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The Effects of Inflammation on Blood-Brain Barrier Permeability and the Ability of Cancer Nanomedicine to Cross the Blood-Brain Barrier

Michelle Zhou

Abstract: There are many membranes around the body, such as the blood-brain barrier (BBB), that stop treatment drugs from reaching targeted disease sites. Inflammation is known to disrupt various membranes in different ways. In this study, inflammation was induced on the BBB, and two assays were performed to measure BBB permeability. In the first assay, the cells were stained to visualize the cell nuclei and adherens junctions, respectively. The results of this immunostaining demonstrated that the cells treated with inflammation had significantly less VE-cadherin expression at the cell junctions, indicating increased BBB permeability. In addition, a transwell assay was used to quantify the movement of particles across a brain endothelial cell layer. Inflammation increased the quantity of particles that were able to cross through. These findings can help to optimize the treatment of brain diseases by increasing BBB permeability and allowing more drugs to be delivered to the brain.

Keywords: blood-brain barrier, permeability, inflammation, nanoparticles, drug delivery

Introduction

The blood-brain barrier (BBB) is a selectively permeable membrane that protects the brain from foreign molecules. While this membrane is meant to protect the brain from harm, it also stops many treatment molecules from reaching the brain and treating brain diseases, such as cancer. However, inflammation can cause the brain to become more vulnerable, making it easier for molecules to pass through the BBB. Thus, inflammation and BBB permeability play a pivotal role in determining the effectiveness of drug delivery to the brain. Furthermore, the rising usage of nanoparticles as a method to transport molecules across membranes has also contributed to increasing the quality of drug delivery to the brain. Nanoparticles pass through the BBB more easily than most other molecules because of their small size, and they have further properties that will be discussed later in this paper that also increase accurate drug delivery across membranes. This study analyzed the effects of inducing inflammation on the BBB while using specific nanoparticles on the extent of drug delivery across the BBB.

Literature Review

Blood-Brain Barrier

The BBB is essential for maintaining homeostasis, promoting neural function, and keeping toxic substances out of the brain (Girdhari & Lal, 2018). Because of its importance in protecting the brain, malfunctioning of the BBB can lead to many harmful consequences and is known to be linked to the causes of neurological infections and diseases such as Alzheimer's disease and multiple sclerosis, as studied by Janigro and Patabendige (2023). When the BBB is damaged, unwanted substances may enter and cause further damage to the brain. Unwanted substances can be bacteria or viruses that cause infections, off-target drugs that create dangerous side effects, and other molecules that may cause harm. Thus, understanding proper BBB functioning is essential in advancing human health and preventing brain damage. While significant research has been done on the BBB, gaps remain in the knowledge base regarding factors that disrupt the BBB and their effects on certain drug delivery methods.

To perform its job as a barrier, the BBB has several components that help to control its structure and its transportation of substances. Bergles et al. (2013) concluded that the BBB is a "complex, dynamic system that involves biomechanical and biochemical signaling between the vascular system and the brain" (para. 1). The BBB is composed of cells, proteins, and nutrients that allow communication between the brain and the external blood to allow essential substances to pass into the brain while restricting harmful substances from invading. However, this means that most artificial molecules also cannot pass. An essential part of the BBB that allows for this selective permeability are the cell-cell junctions that exist between the brain endothelial cells (bECs) that make up the BBB. These junctions are transmembrane proteins that attach bECs to each other; however, the junctions can become damaged through degradation, infection, and other causes, and leak unwanted substances in or out of the brain when disrupted. Disruptions to the junctions are a significant cause of BBB malfunction and may cause many different diseases (Huang et al., 2020). Thus, cell-cell junctions are an essential determining factor in BBB structure and integrity.

Brain Cancer

Brain cancer is a highly damaging and aggressive disease in which fast-growing cancer cells invade the brain and destroy brain tissue. While brain cancer may not be caused by malfunctioning of the BBB, this barrier plays a pivotal role in brain cancer treatment and drug delivery. Because of its highly selective permeability, the BBB does not allow most particles to reach the brain, meaning that treatments for neurological diseases may not be effectively delivered to the brain: "One of the limitations of chemotherapeutic treatment administered intravenously is the high proportion that does not reach the brain and the subsequent side effects that eventuate due to accumulation in off-target tissues" (Bell et al., 2022, para. 1). Common cancer treatments, such as chemotherapy and radiotherapy, mostly require therapeutic drugs to reach the targeted sites of disease to be effective. However, because the BBB is highly protective, these drugs are often unable to reach targeted sites in the brain in adequate amounts for treatment. This leads to ineffective treatment, as well as the accumulation of these drugs in healthy regions of the brain. These drugs induce harmful side effects when reaching off-target sites, and survival rates for brain cancer can be as low as five percent despite multiple treatment attempts (Alomari et al., 2022).

Furthermore, the BBB also "limits their [neuroimaging devices] utility by preventing brain delivery of most targeted molecular probes" (Farrar et al., 2011, para. 1). This means that it is difficult to image the brain and molecules inside the brain due to the surrounding BBB. Thus, the BBB also limits research on brain diseases, such as cancer, because it blocks imaging of the brain, which may be useful in studying different diseases and treatments. Overall, the BBB, while important in protecting the brain, causes many difficulties in brain cancer treatment and research.

