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■ RESEARCH ARTICLE

Development of Mini-brain with Human Brain Cell Lines

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ABSTRACT: The human brain, a complex organ pivotal to the nervous system, orchestrates crucial functions for cognition and behavior. This research advances 3D brain cell modeling, overcoming traditional limitations. While mouse models present challenges in translatability to human brains, the evolution from non-invasive imaging to 3D culture models, specifically brain organoids, holds promise for understanding neurological disorders. Current limitations in 3D brain cell models, particularly the use of pluripotent stem cells, include high costs, extended development times, and a notable failure rate. In response, this study introduces a novel approach utilizing glial and neuron cells, significantly reducing costs and development time while maintaining a low failure rate. Experimental results demonstrate the successful creation and differentiation of 3D brain models, addressing key limitations. The developed mini-brain model, sustained for 21 days, exhibits resilience and responds differentially to glucose treatment, suggesting potential implications for brain morphology. Cell viability assessments confirm the predominantly live nature of the model, providing a foundation for future investigations. While limitations include a focus on two cell types and 21 days, future endeavors aim to diversify cell types and extend the maturation period. This research marks a significant stride in cost-effective, efficient, and ethical 3D brain modeling, laying the groundwork for continued exploration and comparison with established brain models.

KEYWORDS: Cellular and molecular biology; Neurobiology; 3D brain model; Glial cell; Neuron cell.

■ Introduction

The human brain is a complex organ central to the nervous system, which manages many vital functions for human cognition and behavior.[1] Comprising about 86 billion neurons connected by trillions of synapses, the brain is the main center for processing sensory information and facilitating higher cognitive processes such as learning, memory, and decision-making.[2] Neurotransmitters and neural networks are interconnected, forming the basis for thought, emotion, and consciousness. Therefore, understanding the basic interaction of the brain is crucial for understanding the complexities of neurological disorders and advancing interventions to enhance cognitive abilities and mental well-being.[3]

While mouse models have been used in brain research, significant limitations hinder the direct translation of findings from mice to humans. One key limitation is the anatomical and functional differences between the mouse and human brain. [4] The human brain is much larger and structurally more complex, especially in regions associated with cognitive functions regulated by the prefrontal cortex. Moreover, the genetic divergence between mice and humans indicates a significant difference.[5] Many genes associated with human brain disorders do not have direct counterparts in mice. These differences limit the translational relevance of mouse models for studying the human brain.[6]

The first method used for brain research was the non-invasive imaging method, which was limited in revealing the spatial and temporal resolution of the image.[7] Scientists used animal models to understand human brain development and perform tests. However, the model could not resemble the complexity of human brain characteristics, raised high ethical considerations, and failed to translate into human tests. This led to the creation of 2D cell culture systems that supported the growth of neuroscience research but also faced the challenge of mimicking the complexity of cell interaction of the brain *in vitro*.[8] The later development of the new 3D culture method was able to represent a more accurate model of the brain than the previous models. With 3D modeling of the human brain, scientists claim it could also be used for neurodegenerative disease research to prevent its rapid expansion. The 3D cell culture currently uses brain organoids created from pluripotent stem cells (PSCs) that were self-organized to mimic specific brain regions such as the hippocampus, hypothalamus, midbrain, and neocortex.

A common 3D model currently used for 3D brain cell models is pluripotent stem cells.[9] However, a limitation of using pluripotent stem cells for 3D modeling is its high cost, meaning that the stem cell culture media costs a lot. Also, it takes three weeks to one month to develop a 3D model. Lastly, it has a high failure rate because if there has been a little change within the stem cell, it may disturb the process of the stem cell becoming a brain cell model and turn out into an unidentified tissue structure. In this study, the model we created does not require stem cells, which reduces the limitations mentioned before.[10] We used two types of cells to generate the 3D model: glial cells and neuron cells. Cell culture media has a lower cost than stem cells. Also, the process only takes a week to generate a 3D model, which is highly efficient compared to stem cells.

DOI: 10.36838/v6i9.9

There is a low failure rate when using glia cells and neuron cells as the cell percentage of glia and neuron cells is easily managed and is available for easy protocols to use when creating a brain cell model.

