Dexamethasone's impact on LPS-induced inflammation of blood-brain barrier endothelial cells

UNIVERSITY of the
WESTERN CAPE

Department of Medical Bioscience

By

Tershlin Jeftha
BSc Honours Medical Bioscience, UWC

A thesis submitted in partial fulfillment of the requirements for the degree of ***Magister Scientiae (M.Sc.)*** Medical Biosciences in the Department of Medical Biosciences, Faculty of Natural Sciences, University of the Western Cape, South Africa.

Supervisor:
Professor David Fisher

Co-Supervisor:
Doctor Khayelihle Brian Makhathini

15 May 2023

Abstract

The blood-brain barrier (BBB) serves as a protective and regulatory barrier between the blood and the brain. Microvascular endothelial cells provide homeostatic regulation of the central nervous system (CNS). BBB integrity is compromised by inflammation, which contributes to a wide range of neurological disorders. It has been shown that glucocorticoids (GCs) have anti-inflammatory functions on a variety of cells. A particular example is Dexamethasone (Dex), which is used for the treatment of inflammatory conditions and has recently been found to be effective in attenuating COVID-19. **Aim:** The purpose of this study was to determine whether low or high concentrations of Dex can attenuate the inflammatory response induced by LPS on the *in vitro* BBB model. **Method:** bEnd.5 cells were cultured and exposed to LPS (100ng/ml) and subsequently co-treated with Dex to investigate whether selected concentrations (0.1, 5, 10, 20μM) of Dex can modulate the inflammatory effects of LPS on the bEnd.5 cells. Cell viability, cell toxicity, and cell proliferation were investigated, as well as the monitoring of membrane permeability (TEER), and the use of ELISA to identify and quantify the presence of inflammatory cytokines (TNF-a and IL-1β). **Results:** Dex, at a lower dosage (0.1μM), was able to attenuate the inflammatory effects of LPS on bEnd.5 cells. This effect was not seen for Dex at higher dosages (5-20μM). Lower doses of Dex (0.1μM) had no detrimental effects on bEnd.5 cells, while higher Dex doses (5-20μM) decreased bEnd.5 viability, increased bEnd.5 cell toxicity, increased bEnd.5 cell monolayer permeability, and increased pro-inflammatory cytokine secretion. **Conclusion:** Dex at low concentrations (0.1μM), will be more effective than higher concentrations in an attempt to remedy LPS-induced inflammation on bEnd.5 cells.

Declaration

I declare that Dexamethasone's impact on LPS-induced inflammation of blood-brain barrier endothelial cells is my own work, that it has not been submitted for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged by complete references.

Full name: Tershlin Jeftha Date: 15 May 2023

Signed.....

Table of contents

List of abbreviations

Angiotensin II	Angll
Angiotensin-converting enzyme	ACE
Blood-brain barrier	BBB
Brain microvascular endothelial cells	BMVEC
Central nervous system	CNS
Claudin	CLDN
Coronavirus disease 2019	COVID-19
Dalton	Da/kDa
Dexamethasone	Dex
Endothelial cells	ECs
Enzyme-linked immunoassay	ELISA
Glucocorticoids	GCs
Interleukin	Interleukin
Junction adhesion molecule	JAM
Lipopolysaccharide	LPS
Matrix metalloproteinases	MMPs
Neurovascular unit	NVU
Occludin	OCLN
Pericytes	PCs
Tight junctions	TJs
Toll-like receptor-4	TLR4
Trans-endothelial electrical resistance	TEER
TNF	Tumor necrosis factor

List of Figures

Chapter 1: Literature review

1.1 Introduction

An integral part of the brain is a protective barrier called the blood-brain barrier (BBB). The BBB primarily comprises endothelial cells (ECs) (Markowicz-Piasecka et al., 2022) and forms the brain's capillaries. The BBB separates the circulatory system from the brain, restricting the entrance of toxins and other harmful substances (Markowicz-Piasecka et al., 2022). The BBB serves more than just a barrier; it also serves various roles, including, homeostasis, and metabolism, and regulates the entry of immune cells (Abbott et al., 2006; Winkler et al., 2011).

There is a relationship between the BBB and the neurovascular unit (NVU), however, they are distinct concepts (Yu et al., 2020). In terms of physical and functional barriers, the BBB is the barrier between blood circulation and the brain tissue, while the NVU refers to the network of neural cells and structures that plays an important role in maintaining the integrity of the BBB and regulating the permeability of the BBB and capillary blood flow (Yu et al., 2020). The BBB comprises mainly of ECs, pericytes, astrocytes, and tight junctions (Abbott, 2005), while the NVU includes the surrounding neural cells, which engage with the regulation of the BBB, either mainly via the astrocytes or directly via pericytes. A normal functioning central nervous system (CNS) and its ability to respond to injury or disease depend on the communication between these cells (Daneman and Prat, 2015). The NVU is responsible for regulating the accurate provision of nutrients and oxygen to the brain, while also maintaining a high integrity of the BBB. An effective and healthy brain relies heavily on the robust regulation of BBB, since it delivers glucose and other substrates to the brain, as well as removes waste products from the brain to help maintain the healthy functioning of neurons (Kaplan et al., 2020). A dysfunctional neurovascular unit has been linked to various neurological disorders, such as stroke, Alzheimer's disease, and multiple sclerosis (Yu et al., 2020).

The BBB can be compromised by both systematic inflammation and neurological pathologies (Markowicz-Piasecka et al., 2022), which may lead to BBB impairment. As a result of the barriers impairment, neurological pathologies such as epilepsy, stroke, brain oedema, and neurodegenerative disorders can occur (Baeten and Akassoglou, 2011; Raabe et al., 2012; Markowicz-Piasecka et al., 2022; Réus et al., 2015). Additionally, oxidative stress, metabolic dysregulation (Sandoval and Witt, 2008; Michinaga and Koyama, 2015; Rhea and

Banks, 2019), trauma, inflammation, and ischemia often promote brain vascular deterioration, such as stroke, which accounts for millions of medical emergencies (Roy-O'Reilly and McCullough, 2018; Rhea and Banks, 2019).

Sankowski et al., (2015) point out that inflammation is an essential and spontaneous response to infection and tissue damage, but it may also negatively affect brain endothelial cells (BECs) and BBB function. Identifying the underlying cause of inflammation is critical to the success of the treatment. The use of medications, such as nonsteroidal anti-inflammatory drugs (NSAIDs) or steroids such as glucocorticoids , may be necessary to manage inflammation and prevent further damage to brain tissue (Ghlichloo and Gerriets, 2022).

Dexamethasone (Dex) is an established synthetic glucocorticoid steroid with little to no mineralocorticoid action (Brinks et al., 2018; Hench and Kendall 2021; Yasir et al., 2021), authorized by the Food and Drug Administration (FDA) in 1958 as a broad-spectrum immunosuppressant. In addition, it has a longer half-life and is 30 times as potent as cortisone (Giles et al., 2018). In recent events, glucocorticoid steroid, Dex, has been used to treat inflammation and recently as a treatment for COVID-19 (Corssmit and Dekkers, 2019; Lukiw, 2020). Dex is thought to achieve this by preventing neutrophil migration and reducing lymphocyte colony accumulation, which reduced the mortality rate of hospitalized patients who were not critically ill (Lukiw et al., 2020). Dex was demonstrated to be an effective treatment in COVID-19 patients who have been intubated for a short period (Theoharides and Conti, 2020). It was the first pharmaceutical that reduced mortality for hospitalized COVID-19 patients (Lammers et al., 2020). Furthermore, a major advantage of Dex is its ability to reduce the synthesis and secretion of cytokines, which are known to have damaging effects on cells (Chuang et al., 2017). However, its effectiveness in treating brain vascular inflammation at the level of the capillary endothelium has yet to be established.

1.1.1 Over of the blood-brain barrier (BBB)

Germain scientist Paul Ehrlich first conceptualized the BBB in the early 1900s. His experiments on staining biological tissues showed that the brain and spinal cord remained unstained while the peripheral tissues stained blue (Dyrna et al., 2013). Dye injection into the subarachnoid space stained only the CNS, while bodily tissues did not. Using these findings, Ehrlich proposed that blood vessels in the brain differ from those in systemic circulation (Dyrna et al., 2013). In the CNS, the BBB separates circulating blood from the extracellular fluid in

the brain (Abbott et al., 2006). The brain capillaries are lined with ECs, which are connected by tight junctions (TJs) that prevent the passage of harmful substances (Markowicz-Piasecka et al., 2022).

By restricting the entry of harmful substances into the brain, such as toxins, pathogens, and large molecules, the BBB plays an essential role in maintaining the chemical stability of the brain's environment (Winkler et al., 2011). Additionally, it regulates the brain and blood's exchange of nutrients and wastes. A selective permeability characteristic of the BBB means certain substances can pass through it when specific conditions are met. Air, carbon dioxide, and water are small molecules that can pass through the BBB freely (Winkler et al., 2011). Several other molecules are transported across the BBB by specialized transporters, such as glucose and amino acids (Abbott et al., 2006).

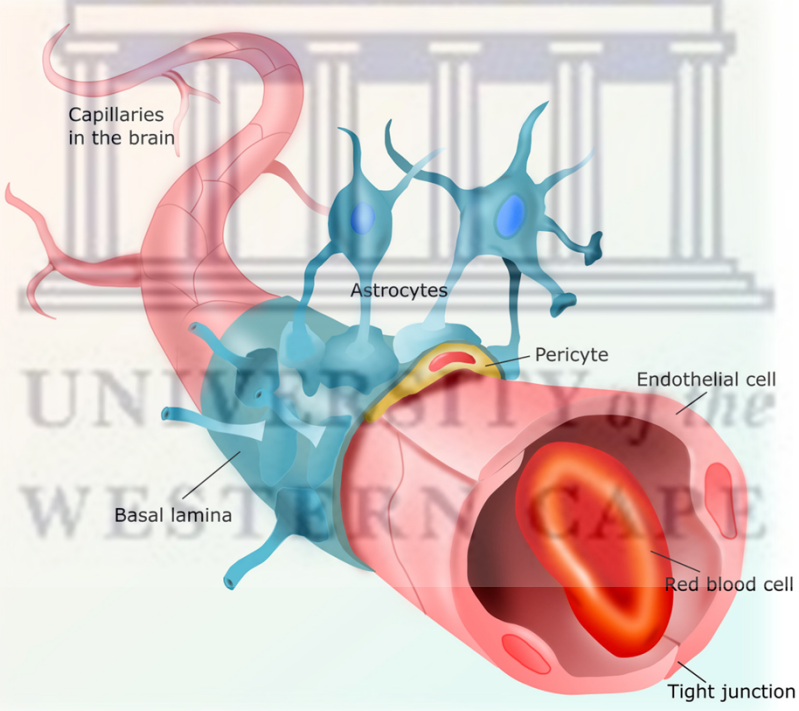

Figure 1: Brain endothelial blood vessel, showing the various components of the blood-brain barrier (Tornabene and Brodin, 2016).

For the BBB to function properly, the functioning of the NVU is crucial. It mainly consists of:

- Endothelial cells: Brain capillaries are lined with ECs forming the BBB. These cells are tightly connected at the apical regions of their paracellular spaces by TJ adhesion molecules, which prevent most molecules from passing through them. The TJs link capillary ECs, which are mainly linked by protein complexes (claudin-5, occluding, and junction adhesion molecules) known as tight junctions. A high degree of selectivity prevents most molecules from passing through these junctions (Liebner et al., 2011).