Effects of Inflammation on Brain Endothelial Cells

However, one way that researchers have been able to increase drug delivery accuracy to targeted sites of brain diseases is by inducing inflammation on the BBB to increase the gap sizes between the bECs that

make up the barrier. Systemic inflammation is a sickness response that helps stimulate the body's healing process. When the BBB experiences inflammation, the barrier becomes more permeable and may be damaged in order to facilitate the healing of a certain wound (Galea, 2021). Therefore, if inflammation is induced on the bECs that make up the BBB, there would be an increase in the size and/or quantity of particles able to cross the barrier into the brain.

Additionally, according to researcher Abbott (2000), who conducted a study of inflammation's effects on the BBB, there are many types of inflammatory mediators (molecules that initiate inflammation) that can be used to force the BBB to become inflamed. For example, the release of interleukin-1 beta (IL-1β), an inflammatory mediator, can disrupt the occludin and ZO-1 (zonula occludens protein 1) junction proteins, which leads to malfunctions between the connections across ECs (Gan et al., 2022). Thus, inflammation of bECs can lead to increased BBB permeability due to the larger size gaps caused by disruptions from inflammatory mediators. Theoharides and Zhang (2011) attested to this phenomenon: "Immune dysfunction and inflammation appear to alter the integrity of the BBB" (para. 5). These malfunctions cause the BBB to weaken and allow more unwanted substances into the brain.

Although the BBB's integrity is essential for protecting the brain, a weakened BBB would allow for more options for treatment drugs to circulate through the brain. In addition, it is possible that inflammation already exists on the BBB in brain cancer patients, because inflammation is a natural immune response. Thus, inflammation of the BBB provides a less invasive treatment strategy as opposed to other commonly used drug delivery methods, such as intrafocal injections that are both highly dangerous and highly invasive.

Nanoparticle Medicine

Furthermore, another effective option for brain disease treatment delivery that can overcome the challenges that the BBB poses are nanoparticles (NPs). Nanoparticles are extremely small molecules that can pass through the BBB, as well as hold treatment drugs and be conjugated with certain antibodies that can target specific sites. "The BBB remains impermeable to nearly all large macromolecules and excludes nearly 98% of small-molecule drugs from the brain, limiting available therapeutic regimens," so nanoparticles have become a significant discovery that expands the options for drug delivery through the BBB (Alomari et al., 2022, para. 3).

In addition, not only is nanoparticle medicine able to cross the BBB, but it is also highly accurate in reaching targeted sites, as well as relatively inexpensive in terms of production costs, as Day and Riley (2017) pointed out. One type of nanoparticle commonly used in research is the Gold nanoparticle (AuNP). With a size range of ten to 30 nanometers, AuNPs are able to cross the BBB and induce "endothelial leakiness," which allows unwanted molecules to enter the brain (Bay et al., 2017, para. 1). Another form of nanoparticle are nanoparticles wrapped in the drug poly(lactic-co-glycolic acid) (also known as PLGA). These nanoparticles are used to target

triple-negative breast cancer cells and can block receptors that signal for cell division, which helps slow cancer cell growth (Dang et al., 2020). Therefore, nanoparticles are highly effective drug delivery tools that can help treat a variety of different diseases in which treatment is difficult due to a highly selective barrier.

Cancer Treatment Enhancement

Generally, by combining the use of inflammatory mediators and nanoparticles, drug delivery across highly selective membranes has become more efficient. Brusini et al. (2020) discovered that the common disease response of inflammation can help "enhance vasculature permeability," (para. 1) and allow nanomedicines to cross blood barriers and recognize targeted sites more efficiently. However, there is limited research on the use of inflammation and certain nanoparticles together on the BBB. Many studies have measured the impacts of potential variations of nanoparticles and environments to optimize drug delivery for specific cancers. For example, Arvanitis et al. (2018) concentrated on the impact of microbubbles (small, gas-filled bubbles) on nanoparticle medicine that targets breast cancer. Similarly, Dang et al. (2019) conducted a study on how nanoparticles were able to block signaling pathways that triggered tumor growth. Overall, many unique studies have been conducted to

test the optimal conditions for effective cancer treatment, but this study will utilize PLGA NPs, which have been understudied when considering inflammation and the BBB.

Research Gap and Purpose

Although there are large amounts of research done on inflammation, nanomedicine, and brain cancer, there is a gap in the body of knowledge regarding the intersection between these three topics. While many studies have identified and reported the ability of different cancer therapy-loaded nanoparticles to cross the BBB, there is a wide range of nanoshells, medications, molecules, and antibodies that can be conjugated together to optimize drug delivery case by case. Additionally, inflammation is an important factor to consider when optimizing drug delivery, because inflammation can impact BBB permeability. Thus, there is a lack of research in observing the effects of inflammation on the BBB and specific drug-loaded nanoparticles that can help treat brain cancer. This helps to pose the question: to what extent does inflammation of the BBB impact the permeability of the BBB and the ability of cancer chemotherapy nanoparticles to cross this barrier? The initial hypothesis was that inflammation would increase BBB permeability by decreasing the number of junctions that bond the bECs together and increasing the number of particles that could pass through this barrier.

The overall purpose of this study is to examine how inflammation can help increase drug delivery to the brain. This study is also extremely relevant in current times because it not only tests the abilities of potential therapies to treat the ongoing issue of cancer, but it also works to optimize drug delivery to the brain, which is a difficult area to treat due to the highly selective BBB. Cady and Curley (2018) identified the problem that "therapeutic delivery is often restricted by the BBB, which prevents transport of clinical compounds to their region of interest" (para. 1). If the hypothesis is correct, more inflammation on the BBB would lead to increased BBB permeability, and more brain cancer chemotherapy nanoparticles would be able to enter the brain to reach targeted sites, because inflammation would increase the gap sizes between bECs that make up the BBB. All in all, this study was conducted to increase the accuracy of brain cancer treatments and improve the survival rate of brain cancer patients.