Methods

Cell culture and maintenance:

Human glia cells (A172) and neuron cells (SHSY5Y) were cultured in RPIM1640 cell culture media supplemented with 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin antibacterial substances. The cells were incubated at 37 ˚C in a CO_2 incubator. For 2D culture, a 6-well culture plate was used to maintain the cells on the surface of the culture plate.

3D culture using the hanging drop method:

After preparing the cell suspension with the desired cell density and volume, the culture plate cover was inverted, and a small droplet of the cell suspension was placed on the underside of the culture plate cover. The cover was placed over a culture plate containing the cell culture media. After the droplet from the cover formed a sphere due to gravity, the EVOS m5000 microscope was used to capture the cell image.

Retinoic acid treatment:

The retinoic acid (RA) was purchased from Sigma. The RA stock solution (50 mM) was prepared with distilled water. The stock solution of RA was stored in a -20 ˚C freezer before being used in the experiments. The cells were treated with RA for seven days to differentiate the cancer cells into normal brain cells.

Cell imaging using a fluorescent microscope:

EVOS M5000 imaging system was used and allowed to warm up for 30 minutes. The cells were stained with Acrydine Orange and Propidium Iodide fluorescent dye. After 10 minutes of incubation, the sample was placed on the EVOS M5000 imaging system stage. The image was captured using the integrated software.

Statistical tests:

Prism 8 was used to conduct a student's t-test to calculate the p-value. A p-value less than 0.05 was considered statistically significant.

Results and Discussion

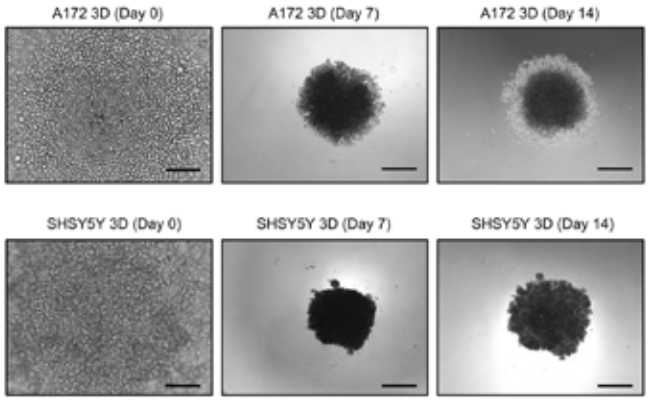

Figure 1: Cell images of 3D brain models were developed by using A172 and SHSY5Y cells individually. Scale bar = 400 μm.

The experiment aimed to see if the individual cells, A172 and SHSY5Y, would become a 3D structured cell. The experiment shown in Figure 1 can show which cell is more suitable for creating a 3D culture model. On Day 0, the cells were prepared for the experiment using a hanging drop method, and photos were taken in that form. After 7 days, the samples were taken photos, and after 14 days. A separate 3D model was prepared for A172 and SHSY5Y to be able to compare their results (Figure 1). On day 0, both cells were dispersed, and no 3D structure was formed. A172 cells were observed to be bigger than AHSY5Y cells. On day 7, 3D structural cells started to be seen through the microscope. From the microscope, SHSY5Y was denser than the A172 3D structure. On day 14, the 3D model size was enlarged compared to the 3D structure on day seven. A172 and SHSY5Y cells can be used for 3D culture, and the 3D model can be maintained for at least 14 days of culture.

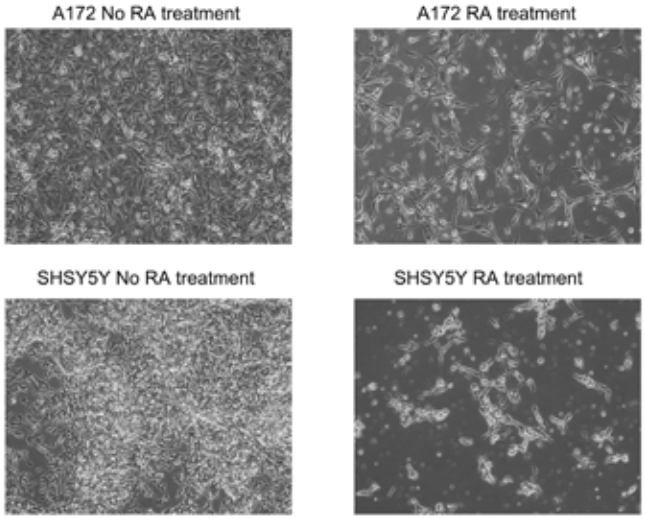

Figure 2: Retinoic acid (RA) treatment successfully differentiated A172 and SHSY5Y cancer cells into normal glial and neuronal cells, respectively.