- Basement membrane: a complex layer of extracellular matrix, which serves as a contact point for the ECs. It surrounds the capillaries separating them from overlying tissue and plays an important role in the morphological polarization of the ECs in apical and basolateral membrane domains (Thomsen et al., 2017).
- Astrocytes: Through their release of signaling molecules, astrocytes regulate the permeability of the BBB and provide structural support. They also play an important interface between the neural elements of the brain and the function of the brain capillary endothelium (Sofroniew and Vinters, 2009).
- Pericytes: The pericytes encircle the ECs of brain capillaries. They maintain the BBB's endothelial structural integrity and regulate capillary blood flow (Shepro and Morel, 1993; Daneman and Prat, 2015).
- Transporters: Certain molecules can pass selectively through the BBB due to specialized transporters. A glucose transporter (glutamine and glutamate), for example, transports glucose across the BBB, while several amino acid transporters (e.g., cationic amino acid transporters, for large and small neutral amino acids) (Zaragozá, 2020), transport amino acids both into and out of the brain across the BBB (Daneman and Prat, 2015).

The most important component of the BBB consists of a single layer of cells, the capillary endothelium, and other protein molecules (figure 1), that protect the brain from harmful substances while still receiving essential nutrients (Zihni et al., 2016). A breach of this barrier can cause a wide range of neurological symptoms, depending on the severity of the substance that entered the brain. Some chemicals can damage, enlarge, and inflame brain tissue, whereas others can disrupt brain activity and cause psychological and physiological changes in the brain (Galea, 2021).

1.2 Systemic Inflammation and Neuroinflammation

The term "systemic inflammation" refers to a widespread, mild inflammatory response throughout the body's bloodstream. Several factors can contribute to this condition, including infections, autoimmune diseases, chronic stress, and exposure to environmental toxins (Galea, 2021).

Systemic inflammation has been proven to significantly affect the BBB (Galea, 2021). As a result of the systemic inflammatory response, immune cells and inflammatory chemicals, such as cytokines, chemokines, and prostaglandins, may enter the bloodstream, affecting the BBB and the brain, causing neuroinflammation and neurodegeneration (Galea, 2021).

Neuroinflammation refers to inflammation in the nervous system and the brain's vascular system and involves the brain's immune system (DiSabato et al., 2016). BBB impairment may be due to the potential consequences of systemic inflammation and neuroinflammation. As visually depicted in Figure 2, the inflammatory response in the BBB causes microglia to become activated and polarized, releasing reactive cytokines, including interleukin-1ß (IL-1ß), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), as well as other chemokines, and growth factors; resulting in the activation of MMPs, which as a consequence makes ECs more permeable by degrading the TJ between ECs (Stamatovic et al., 2016; Shigemoto-Mogami et al., 2018; Subhramanyam et al., 2019; Zhao et al., 2020). It is also worth noting that microglial cells are divided into two types: M1 and M2. By expressing pro-inflammatory cytokines (IL-1ß and TNF-α), M1-microglia have debilitating effects. In contrast to M1-microglia, M2-microglia, have neuro-protective properties by secreting anti-inflammatory cytokines, notably IL-10 and IL-33 (Zhao et al., 2020).

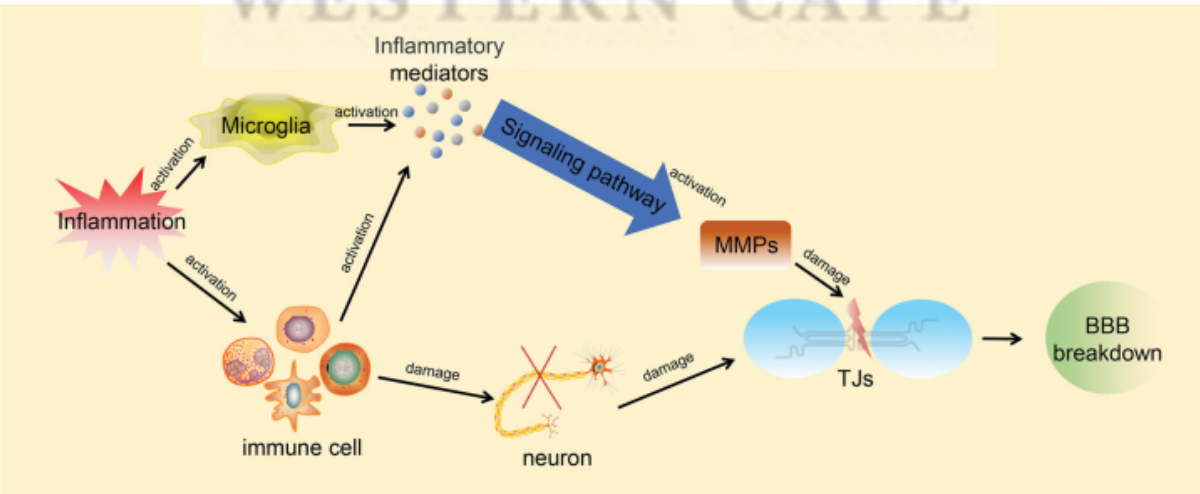

Figure 2: The basic process of inflammation. Diagram depicting the inflammation processes which affect endothelial cells of the blood-brain barrier. From "*Research progress of*

mechanisms for tight junction damage on blood-brain barrier inflammation" by Bo Zhao, Qiyang Yin, Yuxiang Fei, Jianping Zhu, Yanying Qiu, Weirong Fang & Yunman Li. *Archives of Physiology and Biochemistry*. Copyright 2022, reprinted by permission of Informa UK Limited, trading as Taylor & Taylor & Francis Group, (http://www.tandfonline.com).

1.2.1 Inflammation on the endothelial cells of the BBB

The BBB's ECs are susceptible to inflammation, negatively affecting brain health and function (Galea, 2021). A compromised brain endothelial cell may result in an increased permeability of the BBB, which would allow harmful substances like toxins, bacteria, and viruses to penetrate the brain and cause damage. These damages include the activation of immune cells, which release inflammatory molecules, which further damage ECs and increase permeability (Galea, 2021). Additionally, reactive oxygen species (ROS) can be generated during inflammation, which are highly reactive molecules capable of causing cell damage due to oxidative stress (Huang et al.,2020).

Chronic diseases such as diabetes and hypertension, infection, traumatic brain injury, and autoimmune disorders can all lead to inflammation of the BBB and ECs (Rosenberg, 2012). Brain inflammation has been linked to several neurological disorders, namely multiple sclerosis, Alzheimer's disease, Parkinson's disease, and stroke (Stephenson et al., 2018). This may contribute to the development of psychological conditions such as depression and anxiety. Reduced inflammation in the brain is essential for preventing and treating mental and neurological disorders (Miller and Raison, 2015). It is possible to reduce the severity of chronic inflammation through lifestyle changes such as regular exercise, a healthy diet, and medications specifically targeting inflammation or the immune system (Iddir et al., 2020).

1.2.3 *In vitro* Inflammatory model

To study the effect of neuro-vascular inflammation, many studies have used lipopolysaccharide (LPS), an endotoxin generated from the gram-negative bacteria Escherichia coli (Farhana and Khan, 2022). These bacteria cause the stimulation of cytokines in affected cells (i.e. BECs) (Farhana and Khan, 2022). When LPS enters circulation, it can cause a strong immunological response. This usually occurs when gram-negative bacteria are destroyed, and their cell walls degrade, releasing the LPS lipid A component into the blood (Raetz and Whitfield, 2002). The

toll-like receptor-4 (TLR4) is a receptor found on the surface of immune cells that mediates the immunological response to LPS. When LPS binds to TLR4, it causes the release of pro-inflammatory cytokines and chemokines. These chemicals encourage the recruitment of immune cells to the infection site (Raetz and Whitfield, 2002). There is significant evidence that LPS induces the release of cytokines such as TNF-α, IL-1β, IL-6, IL-8, IL-10, IL-12, IL-15, and TGFβ from stimulated cells. It does this by binding to its key receptors (TLR4) on the surface of immune cells (Tucureanu et al., 2017; Farhana and Khan, 2022).

Inherently TLRs receptors are found on cell membranes (Akira et al., 2001); furthermore, Johnson et al., (2018) found that TLR-3 and TLR-4 were also expressed on brain ECs, thus rendering ECs susceptible to LPS-induced disruption. TLRs potentially invoke the MyD88-NF-jB pathway, a well-known inflammatory signaling cascade that generates a wide range of chemokines, and proinflammatory cytokines. This causes a downstream inflammatory response to be activated, stimulating the release of matrix metalloproteinases (MMPs) 2 and 9 to break down TJs (figure 2) (Wang et al., 2015).

1.3 Glucocorticoids on the blood-brain barrier

Glucocorticoids (GCs) are stress-induced steroid hormones by the adrenal cortex (Nicolaides et al., 2018). A vital role of GCs is to regulate various physiological processes in the body, such as metabolism, immune function, and stress response (Marchi and van Eeden, 2021). It has been demonstrated that GC receptors are abundant both in the CNS (Carrillo-de Sauvage et al., 2013) and the peripheral nervous system (PNS). There are numerous brain areas where glucocorticoids bind to glucocorticoid receptors (GRs), including the hippocampus, prefrontal cortex, and amygdala (Hill and Spencer-Segal, 2021). These receptors regulate stress responses and modulate cognitive and emotional functions in the brain. It has been shown that glucocorticoids can influence immune function, inflammation, and tissue repair in the PNS (Wang et al., 2004). In addition, they can block the production of inflammatory cytokines by binding to glucocorticoid receptors on immune cells. By doing so, it prevent excessive inflammation and promotes tissue regeneration (Wang et al., 2004).

Generally, GCs such as Dex can profoundly influence BBB (McMahon, Oakden, and Hynynen, 2020). Dex inhibits immune responses when administered intravenously or systemically to reduce inflammation. This may reduce the disruption of the BBB that may occur as a result of

inflammatory responses. In addition, Dex suppresses cytokine production, a product of inflammation that could potentially disrupt the BBB (Salvador et al., 2013).

Hypothetically, Dex may also directly affect the BBB. A direct result of this may be an increase in the expression of the tight junction proteins that form the BBB's physical barrier (McMahon et al., 2020). As a result, the integrity of the BBB may be repaired, and the leakage of elements that can fuel the inflammatory process may be minimized or prevented (Balasa et al., 2021)

1.3.1 Glucocorticoids Anti-inflammatory Mechanisms

Research has shown that GC receptors are located within the cytoplasm of BBB ECs (Goodwin et al., 2012). Here it plays an important role in regulating the function and permeability of the BBB. For example, GC, such as cortisol (to name a few), bind to these receptors and regulates various physiological processes, including inflammation, metabolism, and stress response (Caratti et al., 2015).

Activation of the GRs in BBB ECs has been shown to reduce inflammation and increase the tightness of the BBB, leading to improved brain function and protection against various neurological diseases (Williams and Ghosh, 2020).

Due to GCs being relatively compact lipophilic compounds, they easily diffuse over cell membranes and into target cells' cytoplasm, exerting most of their effects via binding to intracytoplasmic GCs receptors (Chikanza, 2002). There are two types of GCs receptors: α and β isoforms. There is evidence that GCs bind to the α-isoform of receptors only (Yasir et al., 2021). High levels of the β-isoform have been attributed to GC resistance (Yasir et al., 2021; Chikanza, 2002).
Heat shock proteins are shed in reaction to GCs binding to GC receptors, resulting in GCs creating an activated receptor-GCs complex, which is cleaved away from the receptor and exits the cell. This complex stimulates and suppresses a wide range of gene transcription in the target cell's nuclei by reversibly binding to particular DNA sequences (Yasir et al., 2021).

