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RESEARCH ARTICLE

Exosome-encapsulated miRNAs as Protective Agents against Huntington's Disease

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ABSTRACT: Huntington's disease (HD) kills approximately 2.27 people per million every year and slowly makes everyday activities more difficult for those with the genetic mutation. Key symptoms of HD include chorea, forgetfulness, impaired judgment, personality changes, mood swings, and depression. HD is clinically diagnosed by the HD mutation in the huntingtin gene: overly repeated C-A-G nucleotide sequences. Abnormal sequencing in the gene causes abnormal protein synthesis in the huntingtin protein, the impact of which compounds over time as the proteins create more clumps in the brain, which is why HD is mostly diagnosed in middle-aged individuals - when the proteins have had enough time to cause symptoms. HD is caused by prolonged damage to the striatum caused by the abnormal HD gene and protein which makes brain cells eventually die. The link between miRNAs and HD has been studied before but this study takes a step further by examining how this link can be exploited to create a cure. Using three types of strategically selected miRNAs, this study showed that encapsulating the miRNAs in exosomes for delivery into heavily damaged neurons increased cell proliferation. The 6-hydroxydopamine (6-OHDA)-induced HD model was used to test the impact of three different miRNA types and showed that all three miRNAs increase cell proliferation. Overall, this research focuses on the potential of miRNA with exosome use in therapeutic treatment for blocking neuronal cell death of HD patients. As current techniques evolve, this research can be used for the development of personalized therapy for HD patients.
KEYWORDS: Molecular Biology, miRNA, Huntington Disease, 6-OHDA, Cell Death.

Introduction

HD is caused by a genetic mutation in the Huntingtin gene (IT15) where the C-A-G nucleotide sequence is overly repeated. This discovery has been named the "CAG repeat expansion" where the general population has around 20 repeats in this gene, those with clinically diagnosed HD have over 40. Everyone with this mutation will show symptoms at some point in their lives, but the reason why most patients are middle-aged when first diagnosed is because of the impact the genetic mutation has on huntingtin protein synthesis. The specifics of this protein's function have not been elucidated clearly yet, but scientists assume that the CAG repeat expansion causes abnormal protein function. These proteins are consistently produced over the span of a lifetime and form clumps in the brain causing both physical and neurological symptoms to worsen over time.[1] Although this phenomenon affects the entire brain, the striatum is most heavily impacted. HD does not have a cure at the moment partially because there simply is not enough information on the specifics of what happens to those with the mutation from a scientific standpoint.[2]

Retinoic acid is a small lipophilic molecule derived from vitamin A[3] that plays a key role in cell growth and differentiation.[4] In this experiment, it was used to differentiate the A172 Glioblastoma cell to mimic a neuron cell. Normally, retinoic acid is used to differentiate embryonic stem cells into motor neurons by altering both encoding RNA and miRNA expression[5] and this is the main objective of retinoic acid use in this experiment as well. Cell differentiation is the process in which a cell transforms into a more specialized type. This process changes a cell's shape, size, and energy requirements.[6] Since HD is a neurodegenerative disease that is characterized by the gradual and progressive loss of neurons,[7] an experiment aiming to find protective agents against the disease must take place using neurons, the cell type that's affected by the disease.

Dopamine is a neurotransmitter that sends signals between neuron cells and is an essential part in controlling voluntary movements. An alteration of the dopamine balance in the striatum specifically leads to pathological conditions like HD. Changes in the amount of dopamine and its receptors cause abnormal movements and cognitive deficits. Evidence also shows that an increased level of dopamine release induced chorea, a defining symptom of HD, while a reduction leads to akinesia, the inability to make any voluntary action.[8] Although the reason behind cell loss in HD is unclear, it may be explained by excessive glutamate release from cortical and thalamic terminals or an increased sensitivity of glutamate receptors.[9] Glutamate is another type of neurotransmitter and its receptor's function is controlled by the activation of dopamine receptors. This relationship suggests that an alteration in dopamine function and neurotransmission would significantly impact the motor and cognitive symptoms of HD.[9]

Using this relationship, this experiment uses 6-hydroxydopamine (6-OHDA), a form of dopamine that has been used in Parkinson's disease studies to induce neuronal damage. 6-OHDA causes massive destruction of neurons and experts have been focusing on 6-OHDA's intracellular mechanisms at the striatal level.[10] Therefore, 6-OHDA can be used to mimic

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increased mutated huntingtin (mHTT) expression while suppressing HTT phosphorylation at Ser421, a modification that would have protected it against mHTT accumulation.[11]

Among many of its functions, such as maintaining the division amongst micro and macromolecules, exosomes are also produced by almost every cell in the body and are shown to impact distant cell signaling.[12] Since they are a commonly produced mobile extracellular vesicles, exosomes function as an intercellular communication channel for different proteins and microRNAs (miRNAs) even for distant cells.[13] Recently, many studies have been conducted to find a relationship between exosomes secreted from specific cells and their impact on certain diseases. Such useful functions of exosomes have led scientists to utilize them for therapeutic purposes, one of them being HD.