Methods

Research Approach

For this study, a quantitative approach using the experimental method was ideal. By using a quantitative approach, the specific structures that compose bECs and the quantity of particles that could pass through the BBB were explicitly measured, which yielded results that directly showed the permeability/holes of the BBB. These types of numerical data have also been utilized in similar studies to provide a "quantitative framework for… the development of strategies to treat brain metastases" (Arvanitis et al., 2018, para. 1). Quantitative data can explicitly prove the effectiveness of different levels of inflammation on the BBB, because the amount of junction proteins and nanoparticle flowthrough are both quantifiable variables that can show how inflammation impacts drug delivery.

Research Method

Furthermore, the experimental method was used to prove cause and effect relationships.

This method helped to show and prove the relationship between inflammation and BBB permeability. Many specific procedures and tools were used in this study to quantitatively measure junction proteins and nanoparticle flowthrough, which are both factors that show BBB permeability. One form of measurement of BBB permeability was staining the junction proteins to visualize them and measure their presence using an imaging software called ImageJ, which is a common and accurate measurement tool used in many fields of study. Theoretically, a decreased number of junctions would allow more nanoparticles to pass through the BBB, because the junctions help to hold the cells together. To measure nanoparticle flowthrough, transwell inserts were used to mimic the BBB and quantify nanoparticles that pass through the artificial BBB. Through the experimental method, these procedures illustrated the impacts of varying levels of inflammation and its relationship with the amount of drug delivery across the BBB. This

method allowed for the manipulation and control of all variables in order to determine if inflammation was the variable influencing BBB permeability. The independent variable was the inflammation treatment, and the experimental method helped to prove the treatment's effects on the dependent variables, which were the quantity of junction proteins and nanoparticle flowthrough.

Subject Selection

The human cerebral microvascular endothelial cell line (hCMEC/D3) is a type of brain endothelial cell. These cells were used in this study to model the BBB, which is composed of mostly bECs. The hCMEC/D3 cell line was chosen because it is one of the most commonly used types of cells to model the BBB. In addition, these cells are easy to grow and manipulate, which is why they have been used in various types of studies, including studies that examine inflammation of the BBB. These cells also have drug transport mechanisms similar to those of the central nervous system, which is the brain and spinal cord, so they are relatively accurate in demonstrating the effects of different treatments on the brain.

Validity

Many factors increase this study's validity. Firstly, there are many different types of bECs; however, this study only utilized hCMEC/D3 cells grown at a concentration of 40,000 cells per well for every experiment, thus promoting uniformity and consistency, and adding to the internal validity of this study. In addition, this study was performed in a controlled laboratory setting in which measurement tools and instruments were sterile and free of contamination, thus mitigating the influence of external variables. The measurement tools that will be mentioned later on also have content validity, because they effectively measure fluorescent signals to indicate the presence of junction proteins or particles in a sample to show BBB permeability.

Procedure

Two assays were used to measure BBB permeability: the immunostaining assay and the transwell assay. In both experiments, hCMEC/D3 cells were cultured at 40,000 cells per well. Then, ten nanograms/milliliter of the inflammatory agent tumor necrosis factor alpha (TNF-α) was used to induce inflammation on each well of the treatment group cells. In the permeability assay, cells were grown on a chamber slide, the treated cells were induced with inflammation, and various antibodies that were attached to fluorescent markers were used to visualize the nuclei (used to count the cells), cytoskeleton (used to help outline the cells), and junctions of the cells (see Appendix A for further details). In the transwell assay, the cells were grown in transwell inserts, the treated cells were induced with inflammation, particles were pipetted into the inserts, and the particles that transported into the outer wells were collected at different time points (see Appendix C for further details).

Data Analysis

Statistical analysis was used to analyze the data to determine the effects of inflammation on the BBB. For both assays, a paired two-tailed t-test (see Appendix B for further details) was used to compare the junctions and the nanoparticle flowthrough of the treated and untreated cells. This test measured the average differences between two sets of data in which it cannot be predicted which data set will be greater or less than the other. This allowed for an accurate assessment of the quantitative data, because the analysis compared the averages of the signal of the junction proteins or particles to determine which experimental group had more junctions or particles.

Results

The purpose of this study was to examine the effects of inflammation on the permeability of the BBB. The hypothesis was that inflammation would increase BBB permeability, and there were two assays done to investigate this topic. The experimental method was used to show the cause-and-effect relationship between inflammation and various factors that demonstrate BBB permeability. Quantitative data was collected in order to compare the results of the cells treated with TNF-α with those of the untreated group.