A transient upregulation of transcription factors such as nuclear factor κB [NF-κB], activator protein-1, and interferon regulatory factor-3 causes the down-regulation of proinflammatory cytokines such as IL-1ß, IL-2, IL-6, IL-8, TNF-a and IFN-gamma to name a few (Sharun et al., 2020). In the presence of transactivated transcription factors, such as GC response elements

(GREs), anti-inflammatory cytokines such as IL-10, NF-κB inhibitors, and lipocortin-1 are synthesized (Yasir et al., 2021).

In addition to this, the capillary membrane becomes less porous, thus improving the stability of lysosomal membranes. Dex has been reported to suppress proinflammatory cytokines (Conti et al., 2020) by downregulating gene transcription of inflammatory cytokines (Sharun et al., 2020). Dex has also been linked to increased surfactant levels and better pulmonary circulation. Dex is mostly excreted from the body after being processed by the liver (Johnson et al.,2021). GCs are available in a variety of preparations with qualitatively different efficacy levels. Dex has a biological half-life of 36 to 54hrs (Yasir et al., 2021).

1.4 Conclusive statement

Various studies have found conflicting effects of Dex on the BBB. There is evidence that Dex decreases BBB permeability and prevents misfunctioning of the barrier (Barna et al., 2020) while other studies find no significant effects or even protective effects (Meijer et al., 1998).

Barna et al., 2020 reported that Dex was able to protect their 3-cell co-culture BBB model against kainate (a potent neuroexcitatory amino acid agonist that acts by activating receptors for glutamate) -induced changes. Confirming the clinical benefits of GC Dex in attenuating BBB damage.

The research scope on Dex and brain ECs, allows related scientific questions to be answered since it examines how the drug affects a specific type of brain cell, bEnd.5 endothelial brain cell. By studying the interactions between Dex and brain ECs, you can gain insights into how this drug affects the BBB, which is crucial for regulating the transport of molecules between the brain and the bloodstream. The study could also aid in understanding the potential therapeutic benefits and side effects of Dex, a GC commonly used to treat a wide range of illnesses, such as inflammation, allergies, and cancer. In studying how Dex affects brain ECs, this study provides valuable information for optimizing the use of this drug in clinical settings and identifying any potential risks. Ultimately this study could contribute to answering important clinical questions such as, how long should Dex treatment last to effectively target inflammation of the BBB? Is Dex effective in preventing disruption of the BBB in neuroinflammatory disorders? When used long-term to treat inflammation of brain ECs, does

Dex cause any adverse effects? Answering these questions may provide new insights into Dex's interaction with brain ECs.

It is essential to further investigate the effects of Dex on inflammation and the BBB, as well as its long-term consequences on the integrity and function of the BBB. In addition, to the optimal dosing of Dex and overall efficacy and safety when treating different neurological diseases. Despite the potential benefits of Dex on BBB disruption as a therapeutic approach, it is important to proceed with caution and continue to investigate its impacts on the brain and overall health.

1.5 Hypothesis:

This research study hypothesizes that low concentrations of Dex (0.1-5μM) will be more effective in attenuating the inflammatory effects of LPS on the *in vitro* BBB model than higher concentrations (5-20μM) of Dex; and that Dex would be able to reverse the effects of inflammation on the physiological integrity of the *in vitro* BBB and its cytokine expression.

1.6 Scope of the study

This research study aimed to investigate Dex's ability in attenuating the LPS-induced inflammatory response and further investigate Dex as a potential neuroprotective agent. Furthermore, physiological testing was used to investigate the permeability integrity of the *in vitro* BBB model (TEER) the identification and quantification of selected inflammatory cytokines released to the LPS inflammatory response of cells were measured using ELISA-based experiments. This research study is therefore an experimental account of understanding glucocorticoid Dex potential in remedying the LPS disrupted in vitro BBB model. The study aimed to investigate whether low or high concentrations of Dex can attenuate the inflammatory response induced by LPS in the *in vitro* BBB model. This study sought to generate insight into the development of Dex as a safe and effective treatment for vascular inflammatory conditions (BBB disruption). Furthermore, the inflammatory *in vitro* model was assessed as a useful tool for monitoring the effects of barrier disruption and drug treatments on the BBB *in vitro*.

1.6 Research Aims and Objectives

1.6.1 Aim:

I. This study aimed to determine the optimal LPS concentration for developing an inflammatory *in vitro* BBB model

II. To identify the optimal concentration of Dex to attenuate the LPS inflammatory response of the *in vitro* BBB model

III. To investigate whether Dex could improve the integrity of the inflammatory *in vitro* BBB model

IV. Furthermore, this study aimed to identify and quantify the expression of pro-inflammatory cytokines

1.6.2 Specific Objectives:

I. Trypan blue exclusion assay studies were used to evaluate the percentage of cell viability, the percentage of cell toxicity, and the number of bEnd.5 cells upon co-exposure to LPS (100ng/ml) and Dex (0.1,5,10 and 20μM) relative to control groups (untreated).

II. Trans-endothelial electrical studies (TEER) were conducted to assess barrier integrity and permeability, upon co-exposure to LPS (100ng/ml) and Dex (0.1,5,10 and 20μM) relative to control groups (untreated).

III. ELISA assays were used to identify and quantify proinflammatory cytokines (IL-1ß and TNF-a) expression or inhibition upon co-exposure to LPS (100ng/ml) and Dex (0.1 and 20μM) relative to control groups (untreated).

References

Abbott, N.J. (2005). Dynamics of CNS Barriers: Evolution, Differentiation, and Modulation. *Cellular and Molecular Neurobiology*,25(1), pp.5–23.

Abbott, N. J., Ronnback, L., and Hansson, E. (2006). Astrocyte-endothelial interactions at the blood-brain barrier. Nat. Rev. Neurosci. 7, 41–53.

Akira, S., Takeda, K. and Kaisho, T. (2001). Toll-like receptors: critical proteins linking innate and acquired immunity. *Nature Immunology*, 2(8), pp.675–680.

Baeten, K.M. and Akassoglou, K. (2011). Extracellular matrix and matrix receptors in blood-brain barrier formation and stroke. *Developmental Neurobiology*,71(11), pp.1018–1039.

Balasa, R., Barcutean, L., Mosora, O. and Manu, D. (2021). Reviewing the Significance of Blood–Brain Barrier Disruption in Multiple Sclerosis Pathology and Treatment. *International Journal of Molecular Sciences*, 22(16), p.8370.

Barna, L., Walter, F.R., Harazin, A., Bocsik, A., Kincses, A., Tubak, V., Jósvay, K., Zvara, Á., Campos-Bedolla, P. and Deli, M.A. (2020). Simvastatin, edaravone and dexamethasone protect against kainate-induced brain endothelial cell damage. *Fluids and Barriers of the CNS*, 17(1).

Brinks, J., van Dijk, E.H.C., Habeeb, M., Nikolaou, A., Tsonaka, R., Peters, H.A.B., Sips, H.C.M., van de Merbel, A.F., de Jong, E.K., Notenboom, R.G.E., Kielbasa, S.M., van der Maarel, S.M., Quax, P.H.A., Meijer, O.C. and Boon, C.J.F. (2018). The Effect of Corticosteroids on Human Choroidal Endothelial Cells: A Model to Study Central Serous Chorioretinopathy. *Investigative Opthalmology & Visual Science*, 59(13), p.5682.

Caratti, G., Matthews, L., Poolman, T., Kershaw, S., Baxter, M. and Ray, D. (2015). Glucocorticoid receptor function in health and disease. *Clinical Endocrinology*, 83(4), pp.441–448.

Carrillo-de Sauvage, M.Á., Maatouk, L., Arnoux, I., Pasco, M., Sanz Diez, A., Delahaye, M., Herrero, M.T., Newman, T.A., Calvo, C.F., Audinat, E., Tronche, F. and Vyas, S. (2013). Potent and multiple regulatory actions of microglial glucocorticoid receptors during CNS inflammation. *Cell Death & Differentiation*, 20(11), pp.1546–1557.

Chikanza, I.C. (2002). Mechanisms of Corticosteroid Resistance in Rheumatoid Arthritis. *Annals of the New York Academy of Sciences*, 966(1), pp.39–48.

Chuang, T.-Y., Cheng, A.-J., Chen, I-Ting., Lan, T.-Y., Huang, I-Hsuan., Shiau, C.-W., Hsu, C.-L., Liu, Y.-W., Chang, Z.-F., Tseng, P.-H. and Kuo, J.-C. (2017). Suppression of LPS-induced inflammatory responses by the hydroxyl groups of dexamethasone. *Oncotarget*, 8(30), pp.49735–49748.

Conti P;Ronconi G;Caraffa A;Gallenga CE;Ross R;Frydas I;Kritas SK (2020). Induction of pro-inflammatory cytokines (IL-1 and IL-6) and lung inflammation by Coronavirus-19 (COVI-19 or SARS-CoV-2): anti-inflammatory strategies. *Journal of biological regulators and homeostatic agents*, 34(2).

Corssmit, E.P.M. and Dekkers, O.M. (2019). Screening in adrenal tumors. *Current Opinion in Oncology*, 31(3), pp.243–246.

Daneman, R. and Prat, A. (2015). The Blood–Brain Barrier. *Cold Spring Harbor Perspectives in Biology*, 7(1), p.a020412.

Davson, H. (1989). History of the Blood-Brain Barrier Concept. *Implications of the Blood-Brain Barrier and Its Manipulation*, pp.27–52.

DiSabato, D.J., Quan, N. and Godbout, J.P. (2016). Neuroinflammation: the devil is in the details. *Journal of Neurochemistry*, 139, pp.136–153.

Dyrna, F., Hanske, S., Krueger, M. and Bechmann, I. (2013). The Blood-Brain Barrier. *Journal of Neuroimmune Pharmacology*, 8(4), pp.763–773.

Farhana, A. and Khan, Y.S. (2022). *Biochemistry, Lipopolysaccharide*.

Galea, I. (2021). The blood–brain barrier in systemic infection and inflammation. *Cellular & Molecular Immunology*, 18(11), pp.2489–2501.

Ghlichloo, I. and Gerriets, V. (2022). *Nonsteroidal Anti-inflammatory Drugs (NSAIDs)*.

Giles, A.J., Hutchinson, M.-K.N.D., Sonnemann, H.M., Jung, J., Fecci, P.E., Ratnam, N.M., Zhang, W., Song, H., Bailey, R., Davis, D., Reid, C.M., Park, D.M. and Gilbert, M.R. (2018). Dexamethasone-induced immunosuppression: mechanisms and implications for immunotherapy. *Journal for ImmunoTherapy of Cancer*, 6(1).

Goodwin, J.E., Feng, Y., Velazquez, H. and Sessa, W.C. (2012). Endothelial glucocorticoid receptor is required for protection against sepsis. *Proceedings of the National Academy of Sciences*, 110(1), pp.306–311.

Hench PS; Kendall EC (2021). The effect of a hormone of the adrenal cortex (17-hydroxy-11-dehydrocorticosterone; compound E) and of pituitary adrenocorticotropic hormone on rheumatoid arthritis. *Proceedings of the staff meetings. Mayo Clinic*, 24(8).

Hill, A.R. and Spencer-Segal, J.L. (2021). Glucocorticoids and the Brain after Critical Illness. *Endocrinology*, 162(3).

Huang, X., Hussain, B. and Chang, J. (2020). Peripheral inflammation and blood–brain barrier disruption: effects and mechanisms. *CNS Neuroscience & Therapeutics*, 27(1), pp.36–47.

