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Article

A novel *in vitro* blood-brain barrier model using 3D bioprinter: A pilot study

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SUMMARY

3D bioprinters allow the creation of functional tissues using various cell lines. The blood-brain barrier (BBB) is crucial for maintaining homeostasis in the brain, acting as a protective interface between the cerebral blood vessels and the brain parenchyma. Advancements in tissue engineering have led to the development of in vitro BBB models. However, model accuracy and reproducibility still require improvements. Thus, we hypothesized that producing BBB models using a 3D bioprinter is a novel method to overcome these limitations. We tested the maintainability of habitable conditions for cells and the durability of the 3D-bioprinted BBB model. We printed four different BBB models using a bio-ink composed of Matrigel base and CPA47 endothelial cells: single- and double-layered square and circular scaffolds. The double-layered square scaffold fostered optimal BBB functions as it most effectively maintained a fixed shape and initial cell positions. When A172 glioblastoma cells were placed in the empty square spaces of the square BBB scaffold, most cells were alive and healthy after 96 hours. Although A172 cells remained in their initial cell positions until the first 48 hours, we observed movement across the Matrigel barrier when checked at 96 hours. At 48 hours, A172 cells showed no movement from their initial positions; however, by 96 hours, we observed clear movement of A172 cells across the Matrigel barrier. Due to its relative cost-effectiveness and high reproducibility, our BBB model has several applications, especially for studying brain cancer metastasis past the BBB and developing potential corresponding clinical therapeutics.

INTRODUCTION

Proper brain function requires strict blood, oxygen, and metabolite level regulation. Thus, the blood-brain barrier (BBB) serves to maintain homeostasis (1). The BBB is a highly selective semipermeable membrane that acts as a protective interface between the cerebral blood vessels and the brain parenchyma (1). It prevents solutes in the circulating blood, such as neurotransmitters and neurotoxins, from entering the brain where neurons reside (1). One of the critical structures of the BBB is the endothelial tight junction, where endothelial cells lining the interior of the blood capillary are organized very close together, creating adhesive and tight gaps that control the paracellular entry of substances (2).

Since establishing the BBB depends on different chemical and physical compositions, many *in vivo* and *in vitro* models have been developed (3). Unlike animal models, *in vitro* BBB models enable independent control of cellular components and microenvironments (4). For example, applying a monolayer of brain microvascular endothelial cells (BMECs) with the transwell method simulates the high-throughput screening properties of the BBB (5). However, the transwell method only presents a 2D BBB structure. Thus, more complex models are needed to understand the biological process involving both pathological and physiological responses of the BBB (6). Such complex BBB models should comprise key features, including a 3D presentation, highly selective permeability, uniform single layers of cells, and overexpressed endothelial tight junctions. Moreover, current methods to produce *in vitro* BBB models lack cost-effectiveness and reproducibility (6).

3D printing is one of the most promising techniques for modeling human tissues by assembling biological materials in a complex layout (7). Thus, we hypothesized that using a 3D bioprinter would resolve the limitations of current BBB models and improve cell engineering techniques to accurately mimic the functional properties of the BBB, fostering an inhabitable environment for the cells. To investigate this hypothesis, various BBB models were produced using a mixture of blood endothelial cells and Matrigel. Matrigel is used for bioink for 3D bioprinting to form highly complex 3D tissue models, and enables construction of large-scale tissue models. It has also been used in various *in vitro* assays for angiogenesis, cell invasion, spheroid formation, and organoid formation from a single cell. The four primary components of Matrigel are laminin, collagen IV, entactin, and heparan sulfate proteoglycan perlecan.

Breakdown of the BBB is typical in highly metastatic gliomas, which produce the Blood-Tumor Barrier (BTB) after cytoskeletal remodeling. The BTB is a dysfunctional barrier that enables tumors to enter blood vessels (8). Tumors transform BBB into BTB, which is non-uniform and has high permeability (8). Even though BTB may improve drug delivery across the BBB/BTB, blocking the transformation of BBB into BTB is one of the potential clinical strategies for effective cancer therapy (8). Thus, a double-layered square scaffold with Matrigel and endothelial cells was first produced. Then, we added A172 cells, a glioblastoma (GBM) cell line, into the empty square spaces to confirm that the BBB model transforms into a BTB, allowing penetration of cancer cells.