MicroRNAs are small, non-coding strings of RNA with several functions that heavily impact gene expression which is why their specific role in genetic diseases and the overall human anatomy are being studied extensively. For example, one known function is how miRNAs regulate genes by creating bonds with the three untranslated regions (UTR) of messenger RNAs (mRNAs) to cause deregulation of the target gene's expression, essentially giving them the power to change the expression of multiple genes within a cell. This unique characteristic can be linked to potential positive or negative impacts the lack or abundance of specific miRNAs can have in a genetic disease.[13] Some recent studies have even proposed links between miRNAs and exosomes as there has been speculation around miRNA secretion being controlled by vesicular/exosomal-controlled mechanisms. Either way, exosomes do have a significant role within circulatory miRNA biology which is why exosomes have been used as transportation methods for miRNAs in some studies in order to facilitate gene exchange. Since miRNAs cannot move on their own, scientists have proposed that miRNAs are encapsulated and transported by exosomes when leaving or entering cells.[14]

Methods

A172 Glioblastoma Cell Culture:

A172 glioblastoma cells from homo sapiens brain cells were cultured in RPMI 1640 media supplemented with 10% fetal bovine serum (ATCC) and 1% penicillin and streptomycin. Media was exchanged every three days during cell maintenance. 0.25 % Trypsin-EDTA was used to detach the cells from culture flasks.

Neuronal Differentiation by Retinoic Acid Treatment:

A172 glioblastoma cells were differentiated to neuronal cells by adding retinoic acid. The 50 mM of stock solution of the retinoic acid was prepared. 0, 10, 30, 50 μM concentrations were used to differentiate the retinoic acid.

6-OHDA-derived HD Model:

The 100 mM of stock solution of 6-OHDA, a form of dopamine that has been used in previous Parkinson's studies to induce neuron damage, was prepared. The diluted concentrations of 6-OHDA were prepared and incubated with the A172 glioblastoma cells. IC50 was calculated using the Prism 7 program.

Exosome Isolation :

To isolate the exosomes from the A172 cell, the Total Exosome Isolation Reagent (Invitrogen) and exosome depleted fetal bovine serum (FBS) were used. After harvesting the cell culture media, the cell supernatant was centrifuged at 2000 x g for 30 minutes to remove cells and debris. Then the supernatant was moved to a new tube. The 500 μl of exosome isolation media was mixed with the 1 ml culture media. After the cell culture media and reagents were mixed well by vortexing, the samples were incubated at 4 ºC overnight. Then, the samples were centrifuged at 10,000 x g for 1 hour at 4 ºC. The supernatant was discarded and the pellet containing the exosomes was resuspended by PBS buffer.

miRNA Transfection:

0.5 x 106 cells were prepared in the 6 well culture plate. The miRNA and RNAimax transfection reagent (Invitrogen) were mixed together to 1:3 molar ratio to form a complex. Then the isolated exosomes were added to the mixture and incubated for 10 minutes. Then, the miRNA-RNAimax-exosome complex was added to the prepared cells.

Cell Viability Measurement:

PrestoBlue (Thermofisher) was used to measure the cell viability. After the cells were prepared in the 96 well plate, 10 μl of PrestoBlue reagent was added to each cell containing wells. Then, the cells were incubated in the CO_2 incubator at 37 ºC. After incubation, Epoch microplate reader was used to measure the absorbance at 570 nm wavelength.

Statistical Test:

All statistical tests were performed by the Prism 8 program. For all statistical analyses, a student t-test was used to calculate the p-value. The p-value lower than 0.05 was considered statistically significant.

Results and Discussion

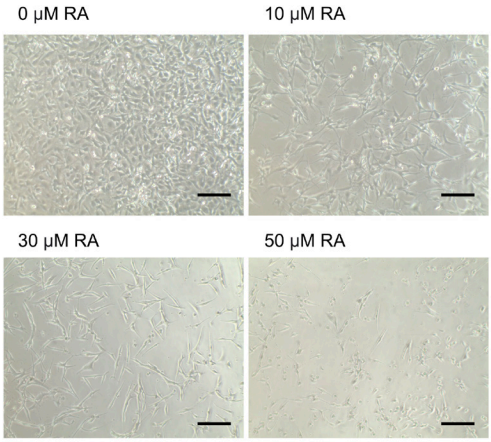

Figure 1: Microscopic images of A172 Glioblastoma cells treated by varying levels of retinoic acid (RA) for 24 hours. This result suggests that 30 μM concentration was the optimal concentration to differentiate A172 cells using RA. Scale bar = 100 μm.