Next, since A172 and SHSY5Y cells are cancer cells, we aim to differentiate A172 and SHSY5Y cells into normal cells by adding RA treatment. A172 and SHSY5Y were observed separately. One of the A172 and one of the SHSY5Y samples were treated with RA for seven days. The cell image was taken seven days after the RA had been treated. The result for A172 and SHSY5Y cells not treated with RA was that the cell confluency was almost 100% and showed no space (Figure 2). However, the A172 cells with RA treatment had 30-40% cell confluency, and the morphology of the cells changed to spindle-shaped cells. SHSY5Y cells with RA treatment were 10-20% confluent, and the morphology of the cells also changed to spindle-shaped cells. Therefore, both A172 (glial cell) and SHSY5Y (neuron cell), which are star-shaped cells, were observed, meaning that the RA treatment has successfully differentiated the cancer cells into normal cells (Figure 2). In conclusion, our RA treatment condition successfully differentiates the cancer cells from normal brain cells.

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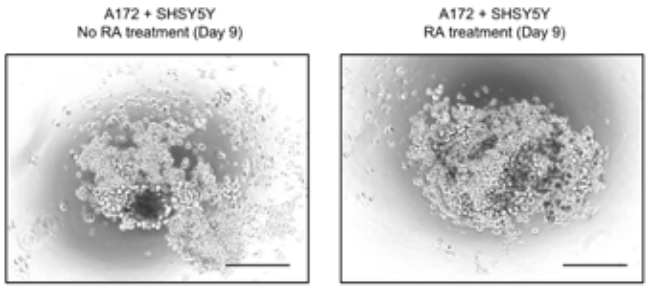

Figure 3: Comparative Analysis of A172+SHSY5Y Mixed Cells in 3D Culture: Impact of RA Treatment. Images captured after nine days of cultivation using the hanging drop method demonstrate distinct morphological outcomes. Scale bar = 400 μm.

Our brain is a multicellular organ that consists mainly of A172 and SHSY5Y cells. We wanted to observe if RA treatment will successfully create a 3D model with mixed cells of A172 and SHSY5Y. The cells were cultured using a hanging drop method to mimic the 3D structure. A microscope imaged mixed cells with RA treatment and without RA treatment after nine days. After nine days of the treatment, a precise observation of a 3D model was made. The mixed cell, A172+SHSY5Y, is heterogeneous and is a cancer cell that, when the cell grows, grows infinitely with a random shape and is distributed all around (Figure 3).

On the other hand, the mixed cell with RA treatment goes through differentiation. The cell becomes a normal cell that behaves like a normal cell. So, the mixed cell with RA treatment creates a round shape. The RA treatment in a mixed cell of A172 and SHSY5Y successfully creates a 3D model by differentiating the cell from a normal cell and maintaining the circular shape.

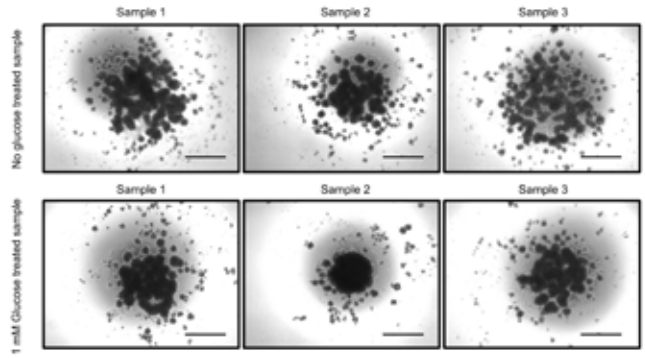

Figure 4: Long-term Performance and Morphological Impact of Glucose Addition in 3D Mini-Brain Models. The experiment involved a 21-day culture of A172 and SHSY5Y cells with RA treatment to develop a mini-brain model. Scale bar = 400 μm.