Iddir, M., Brito, A., Dingeo, G., Fernandez Del Campo, S.S., Samouda, H., La Frano, M.R. and Bohn, T. (2020). Strengthening the Immune System and Reducing Inflammation and Oxidative Stress through Diet and Nutrition: Considerations during the COVID-19 Crisis. *Nutrients*, 12(6), p.1562.

Johnson, D.B., Lopez, M.J. and Kelley, B. (2021). *Dexamethasone*.

Kaplan, L., Chow, B.W. and Gu, C. (2020). Neuronal regulation of the blood–brain barrier and neurovascular coupling. *Nature Reviews Neuroscience*, 21(8), pp.416–432.

Lammers, T., Sofias, A.M., van der Meel, R., Schiffelers, R., Storm, G., Tacke, F., Koschmieder, S., Brümmendorf, T.H., Kiessling, F. and Metselaar, J.M. (2020).

Liebner, S., Czupalla, C.J. and Wolburg, H. (2011). Current concepts of blood-brain barrier development. *The International Journal of Developmental Biology*, 55(4-5), pp.467–476.

Lukiw, W.J., Pogue, A. and Hill, J.M. (2020). SARS-CoV-2 Infectivity and Neurological Targets in the Brain. *Cellular and Molecular Neurobiology*, 42(1), pp.217–224.

Marchi, D. and van Eeden, F.J.M. (2021). Homeostatic Regulation of Glucocorticoid Receptor Activity by Hypoxia-Inducible Factor 1: From Physiology to Clinic. *Cells*, 10(12), p.3441.

Markowicz-Piasecka, M., Markiewicz, A., Darłak, P., Sikora, J., Adla, S.K., Bagina, S. and Huttunen, K.M. (2022). Current Chemical, Biological, and Physiological Views in the Development of Successful Brain-Targeted Pharmaceutics. *Neurotherapeutics*, 19(3), pp.942–976.

Meijer, O.C., de Lange, E.C.M., Breimer, D.D., de Boer, A.G., Workel, J.O. and de Kloet, E.R. (1998). Penetration of Dexamethasone into Brain Glucocorticoid Targets Is Enhanced in mdr1A P-Glycoprotein Knockout Mice*. *Endocrinology*, 139(4), pp.1789–1793.

McMahon, D., Oakden, W. and Hynynen, K. (2020). Investigating the effects of dexamethasone on blood-brain barrier permeability and inflammatory response

following focused ultrasound and microbubble exposure. *Theranostics*, 10(4), pp.1604–1618.

Michinaga, S. and Koyama, Y. (2015). Pathogenesis of Brain Edema and Investigation into Anti-Edema Drugs. *International Journal of Molecular Sciences*, 16(12), pp.9949–9975.

Miller, A.H. and Raison, C.L. (2015). The role of inflammation in depression: from evolutionary imperative to modern treatment target. *Nature Reviews Immunology*, 16(1), pp.22–34.

Muhammad Yasir, Goyal, A. and Sidharth Sonthalia (2021). *Corticosteroid Adverse Effects*. Nih.gov.

Nicolaides, N.C., Pavlaki, A.N., Alexandra, M. and Chrousos, G.P. (2018). *Glucocorticoid Therapy and Adrenal Suppression*.

Raabe, A., Schmitz, A.K., Pernhorst, K., Grote, A., von der Brelie, C., Urbach, H., Friedman, A.,Becker, A.J., Elger, C.E. and Niehusmann, P. (2012). Cliniconeuropathologic correlations show astroglial albumin storage as a common factor in epileptogenic vascular lesions. *Epilepsia*,53(3), pp.539–548.

Raetz, C.R.H. and Whitfield, C. (2002). Lipopolysaccharide Endotoxins. *Annual Review of Biochemistry*, 71(1), pp.635–700.

Réus, G.Z., Fries, G.R., Stertz, L., Badawy, M., Passos, I.C., Barichello, T., Kapczinski, F. and Quevedo, J. (2015). The role of inflammation and microglial activation in the pathophysiology of psychiatric disorders. *Neuroscience*, 300, pp.141–154.

Rhea, E.M. and Banks, W.A. (2019). Role of the Blood-Brain Barrier in Central Nervous System Insulin Resistance. *Frontiers in Neuroscience*, 13.

Rosenberg, G.A. (2012). Neurological Diseases in Relation to the Blood–Brain Barrier. *Journal of Cerebral Blood Flow & Metabolism*, 32(7), pp.1139–1151.

Roy-O'Reilly, M. and McCullough, L.D. (2018). Age and Sex Are Critical Factors in Ischemic Stroke Pathology. *Endocrinology*, 159(8), pp.3120–3131.

Sandoval, K.E. and Witt, K.A. (2008). Blood-brain barrier tight junction permeability and ischemic stroke. *Neurobiology of Disease*, 32(2), pp.200–219.

Sankowski, R., Mader, S. and ValdÃ©s-Ferrer, S.I. (2015). Systemic Inflammation and the Brain: Novel Roles of Genetic, Molecular, and Environmental Cues as Drivers of Neurodegeneration. *Frontiers in Cellular Neuroscience*, 9.

Saunders, N.R., Habgood, M.D., Møllgård, K. and Dziegielewska, K.M. (2016). The biological significance of brain barrier mechanisms: help or hindrance in drug delivery to the central nervous system? *F1000Research*, 5, p.313.

Sharun, K., Tiwari, R., Dhama, J. and Dhama, K. (2020). Dexamethasone to combat cytokine storm in COVID-19: Clinical trials and preliminary evidence. *International Journal of Surgery*, 82, pp.179–181

Shepro, D. and Morel, N.M.L. (1993). Pericyte physiology. *The FASEB Journal*, 7(11), pp.1031–1038.

Shigemoto-Mogami, Y., Hoshikawa, K. and Sato, K. (2018). Activated Microglia Disrupt the Blood-Brain Barrier and Induce Chemokines and Cytokines in a Rat in vitro Model. *Frontiers in Cellular Neuroscience*, 12.

Sofroniew, M.V. and Vinters, H.V. (2009). Astrocytes: biology and pathology. *Acta Neuropathologica*, 119(1), pp.7–35.

Stamatovic, S.M., Johnson, A.M., Keep, R.F. and Andjelkovic, A.V. (2016). Junctional proteins of the blood-brain barrier: New insights into function and dysfunction. *Tissue Barriers*, 4(1), p.e1154641.

Stephenson, J., Nutma, E., van der Valk, P. and Amor, S. (2018). Inflammation in CNS neurodegenerative diseases. *Immunology*, 154(2), pp.204–219.

Subhramanyam, C.S., Wang, C., Hu, Q. and Dheen, S.T. (2019). Microglia-mediated neuroinflammation in neurodegenerative diseases. *Seminars in Cell & Developmental Biology*, 94, pp.112–120.

Theoharides TC;Conti P (2020). Dexamethasone for COVID-19? Not so fast. *Journal of biological regulators and homeostatic agents*, 34(3).

Thomsen, M.S., Routhe, L.J. and Moos, T. (2017). The vascular basement membrane in the healthy and pathological brain. *Journal of Cerebral Blood Flow & Metabolism*, 37(10), pp.3300–3317.

Tornabene, E. and Brodin, B. (2016). Stroke and Drug Delivery— In Vitro Models of the Ischemic Blood-Brain Barrier. *Journal of Pharmaceutical Sciences*, 105(2), pp.398 –405.

Tucureanu, M.M., Rebleanu, D., Constantinescu, C.A., Deleanu, M., Voicu, G., Butoi, E., Calin, M. and Manduteanu, I. (2017). Lipopolysaccharide-induced inflammation in monocytes/macrophages is blocked by liposomal delivery of G_i-protein inhibitor. *International Journal of Nanomedicine*, Volume 13, pp.63–76.

Wang, S., Lim, G., Zeng, Q., Sung, B., Ai, Y., Guo, G., Yang, L. and Mao, J. (2004). Expression of Central Glucocorticoid Receptors after Peripheral Nerve Injury Contributes to Neuropathic Pain Behaviors in Rats. *The Journal of Neuroscience*, 24(39), pp.8595–8605.

Wang, P.-F., Xiong, X.-Y., Chen, J., Wang, Y.-C., Duan, W. and Yang, Q.-W. (2015). Function and mechanism of toll-like receptors in cerebral ischemic tolerance: from preconditioning to treatment. *Journal of Neuroinflammation*, 12, 80.

Winkler, E.A., Bell, R.D. and Zlokovic, B.V. (2011). Central nervous system pericytes in health and disease. *Nature Neuroscience*, 14(11), pp.1398–1405.

Williams, S. and Ghosh, C. (2020). Neurovascular glucocorticoid receptors and glucocorticoids: implications in health, neurological disorders and drug therapy. *Drug Discovery Today*, 25(1), pp.89–106.

Yu, X., Ji, C. and Shao, A. (2020). Neurovascular Unit Dysfunction and Neurodegenerative Disorders. *Frontiers in Neuroscience*, 14.

Zaragozá, R. (2020). Transport of Amino Acids Across the Blood-Brain Barrier. *Frontiers in Physiology*, 11.

Zihni, C., Mills, C., Matter, K. and Balda, M.S. (2016). Tight junctions: from simple barriers to multifunctional molecular gates. *Nature Reviews Molecular Cell Biology*,17(9), pp.564–580.

Zhao, B., Yin, Q., Fei, Y., Zhu, J., Qiu, Y., Fang, W. and Li, Y. (2020). Research progress of mechanisms for tight junction damage on blood–brain barrier inflammation. *Archives of Physiology and Biochemistry*, pp.1–12.

Chapter 2
The effect of dexamethasone on lipopolysaccharide-induced inflammation of endothelial cells of the blood-brain barrier/brain capillaries

The effect of dexamethasone on lipopolysaccharide-induced inflammation of endothelial cells of the blood-brain barrier/brain capillaries

Tershlin Jeftha[1], Khayelihle Brian Makhathini[2], David Fisher[1-*].

Original Research

[1]Co-first author, Tershlin Jeftha, Neurobiology Research Group, Department of Medical Biosciences, Faculty of Natural Sciences, University of the Western Cape, Bellville, Cape Town, 7535, South Africa
Email Address: 3339612@myuwc.ac.za

[2]Co-second author, Dr. Khayelihle Brian Makhathini, Neurobiology Research Group, Department of Medical Biosciences, Faculty of Natural Sciences, University of the Western Cape, Bellville, Cape Town, 7535, South Africa.
Email Address: kmakhathini@uwc.ac.za

Co-first author: Prof. David Fisher, Neurobiology Research Group, Department of Medical Biosciences Faculty of Natural Sciences, University of the Western Cape, Bellville, Cape Town, 7535, South Africa. Adjunct Professor in School of Health Professions, University of Missouri,
Email Address: dfisher@uwc.ac.za

[1]Correspondence: Prof. David Fisher, Neurobiology Research Group, Department of Medical Biosciences, Faculty of Natural Sciences, University of the Western Cape, Bellville, Cape Town, 7535, South Africa.
Email Address: dfisher@uwc.ac.za

Abstract

A protective and regulatory barrier between the blood and the brain is constituted by the blood-brain barrier (BBB), which comprises microvascular endothelial cells providing homeostatic regulation of the central nervous system (CNS). Inflammation compromises the BBB and contributes to many CNS disorders. Anti-inflammatory effects are exerted by glucocorticoids (GCs) on a variety of cells. These GCs include dexamethasone (Dex), which is used for the treatment of inflammatory diseases and recently for the treatment of COVID-19. **Aim:** The purpose of this study was to determine whether low or high concentrations of Dex can attenuate the inflammatory response induced by lipopolysaccharide (LPS) in the *in vitro* BBB model. **Method:** Brain endothelial cells (bEnd.5) were cultured and exposed to LPS (100ng/ml) and subsequently co-treated with Dex to investigate whether selected concentrations of Dex (0.1,5,10,20μM) can modulate the inflammatory effects of LPS on bEnd.5 cells. Cell viability, cell toxicity, and cell proliferation were investigated, as well as the monitoring of membrane permeability (Trans Endothelial Electrical Resistance-TEER), and Enzyme Linked Immune Assay (ELISA) kits were used to identify and quantify the presence of inflammatory cytokines (TNF-α and IL-1β) **Conclusion:** Dex, at a lower dosage (0.1μM, but not higher doses), was able to attenuate the inflammatory effects of LPS on bEnd.5 cells. Lower doses of Dex (0.1μM) had no detrimental effects on bEnd.5 cells, while higher Dex doses (5-20μM) decreased bEnd.5 viability, increased bEnd.5 cell toxicity, increased bEnd.5 cell monolayer permeability, and increased proinflammatory cytokine secretion. These results indicate that treatment of brain vascular inflammation with low doses of Dex should be advocated, while higher doses promote vascular inflammation.