To find the optimal concentration of RA treatment for neuronal differentiation, three different concentrations (10, 30, 50 μM) were treated on A172 cells as shown in Figure 1. After 24 hours of RA treatment, cell morphologies were observed. With increasing concentration of RA treatment, A172 cells elongated, exhibiting neuron-like morphology. However, at

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50 μM concentration, a large number of dead cells were observed, indicating possible toxicity of the substance. As a result of qualitative observation of A172 cells after RA treatment, 30 μM concentration was found to be the optimal concentration to induce differentiation, therefore this concentration will be used for the following experiments. Looking at the cell's morphology, the sample treated with 0 μM of retinoic acid has several small pieces with the occasional dark or white spot. The rest of the samples, treated with 10, 30, and 50 μM of retinoic acid, all have a generally round shape that grows in size as the concentration of retinoic acid increases, showing the progression of cell division. These samples seem to be epithelial with a long, stretched morphology. All cell samples are attached to the bottom layer of the petri dish and the image was taken from a bird's eye view.

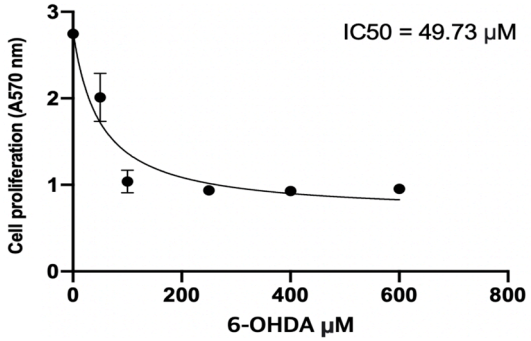

Figure 2: Cell viability of A172 glioblastoma cell under 6-OHDA treatment. IC50 value is indicated in the graph. This experiment result indicates which concentration of 6-OHDA is able to cause 50% of cell death in the A172 cells.

Varying concentrations of 6-OHDA were treated on A172 glioblastoma cells to observe their effects on cell viability by measuring 570 nm wavelength absorbance (Figure 2). Cell viability decreased exponentially upon 6-OHDA treatment. Increasing the concentration above 600 μM did not have any further effect on the proliferation rate. According to the best fit curve, corresponding IC50, which indicates the concentration needed to kill 50 % of the cells, concentration was found to be 49.73 μM. This concentration is used for the following experiments to induce HD-like conditions.

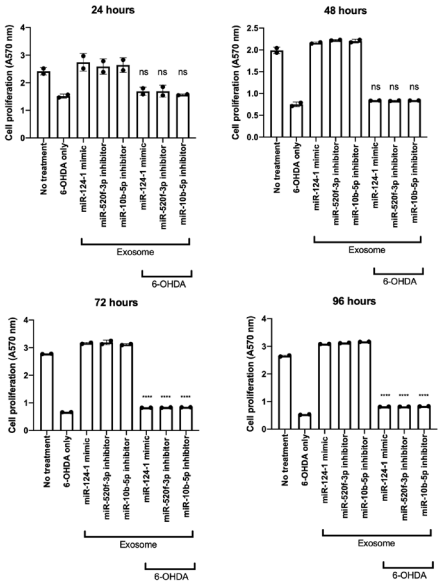

Figure 3: Effect of exosome-derived delivery of three types of miRNA on 6-OHDA-induced cell death during 24, 48, 72, 96 hours incubation. This result shows the long-term incubation of miRNAs and compares the results of manipulating the presence/absence of the miRNAs to 6-OHDA only and 6-OHDA + exosome + miRNA sample. 6-OHDA only sample was set as a control sample and an unpaired student t-test was performed to analyze the statistical significance. $p > 0.05$ (ns), $p < 0.001$ (****).

Effects of three types of miRNA delivered by exosome on A172 cell viability were analyzed over the period of 96 hours (Figure 3). 49.73 μM of 6-OHDA, as found in the previous section, was exposed to cells to mimic the condition of HD. 6-OHDA treatment consistently decreased cell viability with the progression of time, as the lowest value of absorbance was measured after 96 hours. For all three types of miRNA, miRNA and exosome combination seemed to increase the viability of cells for both control group and HD mimic group at all time points. The cell viability did not exhibit any significant differences between the three types of miRNAs after 24- and 48-hours incubation. There is no statistically significant difference in cell proliferation after 24 and 48 hours between 6-OHDA only samples and miRNA transfected samples. For the miRNA combinations to take effect, they must be treated for a minimum of 72 hours, as shown by the p-values derived above.