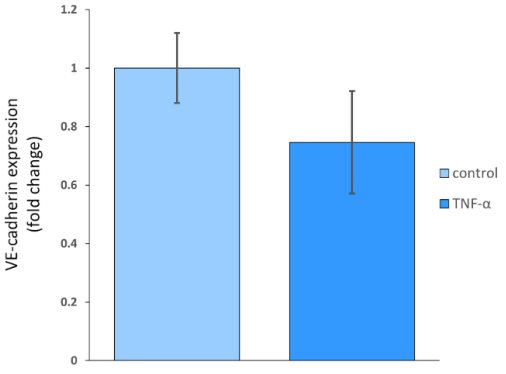
Permeability Assay

The first assay was the permeability assay. bECs were cultured on a slide and split into treatment and no treatment groups. The treatment group was treated with an inflammatory agent. One eight-well slide was used in each trial, and four wells on the slide were treated with TNF-α (the rest were untreated). All other growing conditions and times remained the same across all groups. The same immunofluorescence staining was done on all slides, and images of each well in every slide were taken to quantify the signal of various stains. DAPI (4′,6-diamidino-2-phenylindole) staining visualized the cell nuclei, Phalloidin staining visualized the cytoskeleton, and VE-cadherin staining visualized the junction proteins that hold cells together.

ImageJ was used to count the number of cells in each well by counting the cell nuclei, and this number was used to calculate the VE-cadherin expression per cell in each image. After immunostaining, images were taken at various locations in each well for all eight wells on all three slides. There were also two negative control wells (wells with cells but no staining) to determine the background signal of the VE-cadherin that resulted from the immunostaining (even though there were no bECs on these wells, ImageJ will always pick up at least some signal). The amount of VE-cadherin expression in these controls was averaged to quantify the background signal (33,331,625 arbitrary fluorescence units), which was then subtracted from the total VE-cadherin expression in each of the images from the other experimental wells. This VE-cadherin quantification was then divided by the total number of cells in that image to determine the average VE-cadherin signal per cell in each image. Finally, the VE-cadherin signal per cell in each image was averaged to get the overall VE-cadherin signal for the treated and untreated groups (see Appendix D).

Across the three trials, the cells treated with inflammation exhibited a lower VE-cadherin signal (see Figure 1). The treatment group's VE-cadherin expression was only about seventy-five percent of that in the control group, showing that TNF-α decreases the presence of junction proteins between bECs. These results have statistical significance ($p = 0.0204 < 0.05$).

Figure 1
Ratio of VE-cadherin Expression in Untreated (Control) Group to VE-cadherin Expression in Treated Group



Transwell Assay

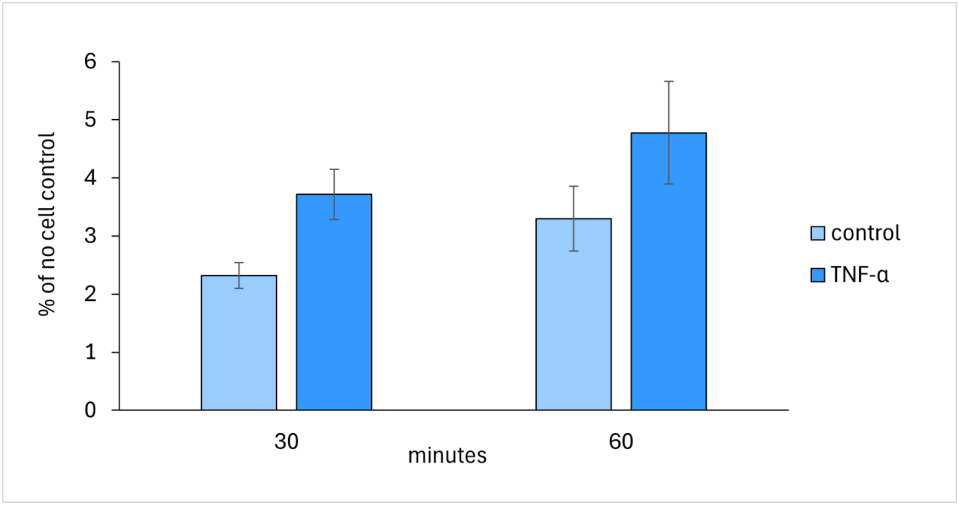
The second assay was the transwell assay. bECs were cultured on transwell inserts, and half of the inserts were treated with TNF-α. Particles were pipetted into the inserts (inner wells), and samples of the media in the outer wells were taken at specific timepoints to quantify the particles that transported across the cells on the inner well. Two different particles were used: FITC-dextran particles and Cy5-PLGA nanoparticles.

FITC-dextran particles are sugars attached to the fluorescent marker FITC (fluorescein isothiocyanate). Flowthrough samples were taken at 30 and 60 minutes. The Agilent BioTek Synergy H1 Plate Reader was used to measure the amount of dextran particles in each sample by reading the fluorescence emission of FITC. The plate reader excited the FITC at 490 nm and quantified the emission of the FITC at 520 nm.

Cy5-PLGA nanoparticles are made from poly(lactic-co-glycolic acid) and attached to the fluorescent marker Cy5 (Cyanine5). Samples were taken at one hour, four hours, and 24 hours, and the plate reader was used to quantify the nanoparticles in each sample by reading the fluorescence emission of Cy5. The plate reader excited Cy5 at 640 nm and quantified the emission of Cy5 at 670 nm.