Keywords: Blood-brain barrier, inflammation, tight junctions, permeability, integrity, central nervous system

1. Introduction

Located within the brain, the BBB serves as a protective barrier that isolates the brain from the rest of the body. The BBB protects the brain from harmful substances, such as toxins and bacteria, while regulating essential nutrients and ions to pass through the blood vessels (1). It is formed primarily by the endothelial cells of the capillaries in the brain, while its function is supported by pericytes, astrocytes, microglia, endothelial tight junctions, and neurons (2). Furthermore, the BBB regulates the exchange of ions and small molecules between the blood and the brain, which helps maintain the proper chemical environment for the brain. A breach of this vital barrier caused by inflammation can compromise its crucial function (2,3). Although inflammation is a vital process in response to infection and injury, but also contributes significantly to the pathophysiology of human disease.

Several neurological pathologies can cause inflammation of the BBB, causing the activation of immune cells and the release of molecules that can damage and compromise the BBB, including multiple sclerosis, lupus, and rheumatoid arthritis (4). There is substantial evidence that certain neurological diseases, including Alzheimer's disease and Parkinson's disease, are associated with inflammation of the BBB (5). It is also possible to cause BBB inflammation due to exposure to toxins such as pesticides, heavy metals, and other pollutants. Inflammation of the BBB enables certain molecules and cells to leak into the brain by causing the normally tight junctions between the cells to be compromised. It makes it possible for immune cells and molecules, such as cytokines and chemokines, to leak through the inflamed BBB, compromising neural function and potentially leading to neurological impairment (2,6).

Inflammation is a spontaneous and essential response to infection or cell damage but on the other hand, might have a detrimental influence on brain endothelial cells (BECs) and the function of the BBB (7). Inflammation can be treated with specific medications and therapies. The most commonly prescribed medication is corticosteroids, an anti-inflammatory molecule that reduces swelling and inflammation in the brain. The partial anti-inflammatory effect of the glucocorticoid, Dex has been reported during the COVID-19 pandemic, before the availability of vaccines. This intervention reduced the mortality rate of hospitalized patients who were not critically ill (8).

Dex is a chemically synthesized glucocorticoid that was authorized by the Food and Drug Administration (FDA) in 1958 as a broad-spectrum immunosuppressant. It is 30 times more

potent than cortisone and has a longer half-life (9). Dex is known for its ability to reduce the synthesis and secretion of cytokines and their harmful effects on cell tissues. According to studies, Dex has been reported to be advantageous when treating severe intubated COVID-19 patients in the short term (10), and it was the first pharmaceutical to demonstrate life-saving efficiency in COVID-19 hospitalized patients (11).

To examine Dex's anti-inflammatory properties related to BECs, LPS was used in an established *in vitro* inflammatory BBB model (12). LPS has been reported to act by attachment to Toll-like receptors (TLRs) that are involved in the recognition of bodily infections and macrophages, as well as the admittance of immune responses. TLRs can detect pathogen-associated molecular patterns (PAMPs) such as LPS. Activated TLRs can trigger an immune response, leading to the expression of proinflammatory cytokines such as TNF-α, IL-1, and IL-12. This activation involves Toll/IL-1R (TIR) adapter proteins, including MyD88, NF-κB-inducing kinase (NIK), and IκB kinase (IKK), which stimulate the downstream molecule NF-κB to move to the nucleus and initiate cytokine generation (13). The CD14/TLR-4 receptor complexes, which are abundant in microglia, astrocytes, and BECs of the CNS, seem to be the primary receptor for LPS binding (14,15). The TLR4/nuclear factor (NF)-B pathway is activated by the downstream signalling of this receptor complex, resulting in the production of proinflammatory cytokines that initiate neuroinflammation (16,17). Furthermore, LPS is known to have proinflammatory effects on bEnd.3 cell-based BBB model, and thus was an optimal candidate to produce inflammation on BBB bEnd.5 cells. Due to the capacity of LPS, a constituent of Gram-negative bacteria's outer membrane, classified as an endotoxin, to affect BECs and stimulate the secretion of proinflammatory cytokines (18–20), it was used to induce inflammation of bEnd.5 cells as part of an inflammatory *in vitro* BBB model.

This study aimed to investigate the anti-inflammatory effect of Dex on BECs using the *in vitro* inflammatory BBB model. This study investigates whether Dex at low and high concentrations could remedy the LPS-induced inflammation in the *in vitro* bEnd.5 BBB model.

2. Methods and Materials

2.1. bEnd.5 Cells and Bio-Reagents

The immortalized mouse brain endothelial cells (bEnd.5) (ECACC 96091930, Salisbury, Wiltshire SP4 0JG, UK) were used in this study. Cells were incubated in Dulbecco's modified Eagle's medium (DMEM, Gibco 31330038.) supplemented with 10% fetal bovine serum (FBS,

Biowest 12010S181G, Rue du Vieux Bourg, 49340 Nuaillé, France); 1% non-essential amino acids (NEAA Gibco 11140050); 1% penicillin/streptomycin (Gibco 15140122), and finally 1% sodium pyruvate solution (Gibco 11360-039). Analytical-grade reagents were used for all experiments. These included: LPS (Sigma L2630, South Africa), Trypan blue solution, (Sigma 125H3668), 0.25% EDTA-trypsin (Gibco 25200-072), and phosphate buffer (Whitehead Scientific BE17-51Q, Stikland South Africa).

2.2 The experimental design:

2.2.1 Establishment of experimental models

The study's primary goal was to investigate how the effects of LPS (a bacterial pyrogen) induced inflammation on bEnd.5 cells by monitoring viability toxicology and live and dead cell numbers (figure 1.1). Once the *in vitro* inflammatory BBB model was characterized, the efficacy of Dex to reverse the inflammation of the primary regulatory component of the BBB, the BEC, was investigated (20,21). A dose-response experiment established the most effective LPS concentration to treat bEnd.5 cell cultures (figure 1.1 and figure 2). Viability, toxicity, live and dead bEnd.5 cells were evaluated after exposure to LPS (figures 2 and 3). Secondly, experiments were devised to evaluate the effects of selected low and higher doses of Dex that would mitigate the LPS-induced inflammation (figure 1.2). Thirdly, we investigated the effects of these treatments on the permeability across monolayers by measuring TEER across bEnd.5 monolayers that were exposed to LPS and Dex. In addition, we measured the effects of these treatments on the secretion of selected proinflammatory cytokines found in the culture media supernatant (ELISA experiments), where we quantified the secretion of selected cytokines (IL-1ß and TNF- α) from bEnd.5 cells exposed to LPS, and combinations of LPS and Dex (figure 1.3).

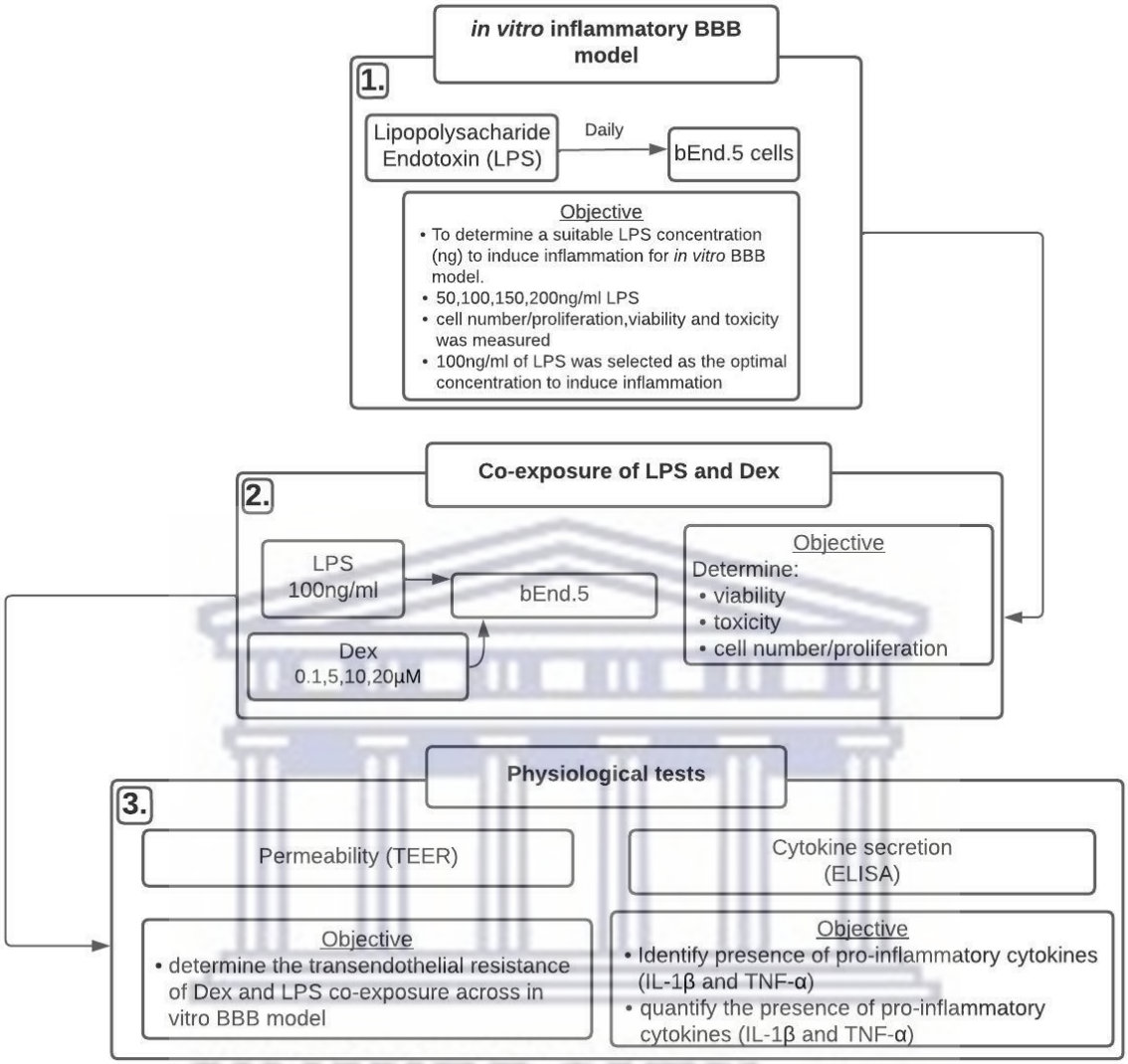

Figure 3: Experimental design: 1: Establishing the *in vitro* inflammatory BBB model: Selected LPS concentrations were used (50, 100, 150, 200ng/ml) on bEnd.5 cells to identify the most appropriate LPS concentration to produce an inflammatory response consistently. Based on these experiments, 100ng/ml of LPS was most effective in inducing inflammation in bEnd.5 cells. 2: Effects of co-exposure of LPS and Dex: Selected concentrations of Dex were used to determine the effects of low and higher Dex concentrations on bEnd.5 cells. 3: Physiological Functionality: Experiments were conducted to determine the TEER of LPS and/or Dex co-exposure across the *in vitro* BBB model (TEER) and to identify and quantify the proinflammatory cytokine expression (ELISA) in LPS exposed cultures of bEnd.5 cells.