The experiment was to see the performance through the long-term cell culture that could lead to a mini-brain model. The shape of the mini-brain and cell viability during the performance have been observed, as well as the impact of adding glucose to the mini-brain. The first three samples have A172 added with SHSY5Y and RA treatment for 21 days. The bottom three samples have the same factor but are added with the 1mM glucose during the 21 days. The long-term experiment, 21 days, has shown that the brain model could maintain their shape in a 3D culture model (Figure 4).

Interestingly, many small aggregates were formed with and without glucose-treated models. Also, the glucose-treated sample showed a more compact mini-brain model than the no-glucose-treated model. This result may indicate that glucose may affect brain morphology through our 3D mini-brain development. Our mini-brain model was successfully developed and could be maintained for at least 21 days.

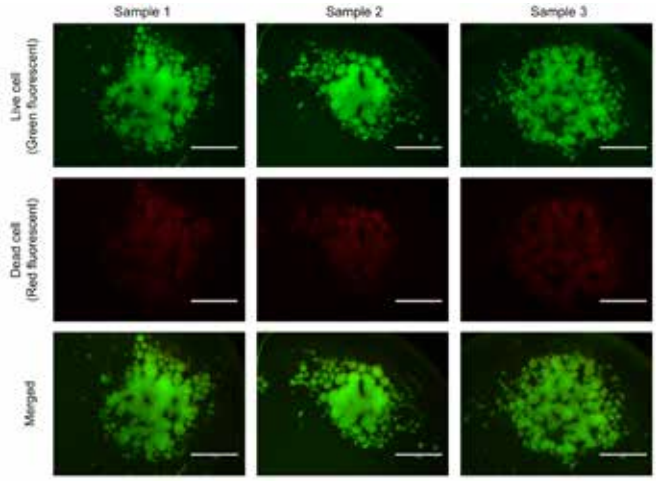

Figure 5: Assessment of Cell Viability in 3D Mini-Brain Model Without Glucose Treatment. A172 and SHSY5Y cells were subjected to RA treatment and cultured in a 3D model for 21 days without glucose. Scale bar = 400 μm.

Next, we sought to check the cell viability of our developed 3D mini-brain model in no glucose-treated samples. The samples mixed with A172, SHSY5Y, and RA treatment were observed for 21 days. To check the cell viability, we added Acridine Orange and Propidium Iodide staining solution to the sample to stain the live (green) and dead (red) cells. Through the fluorescence microscopy images, all the samples had a strong green signal indicating the live cells and a weak red signal indicating the dead cells (Figure 5). Therefore, the merged image shows a stronger signal in green, meaning that most of the brain cells were alive. We confirmed that our brain model mainly consisted of live brain cells.

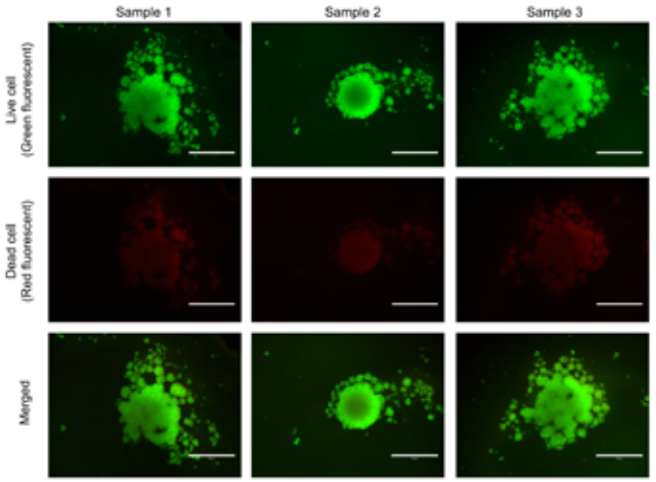

Figure 6: Assessment of Cell Viability in 3D Mini-Brain Model Without Glucose Treatment. A172 and SHSY5Y cells were subjected to RA treatment and cultured in a 3D model for 21 days without glucose. Scale bar = 400 μm.

Next, we sought to check the cell viability of our developed 3D mini-brain model in glucose-treated samples. Three samples added with glucose were observed and added with Acridine Orange and Propidium Iodide staining solution. The solution was to see the cell viability when glucose was added. Three samples had a strong green signal but a weak red signal, indicating more live cells (Figure 6). As the merged image shows, the cells could live even with the glucose added to the sample, as no red signal can be seen from the merged image. Adding glucose to the 3D brain model does not impact the cell viability.