Both the FITC-dextran assay and the Cy5-PLGA assay had four trials each. Each assay had four plates, each plate containing 12 wells: five treatment wells,

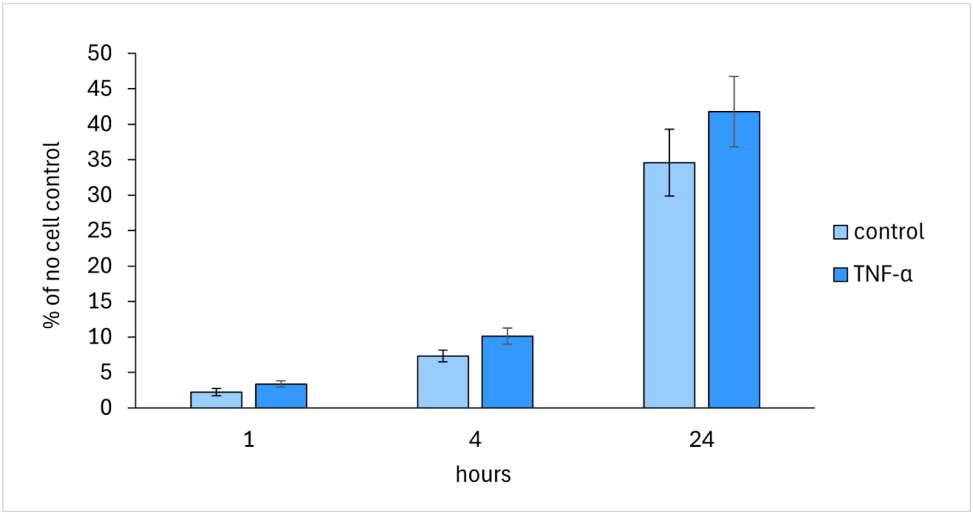
Figure 2
Amount of FITC-dextran Transport Over Time in Cells With and Without TNF-a Treatment



five untreated wells, and two negative control wells with no cells. One sample was taken from each well at each timepoint (see Appendix E).

In both transwell experiments, the wells treated with inflammation had more particle flowthrough (see Figures 2 and 3). The quantified particle flowthrough in each of the experimental wells was divided by the flowthrough of the control wells in that group to determine the percent of particle transport as compared with the negative control, which theoretically had complete (one hundred percent) transfer of particles because there were no cells in the control. These results have statistical significance (p [FITC-dextran] = $0.0039 < 0.05$, p [Cy5-PLGA] = $0.0090 < 0.05$).

Figure 3
Amount of Cy5-PLGA NP Transport Over Time in Cells With and Without TNF-a Treatment



Discussion

Through these results, it can be concluded that inflammation increases BBB permeability, which supports the initial hypothesis. In the immunostaining assay, the VE-cadherin signal was decreased in the cells treated with inflammation. VE-cadherin represents the junction proteins that hold bECs together, so less VE-cadherin signal would indicate less junction proteins.

Because the BBB is composed of mostly bECs, the decreased number of junctions demonstrated by the cells used in the immunostaining assay can be applied to the BBB. With inflammation, the cells had fewer junctions holding the cells together, which weakens the bond between the cells, thus increasing their permeability. This aligns with the current body of knowledge. Past studies, such as one done by Abbott (2000) have proven that "many inflammatory agents increase both endothelial permeability and vessel diameter, together contributing to significant leaks across the BBB" (para. 1).

In the transwell assay, the treated cells had a higher percentage of particles that could pass through the cell barrier on the transwell inserts. At all timepoints in both the FITC-dextran and Cy5-PLGA NP assays, the treated group had a stronger FITC or Cy5 fluorescence signal in the outer well, which indicated that more particles were able to pass through the smaller well (the transwell insert), because the particles were attached to the fluorescent markers (FITC or Cy5). The permeability of a substance is defined by the ability of molecules to pass through, and inflammation increased the ability of particles to pass through the bECs, indicating increased BBB permeability. Inflammation not only increases BBB permeability, but it can also "increase the entry of foreign substances" via other membranes in the body (Asano et al., 2023).

Implications

These conclusions help to further optimize the conditions for drug delivery across the BBB. Inflammation was proven to increase BBB permeability, which makes it easier for drugs to pass through the barrier and reach the brain. This conclusion can be used to increase the dose of drugs delivered to the brain for numerous treatments and diseases. Specifically, the results demonstrated that inflammation increases the number of Cy5-PLGA nanoparticles that can cross the BBB and enter the brain. PLGA nanoparticles are commonly used in delivering chemotherapy drugs to brain tumors; thus, this study has helped to optimize the drug delivery conditions of the treatment of brain cancers. This study fills a gap in the body of knowledge, because of the use of the PLGA nanoparticles. Various studies have examined the effects of inflammation on different membranes in the body, including the BBB. However, this study specifically examines the effects of inflammation on the ability of cancer nanomedicine, which is transported using PLGA NPs, to cross the BBB. PLGA NPs are highly effective in accurately reaching targeted disease sites and releasing drugs to treat the diseases, but in brain cancer specifically, the BBB reduces the effectiveness of the NPs. These conclusions help to decrease the severity of this obstacle and allows for drugs to reach the brain and treat brain cancer more effectively.

Furthermore, because inflammation is a naturally occurring immune response, inducing inflammation to improve the effectiveness of drug transport is a less invasive method of treating brain diseases. Injecting drugs for brain diseases into the bloodstream is less invasive, but the BBB greatly reduces the effectiveness of this drug transport method. Thus, it is essential to determine how to increase BBB permeability and allow less invasive methods to be more effective so that drug delivery to the brain can be safer. This study provided a basis for drug delivery to the brain to become safer by demonstrating that inflammation increases the effectiveness of drug delivery from the bloodstream to the brain, which is less invasive.

Limitations

However, certain limitations exist regarding the results of this study. First, data collection was limited by both time constraints and unexpected obstacles that came up when performing the experiments. A short data collection period limited the amount of data that could be collected, and adjusting the experimental procedure to accommodate for cell growth issues further cut into the time spent on performing the assays. Although at least three trials were done of each assay to demonstrate consistent data, these obstacles hinder the validity of the results. If there had been less limi-

tation in the data collection, more data could be collected to strengthen the results.