2.3 Trypan Blue Exclusion Assay

bEnd.5 were seeded (3500 cells/well) in 12-well plates in triplicate, and were incubated for 24hrs to allow attachment. Thereafter, to enable the counting of bEnd.5 cells, cells were trypsinized with 0.25% EDTA-trypsin. Cells were centrifuged at 2500rmp for 5mins, the supernatant was discarded, and cells were replenished with 500ul of fresh media. Trypan blue dye (20µl) and 20µl of cell suspension were mixed according to a predetermined ratio (1:1). A disposable counting chamber was used to add 10µl of this cell suspension, which was subsequently analysed using the Countess 3 Automated Cell Counter system (Cat. No. AMQAX2000, ThermoFisher Scientific, South Africa). The cell viability (%) was determined by dividing the number of live cells by the total number of cells multiplied by 100. Similarly, cell toxicity % was determined by dividing the number of dead cells by the total number of cells multiplied by 100.

2.4 Induction of inflammation on bEnd.5 Cells: Co-treatment with Dex

To induce an inflammatory response in bEnd.5 cells, cells were first exposed to LPS (100ng/ml) for 12hrs and then co-treated with different concentrations of Dex (0.1, 5, 10, and 20μM) for 24, 48, 72, and 96hrs. A trypan blue assay was conducted to assess viability, toxicology, and cell number (dead and live cells) (as previously described in 2.3).

2.5 TEER

In TEER experiments, 12-well plates were used to culture bEnd.5 cells (5×10^5 cells/insert) on membrane inserts (MF-Millipore, Ser. No. PIHA01250, Germany) until confluence. The culture medium was substituted daily to contain LPS (100ng/ml) and selected concentrations of Dex (0.1, 5, 10, and 20μM) while control samples received fresh media. Using a Millicell epithelial Volt-Ohm meter (Millipore, Ser. No. 57318, 11B, Germany), TEER was performed for 8 days. The quantitative analysis of the recorded TEER values was investigated using the characteristics of an appropriate equivalent circuit, which present the electrical properties across the in vitro BEC monolayer under examination.

2.6 Determination of proinflammatory cytokines using ELISA

The concentrations of IL-1β and TNF-α were determined using ELISA kits (Invitrogen, catalogue- BMS6002 and BMS607-3) from the cell culture supernatant of bEnd.5 cells treated with LPS and Dex (described in 2.4). Only low ($0.1\mu M$) and high ($20\mu M$) concentrations were used. The kits were utilized in accordance with the manufacturer's instructions and included all necessary chemicals for the test to be completed.

2.7 Statistical Analysis

All results are presented as the mean ± SEM. For statistical reproducibility, all tests were carried out in triplicate (n = 3). Graph Pad Prism.9.5.0 (Dotmatics, California, USA) statistical analysis software was used for the statistical analysis. Normality testing was conducted prior to using parametric or non-parametric statistical tests. One-way ANOVA analysis of variance with the Bonferroni post-test was used for parametric data while the Kruskal-Wallis test with the post-test of Dunn's was conducted for nonparametric data. Significant differences between means were accepted at $p<0.05$.

3. Results

3.1 Verifying the *in vitro* LPS-induced inflammatory BBB model:

Treatment of bEnd.5 cell cultures with LPS (100ng/ml) consistently decreased Viability (%) and increased Toxicity (%) over a period of 96hrs (figure 2). This was consistent with the effect of LPS on BECs in established *in vitro* BBB models in the literature (20).

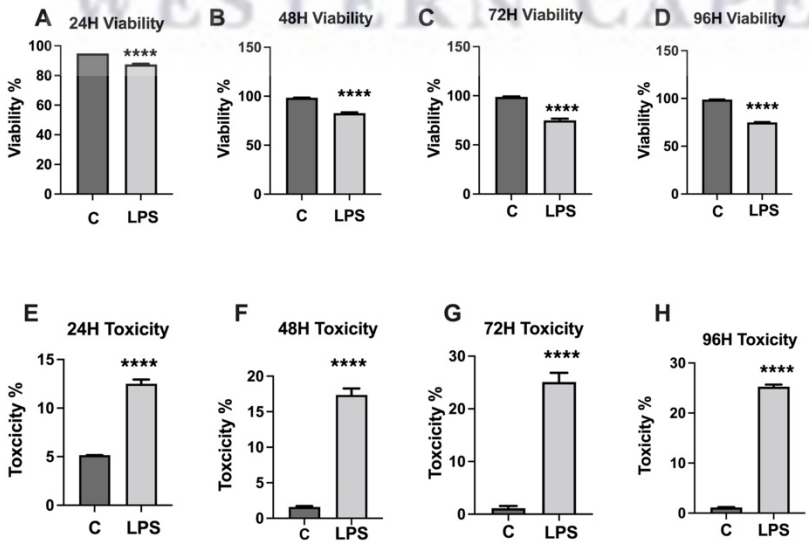

Figure 4: The effect LPS has on viability and toxicity. Graphs A–D indicate the viability of the bEnd.5 cells after exposure to 100ng/ml LPS, whereas graphs E–H illustrate the toxicity after exposure to 100ng/ml LPS. Cell viability and cell toxicity were determined using trypan blue exclusion assays. LPS (100ng/ml) revealed lower viability (graph A-D) and higher toxicity to control (graph E-H). These experiments were foundational in characterizing our inflammatory BBB experimental model. (C=control), (n=3), ****p < 0.0001 versus control group.

Further verification was observed by monitoring the effect of LPS (100ng/ml) on cell proliferation. Here LPS also statistically suppressed live bEnd.5 cell numbers while increasing the numbers of dead bEnd.5 cells over a period of 96hrs (figure 3). This further endorsed the *in vitro* LPS-induced inflammatory BBB model and allowed for the investigation of the effects of Dex on the inflamed bEnd.5 cells.

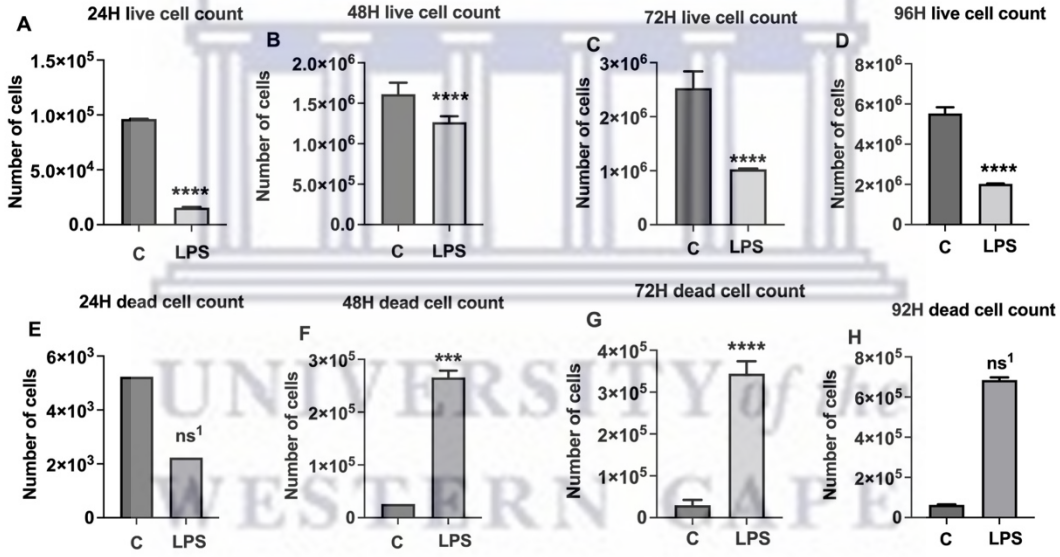

Figure 5: The effect of LPS on bEnd.5 cell proliferation. Graphs A-D shows the live cell count of bEnd.5 cells and graphs E-H shows the dead cell count**.** Live and dead cell count were determined using trypan blue exclusion assays. LPS (100ng/ml) suppressed live cell count throughout the 96 hour experimental time frame (graph A-D) and yielded a higher dead cell count (graph E-H). These experiments characterized the effects of LPS on the bEnd5 cell line and were foundational to our inflammatory BBB model. (C=control; n=3; ***p<0.001 in comparison to the control group; ns[1], no significant difference to control.

3.2. The effect of Dex on reversing inflammatory physiological variables of BECs:

Inflammation of cells has numerous effects on the physiology of cells, which invariably result in decreases in cell population viability, increased cell death (toxicity), decreased cell division, increased cell layer permeability, and release of proinflammatory cytokines. We devised a series of experiments to evaluate the anti-inflammatory effects of Dex on the LPS-induced inflammatory effects on bEnd.5 cells (See figure 1 for the experimental design).