Using our BBB model, we noted that Calf Pulmonary Artery 47 (CPA47) endothelial cells remained alive within the Matrigel environment for at least 96 hours. CPA47 cells are animal endothelial cells derived from cows. Because the only available endothelial cells were from the CPA47 cell line, we decided to use these for our study. We were further able to

observe the invasive nature of A172 glioblastoma cells as they crossed the Matrigel barrier. The aim of our study was to create a 3D BBB scaffold model in desired shapes that maintains living cells. Although we successfully positioned the endothelial cell-Matrigel mixture into various desired shapes, due to the limited precision of the 3D bioprinter used in the study, our BBB models do not fully replicate the physical properties — single-layered, uniform arrangement of cells — of human BBBs. Nonetheless, this method of producing BBB models using 3D bioprinters can provide numerous opportunities to advance our knowledge of BBB and brain cancer therapeutics due to its high accessibility and reproducibility.

RESULTS

We designed a novel model of blood-brain barrier using a 3D bioprinter (**Figure 1**). First, endothelial cells from the CPA47 cell line were cultured. These cells were then mixed with the Matrigel matrix for 3D bioprinting. Commonly used as a scaffold bioink in 3D bioprinters, especially for investigating cancer cell invasion, Matrigel was a suitable choice for the bioink. We then filled the syringe with the Matrigel-cell mixture as the printing material. Then, the 3D bioprinter ejected the matrix to print a scaffold in circular and square shapes; a 0.1 mm nozzle tip was used for printing the circular model, while a 0.05 mm nozzle tip was used to print the square-shaped model. The nozzle tip sizes were different for each design, as the default tip sizes were set by the 3D bioprinter to optimize each shape's stability and function. We chose the circular shape due to its resemblance to the circular shape of the human BBB structure, and we chose the square shape as its standard, grid-like structure made it easy to observe A172 glioblastoma cell movement across the BBB.

In this experiment, we aimed to optimize the shape of our BBB model to most closely mimic the complex structure of human BBB. We printed and observed single- and double-layered square- and circular-shaped Matrigel scaffolds for three repetitions each. Because all three experimental trials yielded almost identical results, we included only one photo for each scaffold design. For all single-layered scaffolds, we found that the Matrigel scaffold did not form continuously as both the circular and square shapes showed a very thin and unstable structure (**Figure 2**). Further, we evaluated that precisely ejecting and equally distributing cells into the circular scaffold was difficult due to its roundness and would hence produce experimental errors. Thus, we concluded that the double-layered square-shaped Matrigel scaffold would provide an optimal imitation of the BBB. The double-layered square scaffold showed the sturdiest BBB structure and was most reproducible. However, we did notice that areas where Matrigel ejections overlap, such as the square corners, were flattened out due to the additional weight and fluidity of the Matrigel.

The purpose of this experiment was to confirm that CPA47 cells remained healthy after being placed into the Matrigel for scaffold printing. We also tested for the induction of BBB to BTB structural breakdown by adding the A172 glioblastoma cells inside the square-shaped scaffold after it was printed. Because the CellTracker is better retained in live cells, we deduced that the blue-stained CPA47 cells were alive after BBB scaffold printing. After the double-layered 10-squares x 10-squares scaffold was generated, the A172 cells (pre-

