-Western medicine, specifically in the context of over-the-counter supplements. Targeting specifically off-the-counter ASH supplements due to their ties with improved mental health, availability, and affordability, this study aims to deliver enhanced results for communities that may experience augmented benefits. Some of these groups that experience difficulty accessing mental health care include youth, low-income communities, rural communities, and non-Western regions with hesitation toward psychiatric prescription drugs. This is not to say that the studied Chitosan-ASH complex can serve as a replacement for psychiatric prescription drugs, but rather a more accessible, alternative option to prescription drugs. This enhanced BBB delivery efficiency of the ASH-infused CNPs can result in lower dosages of ASH required to produce similar results, thereby reducing possible side effects associated with higher dosages of the evergreen shrub. In addition, this complex is safe for human consumption, as those with allergies—even shellfish allergies—can safely consume chitosan. The Chitosan-ASH complex is easily digestible by the human body, as it fully decomposes, and is non-toxic. As chitosan is positively charged, it can successfully be attracted to other negatively charged substances through electrostatic interaction; this poses an alternative research direction for chitosan complexes with other substances than ASH. Through these benefits of the complex investigated in this study, reservations surrounding the efficacy of non-Western traditional medicine can be challenged, further encouraging subsequent research of alternative medicine that may better cater to the needs of less-represented groups.

■ Conclusion

Previous studies have highlighted ASH's ability to relieve stress and improve cognitive functions. However, ingestion of over-the-counter ASH supplements poses limited benefits due to their restricted ability to penetrate the BBB. Therefore, this study leverages CNPs to formulate a complex with ASH to minimize this limitation. A transwell BBB system was developed to observe the impact of the Chitosan-ASH complex's BBB permeability and protective abilities against hydrogen peroxide stress. Our main result indicates that this infusion of ASH in CNPs augments its benefits through statistically significant increases in neuron cell viability. This result provides a new avenue for more effective over-the-counter ASH supplements, increasing the accessibility and maintaining costs of CAM for those with limited access to quality mental health

care, including cultural and ethnic minorities, communities of lower socioeconomic status, and those in rural areas.

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Mirae Do is an interdisciplinary researcher with a particular interest in combining her advocacy for mental health literacy and minority populations with neuroscience and psychology. By exploring ways to increase accessibility and inclusivity within mental healthcare through scientific research, she aims to foster a system that is considerate of one's unique needs.

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