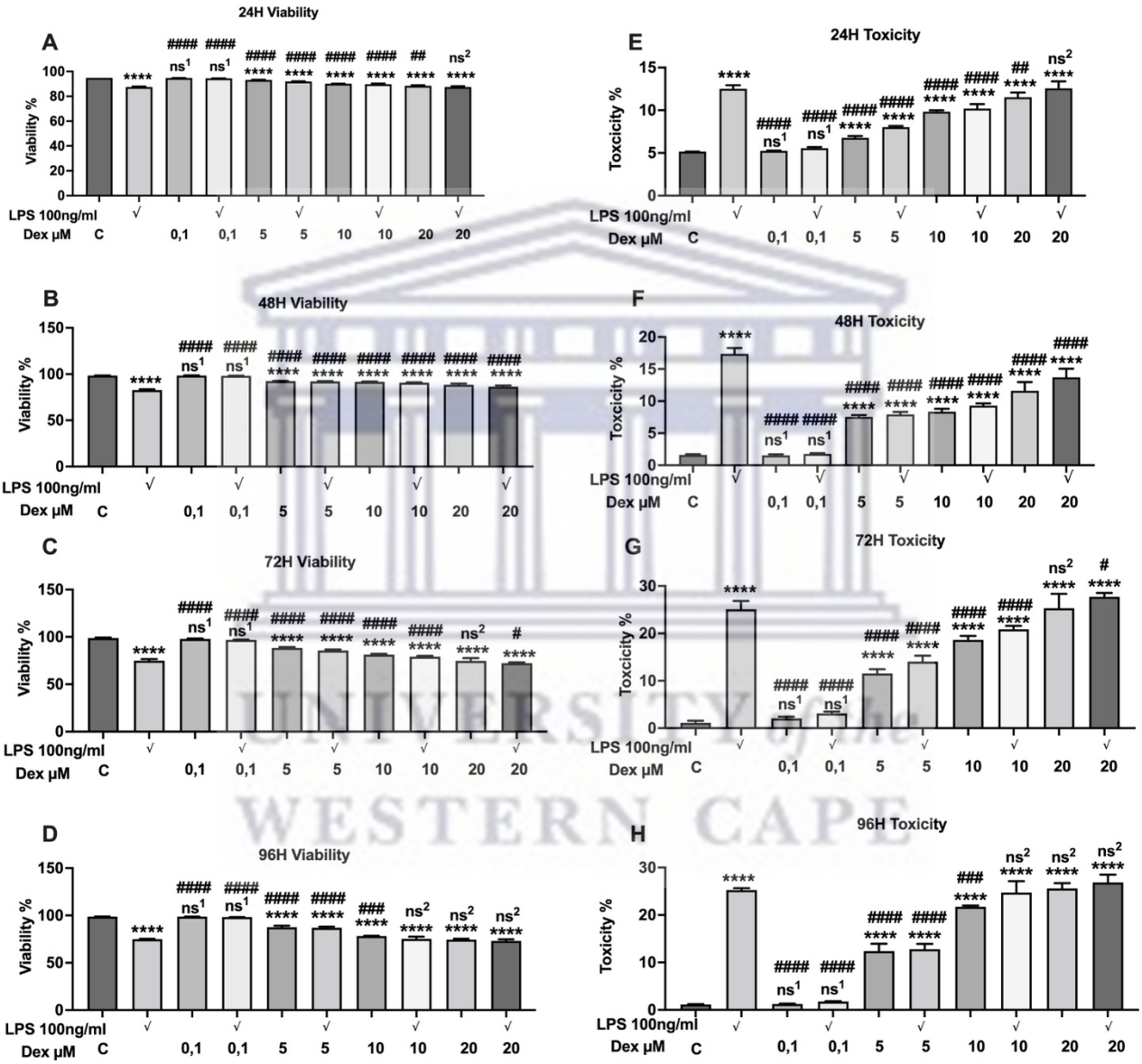

Figure 6: The effect of Dexamethasone (Dex) on the LPS-induced changes to bEnd.5 cell culture viability and toxicity. Graphs A-D show the viability and graph E-H show the toxicity after co-exposure of 100ng/ml LPS on bEnd.5 cells and a range of Dex concentrations (0.1, 5, 10, 20μM). Trypan blue exclusion assay was used to determine cell viability and cell toxicity. Graphs A-E revealed that the effects of LPS on cell viability was reversed at low concentrations

of Dex (0.1 ***μM***) but not at higher concentrations of Dex (**5 to 20*μM*)**. The LPS-induced increase in cell toxicity in graphs E-H, was reversed at low concentrations of Dex (0.*1 μM*) but not at higher concentrations of Dex **(**5 to 20*μM*)**.** Note that treatment of bEnd5 cells with higher concentrations of Dex *(5 to 20μM*) alone were detrimental to both cell viability and to toxicity levels. (**C**=control; n=3; ****$p < 0.0001$ versus control group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ and $^{\#\#\#\#}p < 0.0001$versus LPS group; ns[1], no significant difference to control; ns[2], no significant difference to LPS).

3.2.1 Cell viability and cell toxicity following co-exposure to LPS and Dex**.**

Viability: Cultured bEnd.5 cells were exposed to LPS (100ng/ml) for 12 hrs and thereafter treated with Dex (0.1, 5, 10, 20*μM*) for 24-96 hrs. LPS throughout the experiment time frame was shown to negatively affect viability when compared to the control ($p < 0.0001$). Viability (figure 4: A-D) was decreased in a dose-related manner with the increase in Dex concentrations. Dex (figure 4A -D) induced a steady decline in viability as the concentration of Dex increased. The trend of decreasing viability with Dex was significantly more pronounced during 72hrs and 96hrs. Treatment of bEnd.5 cells with low concentrations of Dex (0.1*μM*) had no statistical effect on viability and was the only concentration of Dex that was able to consistently reverse LPS-induced decreases in viability (figure 4 A-D).

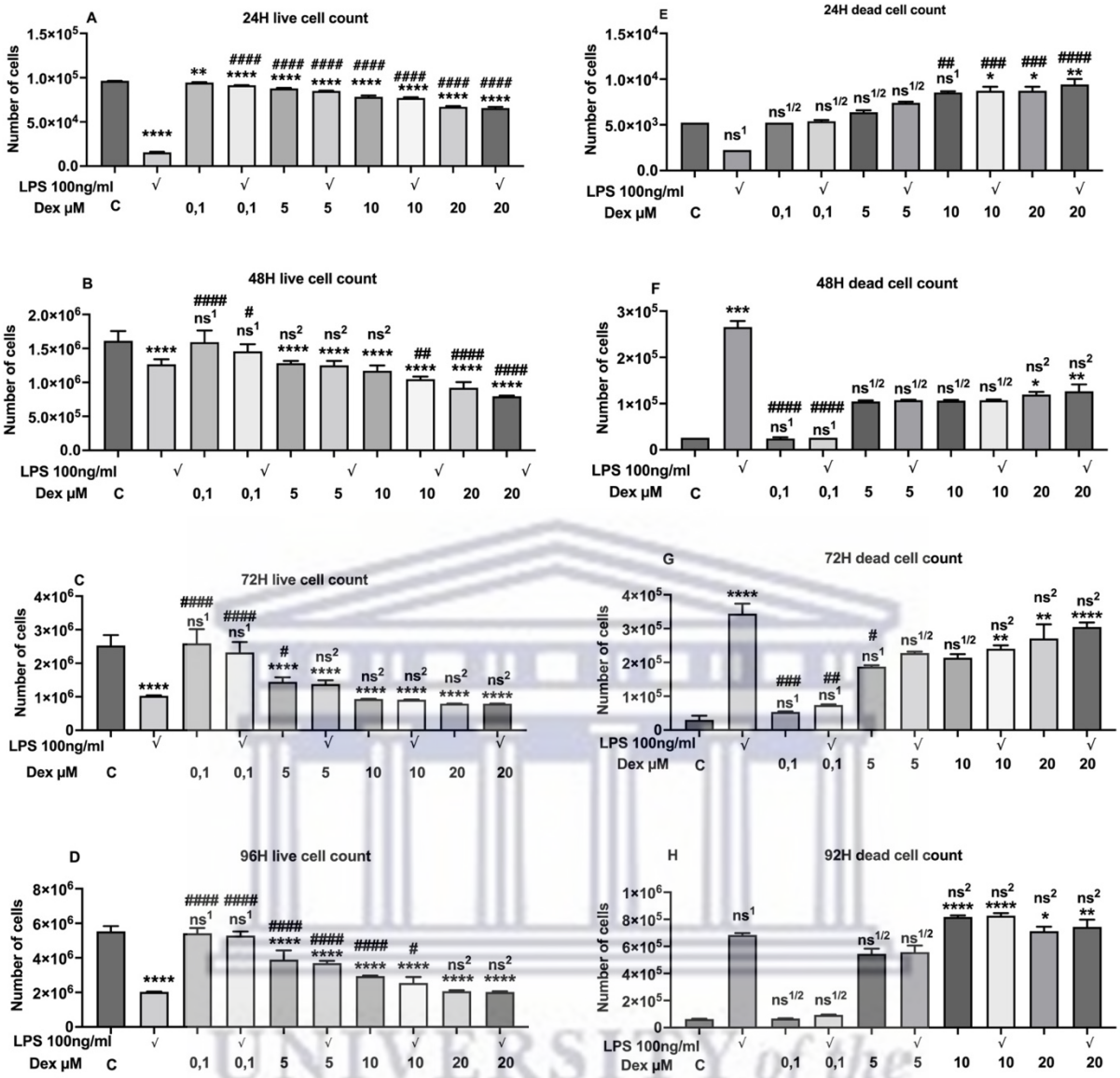

Figure 7: Effects of Dex on LPS-induced effects on bEnd.5 cell proliferation. LPS (100ng/ml) was co-exposed with concentrations of Dex (0.1, 5, 10, and 20μM) to bEnd.5 cell cultures. Trypan blue exclusion assay was used for the identification and enumeration of live and dead cells. Graphs A-D shows that Dex at low concentrations could reverse the LPS-induced suppression of cell division and cell toxicity, but at higher concentrations of Dex *(*5 to *20μM*) it was not able to reverse the LPS effects on cell proliferation (graphs A-D). Dex at low concentrations were able to reverse the LPS-induced toxicity on bEnd5 cells, but not at higher concentrations (graphs E to H). Note that treatment of bEnd5 cells with higher concentrations of Dex alone *(5 to 20μM*) were detrimental to both cell proliferation and to toxicity levels. (**C**=control),(n=3),*$p < 0.05$, **$p < 0.01$, and ****$p < 0.0001$ versus control group; $^{\#}p < 0.05$,),$^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ and $^{\#\#\#\#}p < 0.0001$versus LPS group; ns^1, no significant difference to control; ns^2, no significant difference to LPS).

Toxicity: The toxicity effects were paralleled by the viability trends. An increase in cell toxicity as the concentration of Dex increased was observed, showing a dose-related relationship. LPS throughout all hours showed increased cell toxicity when compared to the control ($p < 0.0001$). Low concentrations of Dex ($0.1\mu M$) had no statistical effect on toxicity compared to control toxicity levels, while higher Dex concentrations ($5\mu M$-$20\mu M$), with or without LPS, significantly ($p < 0.0001$) increased toxicity across all measuring hours (figure E-H).

Cultures of bEnd.5 cells were exposed to LPS (100ng/ml) for 12hrs and thereafter co-treated with Dex (0.1, 5, 10, $20\mu M$) for 24-96 hrs. LPS (100ng/ml), throughout the experimental time frame significantly ($p < 0.0001$) decreased live cell count (figure 5A-D). Higher Dex concentrations ($5\mu M$ -$20\mu M$) with or without LPS, decreased live cell number significantly across 96hrs (figure 5A-D), while treatment with low concentrations of Dex ($0.1\mu M$) was not significantly different from control means, and was able to reverse LPS induced suppression in cell numbers (figure 5 A-D: $p < 0.05$, $p < 0.0001$). Low concentrations of Dex ($0.1\mu M$) were not toxic to bEnd.5 cells (figure 5E-H), while co-treatment with LPS-treated bEnd.5 cells prevented the LPS-induced toxicity. Higher concentrations of Dex (5, 10, $20\mu M$) were not able to reverse LPS-induced bEnd.5 toxicity, while on their own these concentrations of Dex induced statistically increased levels of cell death (figure 5E-H).

3.3. ELISA

An ELISA was conducted to identify and quantify the pro-inflammatory cytokines (IL-1β and TNF-α) released by bEnd.5 cells into the media supernatant in response to LPS and/or Dex treatment. Cultures of bEnd.5 cells were exposed to LPS (100ng/ml) for 12hrs and thereafter treated with Dex ($0.1\mu M$, $20\mu M$) for 96hrs.