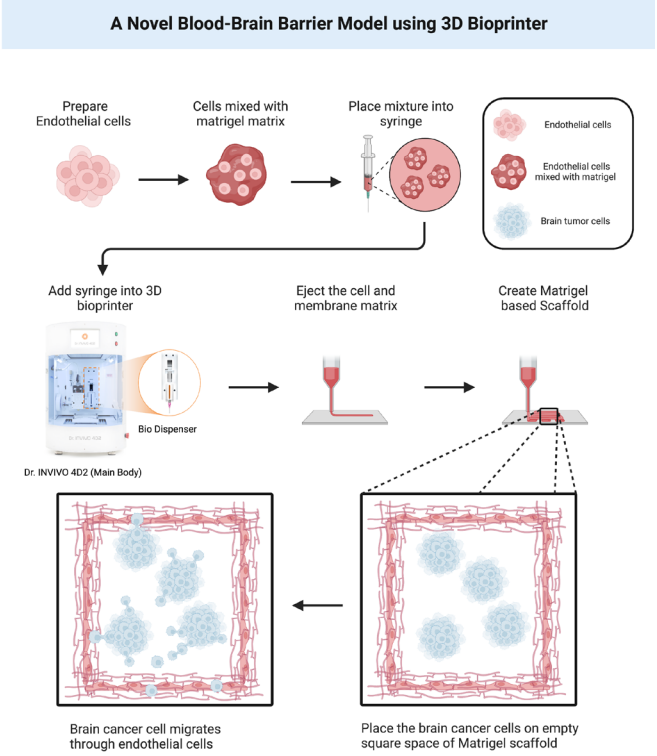

Figure 1. Developing a novel Blood-Brain Barrier (BBB) model with Cell-Matrigel Complex using 3D Bioprinter. The overall step-by-step method used to create the BBB model. CPA47 endothelial cells were used for the Matrigel complex, and A172 glioblastoma cells were used to observe cell penetration across the BBB. Created with BioRender.com.

stained with red fluorescence) were ejected into the middle of each square. As each dot in the image represents an individual cell, the position of the blue fluorescence after 48 hours shows that CPA47 cells mostly remained in their initial cell positions, and the position of red fluorescence shows that A172 cells remained within the Matrigel barrier (**Figure 3A**). The experiment was conducted only on one square region of the BBB model (**Figure 3**). We conducted this experiment twice, and both attempts yielded very similar results, thus supporting its reproducibility. These results suggest that the 3D-printed Matrigel scaffold successfully maintains cell structure and position.

Next, we confirmed that CPA47 cells remained alive under experimental conditions inside the Matrigel scaffold after 96 hours (**Figure 3B**). The change in position of red fluorescence indicated by the white arrow displays that some A172 cells were able to penetrate the Matrigel barrier and disperse from the initial positions (**Figure 3B**). This shows the invasive potential of A172 cells to pass the endothelial BBB. We also observed that the fluid Matrigel scaffold droops down and does not hold its structure stably after 96 hours of incubation in cell culture medium. Therefore, we speculate that the changes in the position of blue fluorescent cells after 96 hours are due to the spreading out of the Matrigel barrier itself over time. Overall, these results from our artificial BBB model indicated that we successfully optimized the 3D printing conditions to allow the cancer cells to penetrate the BBB.

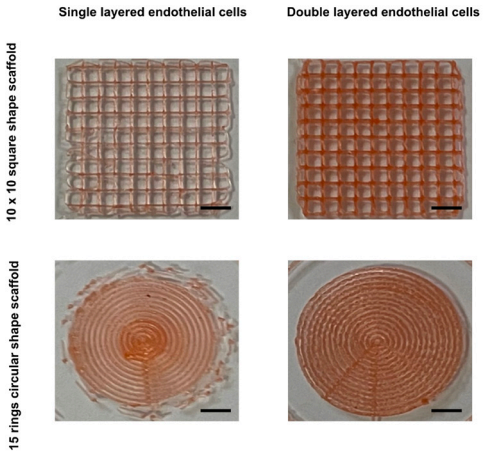

Figure 2. Image of Matrigel-based scaffolds containing CPA47 cells to create artificial BBB (N=2). After a syringe containing Matrigel mixed with CPA47 was installed in a 3D bioprinter, two 10 x 10 square scaffolds, with final dimensions 20 mm x 20 mm, and two circular scaffolds, with a final diameter of 20 mm, were printed on the cell culture dish. Producing single- and double-layered Matrigel-based scaffolds, both square and circular identified that a double-layered square scaffold most effectively maintains a fixed shape. Scale bar = 5 mm.