Furthermore, growing the cells to a consistent confluency also posed an issue. Not only did the cells grow at different rates at some points, but they also grew in many layers, making it difficult to see the separate cells and measure the gap sizes between the junctions. To accommodate for this, the number of junctions was quantified instead of the gap sizes to show the permeability of the BBB in a different way.

Another limitation in these results is that although increasing BBB permeability is a positive outcome in terms of drug delivery, the barrier is still essential in protecting the brain and increased BBB permeability could cause harm and disease. While this study has implications in creating a more effective method of drug delivery to the brain, the results of this study alone are not enough to start using inflammation to increase drug delivery. Further research must be done to examine the extent to which inflammation can be used to increase BBB permeability to ensure that this drug delivery method does not do more harm than it helps.

Future Research

Many further studies can be done regarding inflammation and BBB permeability. For example, similar assays can be performed *in vivo* to further display the effects of inflammation. This study was performed *in vitro* by modeling the BBB using bECs. While the results proved to be statistically significant, a stronger argument regarding inflammation and BBB permeability can be made with further *in vivo* studies. Furthermore, inflammation can be highly damaging to the BBB and an individual's health, so further research must be done to examine the limit to the amount of inflammation that can be used to effectively increase BBB permeability while still ensuring the safety of the patient.

Another path to explore could be targeted drug delivery across the BBB. This study only examined how the particles could reach the brain, but there are also many factors that influence whether the drugs can actually reach the targeted site. For example, many drug particles are attached to antibodies that can target specific structures. However, these antibodies can bind improperly or not bind at all to the structures for various reasons, which reduces the effectiveness of the treatment.

Conclusion

The results of this study suggest that inflammation increases BBB permeability, which allows drugs to be transported to the brain more easily. In both assays, the inflammatory agent TNF-α increased BBB permeability through impacting the bECs in various ways. The immunostaining assay focused on the junctions between the cells and how inflammation decreased their prevalence. In the transwell permeability assay, inflammation increased the ability of certain particles to pass through the BBB. Specifically, dextran and PLGA particles showed increased transport across the cells that were treated with TNF-α. The use of PLGA nanoparticles in the transwell assay helped to fill the research gap regarding inflammation and specific cancer nanomedicine transport across the BBB. The quantitative data resulting from this study have many implications in the healthcare field, such as optimizing drug delivery conditions to the brain. While it is possible that other factors influenced BBB permeability, optimizing the experimental procedure and conducting these experiments in a sterile laboratory setting helped to limit these external influences and increase the validity of the results. In the future, further research can be done to examine inflammation's impacts on the BBB *in vivo* to strengthen the results of this study.

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Appendix A

Inflammation Induction and Immunofluorescence Staining Procedure:

Plate and Treat Cells

1. Plate hCMEC/D3 cells at 40,000 cells/well (400 uL of media) and incubate at 37°C, 5% CO_2 for 4 days
2. Remove media and incubate with 10 ng/ml TNF-α (400 uL of media) for 24 hours

Fix and Permeabilize Cells

3. Prep 4% formaldehyde before starting wash steps
4. Gently rinse cells 3 times with 300uL of 1xPBS to remove TNF-α
5. Fix with 150uL of 4% formaldehyde in 1xPBS for 10 minutes at room temperature
   Prepare permeabilization solution during cell fixation
6. Wash cells 3 times with 300uL of 1xPBS for 5 minutes
7. Permeabilize cells with 100uL of 0.2% Triton-X in 1xPBS for 10 minutes at room temperature
8. Wash cells 3 times with 300uL of 1xPBS for 5 minutes

Primary and Secondary Antibody

9. Block with 100uL of 3% BSA in 1xPBS for 30 minutes at room temperature
10. Incubate cells with 100uL of VE-cadherin antibody at 1µg/mL (1:200) in PBST overnight on rocker at 4°C
11. Wash samples three times with 300uL of 1xPBS for 5 minutes
12. Incubate cells with 100uL of goat anti-mouse secondary antibody AlexaFluor 594 at 2µg/mL (1:1000) in PBST for 1 hour at room temperature in the dark
13. Wash samples three times with 300uL of 1xPBS for 5 minutes

Prep Chamber Slides

14. Remove chamber walls
15. Mount slide with VectaShield DAPI mounting media (~1 droplet per well)
16. Seal slide with coverslip & nail polish: Let the slide dry ~30 min (check for any leaks) before imaging

Image Slides (EVOS m5000 Scope)

17. Image via Fluorescence Microscopy (20x Objective)
    DAPI (nuclei)
    Texas-Red (VE-cadherin)

Analyze Images (ImageJ)

18. Analyze via ImageJ (VE-cadherin intensity/cell)

Appendix B

Quantification of VE-cadherin in Images Using ImageJ

Procedure:

Quantification in ImageJ

1. Open ImageJ and open image files – VE-cadherin channel and DAPI channel in separate images
2. For DAPI image:
   a. Threshold DAPI image to make black and white → Image > Adjust > Threshold or Ctrl+Shift+T
      - Method: Percentage
      - Press Apply
   b. Add 1-pixel lines in between cells → Process > Binary > Watershed
   c. Perform automated analysis: Analyze>Analyze Particles
      - Check off "Pixel Units," Adjust Size (inch2): 600-infinity
      - Modify Circularity: 0.00-1.00
      - Show: Outlines
      - Check off Display results, Clear results, Exclude on edges
      - Press OK when all parameters set
3. For VE-cadherin image:
   a. Analyze > Set Measurements → check off Area, Integrated density, and Mean gray value
4. Output and Results of the analysis
5. Copy results into Excel spreadsheet
6. Measure background values (VE-cadherin negative control (no primary antibody) image)
7. Perform analysis in Excel
   a. Calculate average fluorescence of the background by taking the average of the Integrated Density column
   b. Calculate CTCF (corrected total cell fluorescence):
      - CTCF = Integrated Density – Average fluorescence of background readings
   c. Average the CTCF for all cells present in images for each sample

Statistical Analysis

8. One-tail paired t-test (Excel) was performed to compare the mean CTCF of TNF-α treated and non-treated groups. The threshold for statistical significance was set to * $p < 0.05$.

Appendix C

Inflammation Induction and Transwell Permeability Assay

Procedure:

Plate and Treat Cells

1. Coat the inserts with 50uL of 150ug/mL collagen in incubator for at least an hour; aspirate the excess liquid
2. Fill the 24-well plate with 600uL of media/well
3. Transfer the inserts into wells
4. Plate hCMEC/D3 cells at 200,000 cells/insert (100uL of media) and incubate for 2 days
5. Remove media from the top and bottom chamber and incubate with 100ng/mL TNF-α (100uL of media for top and 600uL of media for bottom) for 24 hours

Transwell Assay

6. Change media in the bottom chamber (600uL)
7. Remove media from the inserts and add 100ul of media containing FITC-dextran or Cy5-PLGA NP in the top chamber and incubate
   - 250ug/mL FITC-dextran (70kD)
   - 1mg/mL Cy5-PLGA NP
8. Sampling: remove 100uL of sample from the bottom chamber at below time point and add 100ul of media to the bottom well after sampling
   - FITC-dextran: 30, 60 minutes
   - Cy5-PLGA NP: 1, 4, 24 hours
9. Fluorescence of the samples was measured using a plate reader
   - FITC-dextran: excitation at 490nm and emission at 520nm
   - Cy5-PLGA NP: excitation at 640nm and emission at 670nm Statistical Analysis
10. Permeability is calculated as percent of control (insert with no cells). Two-tail paired t-test (Excel) was performed to compare the mean permeability of TNF-α treated and non-treated groups. The threshold for statistical significance was set to * $p < 0.05$.

Appendix D

Immunostaining Permeability Assay - Raw Data (Three Trials)

Treatment	Well #	Location #	# of Cells	Total Density	Density per Cell
Not Treated, No Stain	1	1	1046	34886261	N/A
Not Treated, No Stain	1	2	1030	33242326	N/A
Not Treated, No Stain	1	3	1004	33194783	N/A
Not Treated	2	1	1002	262107699	228319.4351
Not Treated	2	2	1014	275829474	239149.7525
Not Treated	2	3	1001	236555245	203020.5994
Not Treated	2	4	1020	251457290	213848.6912
Not Treated	5	1	952	290375460	270004.0284
Not Treated	5	2	1056	251708247	206796.0436
Not Treated	5	3	1036	327840725	284275.1931
Not Treated	5	4	992	309508074	278403.6784
Not Treated	6	1	1020	279967911	241800.2804
Not Treated	6	2	1014	271110044	234495.4822
Not Treated	6	3	1065	281568364	233086.1399
Not Treated	6	4	1039	290995258	247991.9471
Treated, No Stain	3	1	944	34720187	N/A
Treated, No Stain	3	2	901	32925503	N/A
Treated, No Stain	3	3	927	31020692	N/A
Treated	4	1	876	187289889	175751.4429
Treated	4	2	903	200340306	184948.7054
Treated	4	3	854	154443964	141817.7272
Treated	4	4	878	176526191	163091.7608
Treated	7	1	940	192756730	169601.1755
Treated	7	2	900	271941784	265122.3989
Treated	7	3	938	241065013	221464.1663
Treated	7	4	971	264118504	237679.587

Treated	8	1	838	253665091	262927.7637
Treated	8	2	891	222059065	211815.3086
Treated	8	3	831	224778090	230380.8243
Treated	8	4	843	226896227	229614

Treatment	**Well #**	**Location #**	**# of Cells**	**Total Density**	**Density per Cell**
Not Treated	1	1	862	191438543	183418.6984
Not Treated	1	2	853	224271442	223845.0375
Not Treated	1	3	871	224750530	219769.1217
Not Treated	1	4	863	228500798	226151.9965
Not Treated	2	1	864	186560082	177347.7512
Not Treated	2	2	871	192467216	182704.4673
Not Treated	2	3	878	176502160	163064.3907
Not Treated	2	4	930	181303698	159109.7559
Not Treated	5	1	905	185333285	167957.6354
Not Treated	5	2	856	187007226	179527.5713
Not Treated	5	3	847	181702011	175171.6482
Not Treated	5	4	881	191082278	179058.63
Not Treated	6	1	964	182225126	154453.8392
Not Treated	6	2	854	209579456	206379.1932
Not Treated	6	3	878	202754706	192964.7847
Not Treated	6	4	891	238678931	230468.3569
Treated	3	1	858	146037192	131358.4697
Treated	3	2	806	132812511	123425.4169
Treated	3	3	785	133920804	128139.0815
Treated	3	4	862	139103943	122705.7053
Treated	4	1	854	164678224	153801.6382
Treated	4	2	873	135836071	117416.3184