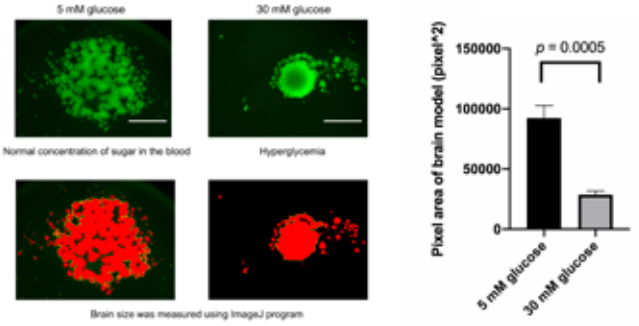

Figure 7: Glucose decreased the size of our 3D mini-brain model. The pixel area of the brain model was quantified using the ImageJ program (left). Bar graph representing the mean and standard deviation of the pixel area of the brain model (right). Student t-test was performed to calculate the p-value.

Finally, extended research was conducted to see the impact of the amount of glucose treatment on the 3D brain model. One sample had a normal sugar concentration of 5 mM glucose, and the other had 30 mM glucose, which mimicked hyperglycemia. The samples were then imaged using an ImageJ program that helps measure the brain size. The program shows the graph on the right, which shows that the 5 mM glucose sample has a larger pixel area than the 30 mM glucose sample, and importantly, hyperglycemia leads to a decrease in brain size (Figure 7). Therefore, we found that when the amount of glucose added to the sample exceeds, there is an impact on the brain size.

Conclusion

A172 and SHSY5Y cells were used to create a 3D brain model. The research was done to see if A172 and SHSY5Y cells could create a 3D structure model. First, it was discovered that A172 and SHSY5Y cells could create a 3D structure and mimic the brain model. However, A172 and SHSY5Y cells are cancer cells and had to be differentiated into normal cells to mimic the brain cells accurately. Then, RA treatment was added to the separate samples, and the cancer cells were successfully differentiated into normal brain cells. We performed additional research where we created mixed samples with A172 and SHSY5Y cells, as our brain consists mainly of those two cells. We added RA treatment, successfully creating a 3D model and differentiating the cell into a normal one. We continued using the mixed sample and created a mini-brain model with both samples added with glucose and no glucose treatments. Finally, we checked the cell viability of the 3D mini-brain model as a final step. The 3D brain model we mainly created consisted of the live brain cells for the no glucose-treated and glucose-treated samples. As a final research result, we experimented with adding 5 mM glucose and 30 mM glucose to the samples to see if a normal concentration of glucose in blood and hyperglycemia would impact the 3D brain model. In conclusion, the amount of glucose added to the brain results from brain size changing, where when more glucose is added, it leads to the brain shrinking.

The limitation of the research was that we only tested with two types of brain cells, and there are other brain cell types, such as astrocytes, oligodendrocytes, and endothelial cells, apart from glia and neuronal cells, that can be used for the investigation. The longest period we generated for the 3D brain model was 21 days. Therefore, we plan to develop a 3D brain model using more cell types and investigate a more extended period, at least two months, of incubating the 3D brain model to mature our 3D model. Compared to the previous stem cell culture method, our model was incubated for a similar period and produced successful results. However, incubating for a longer time will allow us to observe the changes in the complex brain more closely. Furthermore, the impact of glucose concentration on the 3D brain model concerning brain damage should be further investigated.

Through the research, we have successfully developed a mini-brain model with two types of cancer cells: A172 and SHSY5Y cell lines. Our generated mini-brain model used a new method that did not require any stem cells when creating it and required less effort and cost than previous brain models. When we compare the shape and size of the mini-brain model with the previously developed models, the similarity of shape and size was observed and maintained. However, further investigation is needed to compare the characteristics of the mini-brain with the previous brain model.

Acknowledgment

I would like to express my gratitude and recognize Prof. Lee and my parents for their contributions to this project. They provided guidance, support, and assistance throughout the process.

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DOI: 10.36838/v6i9.9

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