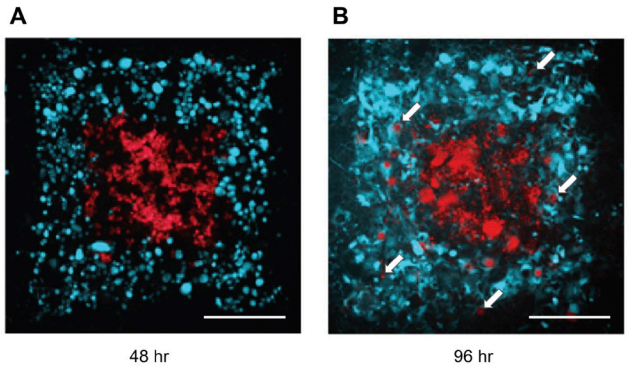

Figure 3. Fluorescent microscopy image of A172 and CPA47 cells within a 3D bioprinted artificial BBB section, depicting the penetration of A172 cells through the BBB after 96 hours. A172 and CPA47 cells were treated with CellTracker Red and CellTracker Blue, respectively, for 2 hours. Then, CPA47 cells were mixed with the Matrigel to generate a 10 x 10 3D square scaffold, with final dimensions 20 mm x 20 mm (see Figure 2). A172 cells were then placed in the center of the one empty square. We successfully created an artificial BBB to observe the movement of endothelial cells and brain tumor cells when present together, as cell positions were initially fixed by 3D Bioprinter structure. (A) A172 cells (red fluorescence) and CPA47 cells (blue fluorescence) shows the change in cell positions within 48 hours. (B) A172 cells and CPA47 cells shows a change in cell positions 96 hours after first placement. The white arrows indicate the A172 cells that have penetrated the Matrigel barrier. Scale bar = 1.0 mm.

DISCUSSION

In this study, we developed a novel BBB model using a 3D bioprinter. After printing four different BBB models, including single- and double-layered square and circular shapes, we identified that the double-layered square scaffold fostered optimal BBB functions for two reasons. Firstly, it most effectively maintained a fixed shape (**Figure 2**). Secondly, the two layers provided sufficient height to prevent the A172 glioblastoma cells from passing into an adjacent square during cell ejection. The use of CPA47 endothelial cells in the lining of a double-layered square BBB model and A172 glioblastoma cells in the empty square spaces showed that most cells remained healthy in their initial positions after 48 hours (**Figure 3**). After 96 hours, the movement of A172 cells across the Matrigel barrier showed the effectiveness of the BBB model, as cancer metastasis was carried out normally (**Figure 3**).

One of the most crucial functions of the BBB is its high selectivity regarding the materials that can pass into the brain. Substances that can cross the BBB have characteristics such as high lipophilicity, small size, and low molecular weight. Thus, it is important to confirm that our BBB model is also semipermeable and filters the entry of substances. Because this could not be confirmed in our study, future experiments should test the blocking ability of non-lipophilic molecules with high molecular weights. Whilst the human BBB is made of a single layer of blood endothelial cells, our BBB model is made of multiple layers of cells with irregular structures (9). Due to this difference, results derived from our artificial BBB model may not correspond to the actual BBB tissue functions. The BBB is also composed of a variety of cells, such as glia and mural cells, beyond just endothelial cells (10). This limitation of our BBB model could be overcome by co-culturing endothelial cells with primary glia and mixing them with Matrigel for printing. Further, to understand the cruciality of the endothelial tight junction structure in BBBs, the tight junction proteins should be investigated in CPA47 cells in our BBB model through immunostaining. Another limitation is that, for each of the endothelial and brain tumor cells, only one cell line was used. Therefore, further research is needed to confirm that this method can be used to develop BBB models with other endothelial and brain cancer cell lines. Furthermore, the low viscosity of the Matrigel base limits the number of hours the BBB structure can maintain its shape. Another large limitation of Matrigel is its batch-to-batch variability resulting from being animal-derived. By affecting cell behavior and possibly printability, this limits the reproducibility of our BBB model. To overcome these drawbacks, other bioinks should be tested. A previous study demonstrated that agarose-based hydrogels, which display characteristics of high strength and rigidity, have been successfully used for 3D bioprinting cartilage tissue, and therefore may be an alternative bioink for BBB models (11).
Since the CellTracker fluorescent staining method stains dead cells as well as living cells, the observed blue fluorescence may not indicate that all visible cells are alive. Thus, an alive or dead staining method should be conducted on the CPA47 blood endothelial cells and the A172 brain tumor cells to confirm that they are alive after BBB scaffold printing. Another limitation is that although brain cancer cell penetration through the BBB scaffold was observed, this may have been achieved through the Matrigel itself. Therefore, a control experiment using only Matrigel should be performed in the future. To show that the BBB scaffold serves its function, a control scaffold should be created with only Matrigel, without CPA47 cells. The ability of brain cancer cells to penetrate the control scaffold should be measured and recorded. Further,

our observation of A172 cell movement is ambiguous in that it may either represent cancer metastasis or simply the migration of cells via floating in the liquid medium. Thus, we should perform the experiment using negative control cells that migrate but do not penetrate the BBB to validate our conclusion.