Treated	4	3	872	132688674	113941.57
Treated	4	4	863	142931799	126999.0429
Treated	7	1	779	133433200	128500.0963
Treated	7	2	825	126501850	112933.6061
Treated	7	3	823	148036847	139374.5103
Treated	7	4	725	129194527	132224.6924
Treated	8	1	734	124828716	124655.4373
Treated	8	2	864	147366191	131984.4514
Treated	8	3	831	136140799	123717.4176
Treated	8	4	818	164255905	160054.132

Treatment	**Well #**	**Location #**	**# of Cells**	**Total Density**	**Density per Cell**
Not Treated	1	1	995	274577772	242458.4392
Not Treated	1	2	1042	308301372	263886.5134
Not Treated	1	3	983	314249174	285775.7365
Not Treated	1	4	1004	240154868	205999.246
Not Treated	2	1	1059	267445761	221070.95
Not Treated	2	2	1029	270778364	230754.8484
Not Treated	2	3	995	236018628	203705.5307
Not Treated	2	4	1027	310011681	269406.0915
Not Treated	5	1	912	306325231	299335.0943
Not Treated	5	2	965	258545262	233382.0073
Not Treated	5	3	998	246692501	213788.4529
Not Treated	5	4	969	265504916	239600.9195
Not Treated	6	1	1015	308182041	270788.5872
Not Treated	6	2	984	278500739	249155.6037
Not Treated	6	3	975	259656321	232127.8933
Not Treated	6	4	992	284151550	252842.6663

Treated	3	1	892	176686378	160711.6065
Treated	3	2	918	214619792	197481.6634
Treated	3	3	892	177114030	161191.037
Treated	3	4	882	191122744	178901.4955
Treated	4	1	930	137938986	112481.0333
Treated	4	2	828	129114443	115679.7319
Treated	4	3	922	141752780	117593.4436
Treated	4	4	861	126599707	108325.2985
Treated	7	1	910	173673420	154221.7527
Treated	7	2	918	177346365	156878.8017
Treated	7	3	914	190179720	171606.2309
Treated	7	4	900	168214047	149869.3578
Treated	8	1	886	181977201	167771.5305
Treated	8	2	867	151159437	135902.8973
Treated	8	3	921	173902293	152628.304
Treated	8	4	870	164522161	150793.7195

Appendix E

Transwell Permeability Assay - Raw Data

FITC-dextran Data

Trial	Timepoint (minutes)	Treatment	Average Fluorescence	% Fluorescence
1	30	Not Treated	5375.6	2.580007295
1	30	Treated	7744.4	3.7169076
1	30	No Cells	208356	N/A
1	60	Not Treated	8732.4	3.668451377
1	60	Treated	11561	4.856736564
1	60	No Cells	238040.5	N/A
2	30	Not Treated	6517.8	2.162597984
2	30	Treated	10107.2	3.353556468
2	30	No Cells	301387.5	N/A
2	60	Not Treated	9340.8	2.467354596
2	60	Treated	13683.8	3.614549806
2	60	No Cells	378575.5	N/A
3	30	Not Treated	4462.6	2.433811359
3	30	Treated	7931	4.32540624
3	30	No Cells	183358.5	N/A
3	60	Not Treated	7131.2	3.558314351
3	60	Treated	11528.8	5.752621507
3	60	No Cells	200409.5	N/A
4	30	Not Treated	8850.4	2.10705749
4	30	Treated	14606.2	3.477368607

4	30	No Cells	420036	N/A
4	60	Not Treated	16676.8	3.496646308
4	60	Treated	23312.8	4.888025043
4	60	No Cells	476937	N/A

Cy5-PLGA NP Data

Trial	Timepoint (hours)	Treatment	Average Fluorescence	% Fluorescence
1	1	Not Treated	46.6	2.937283328
1	4	Treated	64	4.034037189
1	24	No Cells	1586.5	N/A
1	1	Not Treated	206.8	7.956906503
1	4	Treated	297	11.4274721
1	24	No Cells	2599	N/A
1	1	Not Treated	1748.2	34.88028731
1	4	Treated	2184.2	43.57940942
1	24	No Cells	5012	N/A
2	1	Not Treated	32.4	1.856733524
2	4	Treated	54.6	3.128939828
2	24	No Cells	1745	N/A
2	1	Not Treated	175.8	6.356897487
2	4	Treated	244.4	8.83746158
2	24	No Cells	2765.5	N/A
2	1	Not Treated	1414.2	31.18412348
2	4	Treated	1698	37.44211687
2	24	No Cells	4535	N/A
3	1	Not Treated	36.4	2.08297568
3	4	Treated	54.4	3.113018598
3	24	No Cells	1747.5	N/A
3	1	Not Treated	185.4	6.933433059
3	4	Treated	253.2	9.468960359
3	24	No Cells	2674	N/A

3	1	Not Treated	1475.8	31.05639731
3	4	Treated	1811.2	38.11447811
3	24	No Cells	4752	N/A
4	1	Not Treated	37.6	1.987315011
4	4	Treated	59.6	3.150105708
4	24	No Cells	1892	N/A
4	1	Not Treated	223	8.068017366
4	4	Treated	296.2	10.71635311
4	24	No Cells	2764	N/A
4	1	Not Treated	2085.2	41.17693523
4	4	Treated	2430.8	48.00157978
4	24	No Cells	5064	N/A