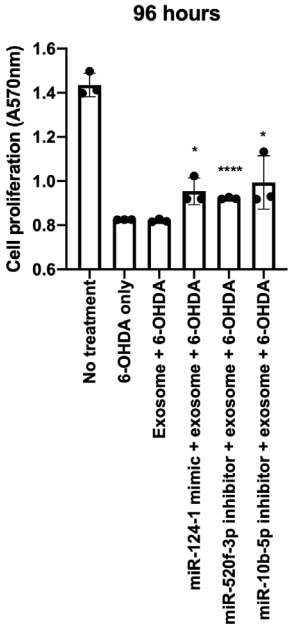

Figure 4: Effect of exosome and exosomal-miRNA combination on A172 cell viability in 6-OHDA-derived HD model after 96 hours. This figure shows the protective effect of miRNA on A172 cells, indicating that the protective effect of miRNA and exosome complexes in Figure 3 was not caused by the exosomes. Exosome + 6-OHDA sample was set as a control sample and an unpaired student t-test was performed to analyze the statistical significance. $p < 0.05$ (*), $p < 0.001$ (****)..

Data from Figure 4 shows there was a significant positive impact of the miRNA + exosome + reagent on cell proliferation after they were treated with 6-OHDA. However, the positive effect on cell proliferation may be induced either by exosomes with reagent or miRNA itself. To find out which factor derives the protective effect against 6-OHDA induced cell death, two conditions were tested with the addition of 6-OHDA:

1) exosomes with reagents 2) exosomes with reagents + miRNA.

Figure 4 shows that there is almost no difference in cell proliferation between cells treated only with 6-OHDA versus those treated with the exosome + reagent combination while there's a clear increase in cell proliferation in samples treated with the miRNA types as well showing that the impact of the last three bars is solely due to the addition of different miRNA types. Also, the error bars in the second and third bar graphs are almost zero, meaning this data is very reliable, again showing that the exosome + reagent combination had no positive impact on cell proliferation. On the other hand, the error bars for the fourth and last columns are quite significant but still show an increase in cell proliferation since the lower and upper bounds of all error bars still have a higher level of cell proliferation when compared to the second, 6-OHDA only bar. Therefore, the protective abilities of all three miRNA types against cells dying from 6-OHDA have been demonstrated.

Conclusion

Prior to the experiment, it was predicted that the insertion of certain miRNA types would increase proliferation in cells dying from HD, mimicked using 6-OHDA. Since the miRNA types were chosen based on pre-existing knowledge about miRNAs that are types impacted in the brains of HD patients, an experiment targeted towards ameliorating this shortcoming should have positively impacted cell growth that was already dying from HD. This hypothesis was supported as shown by Figures 3 and 4 as they clearly show that all three miRNAs significantly enhanced cell viability under 6-OHDA treatment.

While the change may seem trivial, considering that this experiment is exploring unknown topics with a lot of room to grow, this initial data is promising. Not only does it use 6-OHDA to mimic HD, but it also shows that miRNAs are effective in protecting cells from neuronal damage. This study is one of the first to explore how miRNAs can be used as a therapeutic method against HD and, unlike others that focus on one specific type, miR-124, this study explores the possibility of three additional types. One extension this research can take is to use combinations of the three types tested above. Since all three miRNAs have shown a positive impact on cell proliferation, their combination may further enhance the effect. If miRNAs prove to be an effective therapeutic in fighting HD, this disease might have its first cure. Granted, not every patient has the same circumstances so there cannot be just one solution, but by finding protective miRNAs and potentially using different combinations for each patient, this can be a potentially effective way to at least slow down disease progression.

In other studies that have used miRNAs and exosomes to discover more about HD, most have used cells from another species to transfect into a miRNA vector. For example, a previous study used genetic cloning technology to replicate their target miRNA into a plasmid to create miRNA vectors. They then transfected HEK 293 human cells with the miRNA vector and the resulting cells were put into a medium where scientists later chose cells that overexpressed the target miRNA.[15] The chosen cells were cultured into an exosome-free medium where the exosomes in the cell were isolated and the level of target miRNA left was quantified. To finish, the scientists injected the exosomes they harvested, that now had their target miRNA, into transgenic mice using a syringe.

This experiment, on the other hand, has differences that improve the efficacy and accuracy of the previously mentioned process. First, this experiment eliminates the need for vectors and cultured cells, allowing for the target and origin cell for exosome isolation to be from the same subject. Much like the idea behind organ transplants, it is much more preferable and beneficial if the original cell where an exosome was derived has a similar genetic makeup as the target cell as there is a significantly lower possibility of the target cell rejecting the exosome. However, this concept is not applied to the current, widespread method of miRNA delivery which is why this study proposes a method that theoretically would work. Therefore, this study will be able to explore the possibility of a method that can produce remarkably more accurate results thanks to the use of native cells.

As a whole, discovering effective miRNA types to recover and heal the neuron cells damaged from HD will be the first plausible cure/management method for the disease. While the specific method for injecting miRNAs into living human brains can be a future extension, this study shows the strong possibility of this treatment method panning out theoretically.

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