3D bioprinting has made substantial progress in replicating functional tissues, including the BBB (12). Due to its relative cost-effectiveness, minimal requirement of resources, and efficiency, producing BBB models using a 3D bioprinter has high reproducibility. This opens many new opportunities for scientific discoveries from enhanced studies on the BBB's elaborate mechanisms to the behavior of brain cancer cells. Overall, this study demonstrates the promise of bioprinted BBB models, which may be applied in the future to develop potential groundbreaking clinical therapeutics, especially for brain cancers.

MATERIALS AND METHODS

Creating Matrigel-based scaffold using 3D bioprinter

The 3D layout of the Matrigel-based scaffold (356234, BD Biosciences) was designed using NewCreatorK V1.57.71 (ROKIT healthcare). Two types of Matrigel-based scaffold were prepared: square shape (0.05 mm nozzle) and circular shape (0.1 mm nozzle). After the 3D layout was prepared, the CPA47 endothelial cells were detached from the cell culture plate using trypsin Ethylenediaminetetraacetic acid (EDTA) buffer (Gibco). After the cells were completely detached from the cell plate, the cell suspension was moved to a 1.5 mL tube. CPA47 cells were cultured in Roswell Park Memorial Institute Medium 1640 (RPMI1640, Gibco) supplemented with 10% fetal bovine serum (Thermofisher) and 1% penicillin and streptomycin (Gibco) for 48 hours, then about 8 x 10^6 CPA47 cells were mixed with 5 mL of Matrigel solution. The mixture of blood endothelial cells with Matrigel was moved to the 3D printer syringe. Then, the 3D bioprinter was sterilized for five minutes prior to printing. During this time, the High Efficiency Particulate Air (HEPA) filter and UV lamp inside the Bioprinter were turned on to remove contaminants. Then, a 100 mm culture plate was placed under the 3D extrusion bioprinter (Dr. InVivo 4D2, ROKIT Healthcare) and was fixed onto the middle of the printing position by four metal holders. On the settings of the bioprinter software, the pressure of the printing nozzle was controlled for the flow rate optimization of the Matrigel mixture. After the first layer of the scaffold was printed, a second layer was printed on top.

Modeling the penetration of brain cancer cells through Matrigel-based scaffold

Before producing a Matrigel-based scaffold with CPA47 cells, the cells were stained with 1:1000 diluted 25 mM CellTracker Blue CMAC Dye (C2110, Thermo Fisher Scientific) for 2 hours at 37°C. After removing the staining solution, the cells were washed with Phosphate-Buffered Saline (PBS) three times. Then, the stained cells were mixed with the Matrigel solution to create a 1.2 x 106 cells/mL concentration. The mixture solution was added to the 3D printer syringe. A172 human glioblastoma cancer cells were stained with 1:1000 diluted 25 mM CellTracker Red CMAC Dye (C34552, Thermo Fisher Scientific) for 2 hours at 37°C. To dissociate cells from the cell culture plate, 500 µL of trypsin EDTA. The cell suspension was then removed from the culture plates and mixed with 500 µL of RPMI1640 medium. Then, 100 µL of cell suspension was placed onto each corner of the square-shaped scaffold (total 400 µL), as well as on the center of the circular scaffold. When all the cells were attached to the surface of the culture plate in 24 hours, 10 mL RPMI cell medium was added to each plate to cover the Matrigel scaffold matrix fully. To visualize the fluorescence, an EVOs™ M5000 Imaging System (AMF5000, Invitrogen by Thermo Fisher Scientific, Spain) was used. This experiment was successfully replicated twice.

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