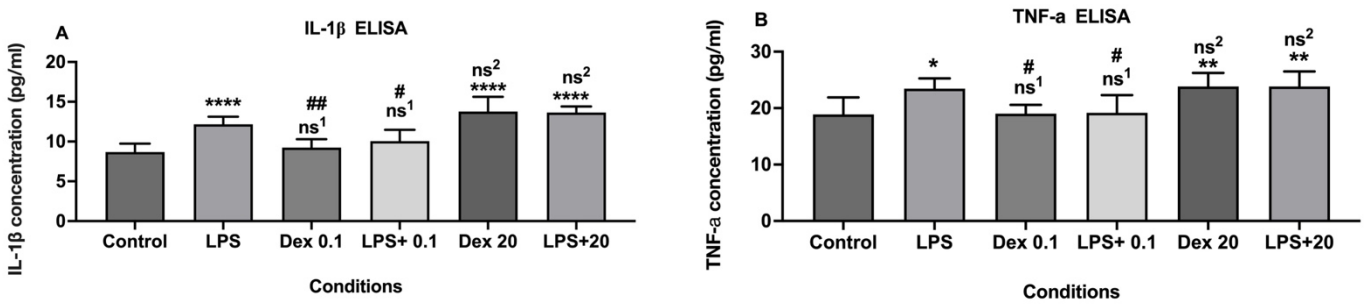

Figure 8: The secretion of IL-1β and TNF-α by bEnd.5 cells. ELISA was used to identify and quantify cytokines released in response to LPS exposure. LPS increased IL-1β and TNF-α cytokine expression (graphs A and B respectively). Low concentrations of Dex ($0.1\mu M$)

maintained cytokine expression to control levels and were able to reverse LPS-induced cytokine secretion. The higher concentration of Dex ($20\mu M$) increased cytokine expression of both IL-1β and TNF-α, and was not able to reverse LPS-induced cytokine secretion. (n=9),*p < 0.05, **p < 0.01, and ****p < 0.0001 versus control group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$; ns^1, no significant difference to control; ns^2, no significant difference to LPS.

ELISA kits were used to identify and quantify the presence of inflammatory cytokines IL-1β and TNF-α secreted into the media supernatant by bEnd.5 cultures after co-exposure of LPS (100ng/ml) and Dex ($1\mu M$ and $20\mu M$). Compared to the control, LPS (100ng/ml) caused a statistically significant increase in IL-1β and TNF-α secretion from bEnd.5 cells. Dex only reduced the expression of IL-1β and TNF-α at low doses ($1\mu M$), while it increased the levels of both cytokines at high concentrations ($20\mu M$) ($p < 0.0001$; $p < 0.01$)). Low concentrations of Dex ($0.1\mu M$) exhibit significance against LPS (figure 6, A/B: $p < 0.05$; $p < 0.01$).

3.4 Effects on TEER: Permeability

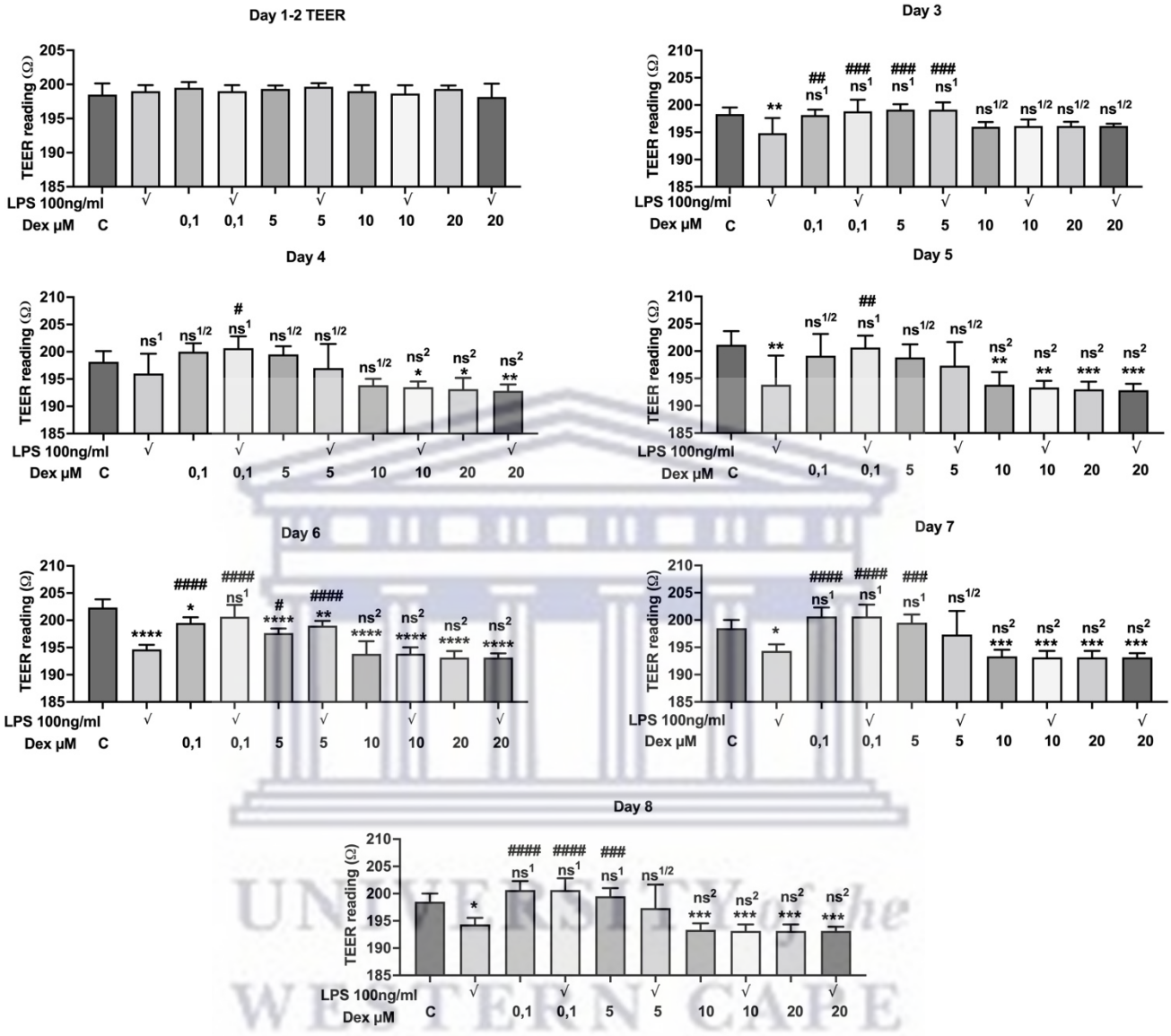

Figure 9: The effects of Dex and LPS on TEER-across confluent monolayers of bEnd.5 cells. TEER was used to determine the electrical resistance of bEnd.5 cells in response to Dex and LPS exposures. TEER in graphs B-E showed lower TEER at high concentrations (10 and 20*µM),* and a higher TEER at low concentrations (0.1 and 5*µM)* of Dex. Monolayers of bEnd.5 cells were either exposed to LPS (100ng/ml) or co-exposed to LPS (100ng/ml) and/or selected concentrations of Dex. (**C**=control),(n=3),*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$ versus control group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ $^{\#\#\#}p < 0.001$ and $^{\#\#\#\#}p < 0.0001$ versus LPS group; ns^1, no significant difference to control; ns^2, no significant difference to LPS).

TEER across bEnd.5 endothelial monolayers was conducted to investigate any change in the integrity and permeability of the confluent bEnd.5 monolayers after treatment with LPS and/or Dex. Cultured bEnd.5 cells were exposed to LPS (100ng/ml) for 12hrs and thereafter treated with Dex (0.1, 5, 10, 20μM) throughout the 8-day experimental time frame. TEER presented a dose-related trend across the experimental time frame: as the concentration of Dex increased, TEER decreased (figure 7). Lower TEER reflected higher permeability across the confluent mono layers of bEnd.5 cells and vice versa. Treatment with 0.1μM Dex resulted in the statistically significant TEER recovery from the effects of LPS-reduced TEER (increased permeability) across bEnd.5 monolayers in comparison to higher Dex concentrations (5, 10, 20 μM), which decreased TEER (figure 7). Higher Dex concentrations (5, 10, 20μM) were not effective in reversing the LPS-induced decrease in TEER consistently across the experimental time frame.

Discussion

Brain inflammation, also known as encephalitis, can be brought about by several causes which include viral and bacterial infections (22), autoimmune pathologies such as Lupus (4), exposure to toxins (e.g. insect bites), poisons (industrial or animal), industrial chemicals/pesticides or heavy air pollution (e.g. in dense cities), lifestyle diseases (Diabetes and high blood sugar, poor circulation, lack of exercise, chronic stress, heart failure, respiratory issues) (23), and sometimes by unknown factors (24). Chronic brain inflammation is greatly damaging to the nervous system and has been linked to numerous neurodegenerative diseases (Alzheimer's disease, Parkinson's disease, etc.) ((5) and several psychiatric disorders, including depression, anxiety, schizophrenia, etc (25). Most times, inflammation of the brain implicates the cerebral blood vessels and especially the capillaries. These capillaries are the primary component of the BBB, and are responsible for maintaining the homeostasis of the neuronal environment (1). Their endothelial cells are responsible for maintaining robust regulation of brain capillary permeability to ensure homeostatic control of substances entering and leaving the brain (26,27).

In this study, we established the inflammatory BBB model by exposing a selected concentration of the bacterial pyrogen, LPS, to cultures of the brain endothelial cell line, bEnd.5. LPS is commonly used as a key component in cytokine and chemokine production as well as being a potent activator of inflammation (28). We established that LPS induced increases in the pro-inflammatory cytokines IL-1β and TNF-α (figure 6), which was supported

by suppression of cell division (figure 5A-D) and increases in toxicity (figure 4E-H). These data were endorsed by suppression of bEnd.5 viability and increased toxicity (figure 4A-H). The BBB-LPS-induced inflammatory model is endorsed by the work of (20) who showed that LPS (100ng/ml) was sufficient to induce an inflammatory response in bEnd.3 BECs. They also showed that LPS induced increases in the secretion of cytokines IL-1β and TNF-α. Our data, therefore, endorses the *in vitro* LPS-induced inflammatory BBB model using the bEnd.5 cell line, for the study of vascular inflammation of brain capillaries.

Early in the COVID-19 pandemic, before vaccines were available, reports of the partial anti-inflammatory success of low doses of the glucocorticoid, Dex, were shown to decrease the mortality of hospitalized patients who were not critical (8). Glucocorticoids, such as Dex, is thought to act directly on cell membrane receptors, regulating the immune system through a nongenomic mechanism, to bring about anti-inflammatory actions (29). We wanted to investigate whether Dex, at low or high concentrations, could be beneficial in attenuating LPS-induced inflammation in the *in vitro* BBB-inflammatory model.

Therefore, a dose-response experiment was used to establish which LPS (50 to 200ng/ml) concentration would best produce a consistent inflammatory effect over 96hrs (figure 4). LPS (100ng/ml) served as the optimal concentration to represent the LPS effect on bEnd.5 cells, as it was the lowest concentration to produce consistent statistical significance over the experimental time frame. Furthermore, this concentration of LPS (100ng/ml) was supported in the literature for producing inflammatory effects of LPS (20) on a similar cell line (bEnd.3 cells) (20) who reported significant effects on proinflammatory cytokine secretion, as well as on the viability, toxicity, live and dead bEnd.3 cells.

LPS produces both a toxicity effect, as evident by the increase in dead cells (figure 5), as well as a suppression of cell division. Compare to the control, the number of dead cells does account for the difference in the live cell number found in LPS-treated cells. Therefore, the discrepancy between treated and control live cell numbers have to be due to both the toxicity effect of LPS (12.24%) and the suppression of live cell division (36.25%) (figure 8). This infers that the mechanism by which LPS treatment decreases cell number is brought about by the suppression of cell division and by increased cell death (toxicity). This inflammatory effect is well documented in the literature and can be ascribed to the ability of LPS to render its target cells

more permeable (30), thus compromising normal cell signalling, and hence normal cell division (30). LPS pyogenic effect is also evident in the bEnd.5 cell viability (%) (figure 4 A-D), where the dose-related effect is mostly due to the ratio of live cells to increased dead cells. This is also reflected in the toxicity %, which shows a dose-related effect on cell toxicity (increased dead cells) (31).

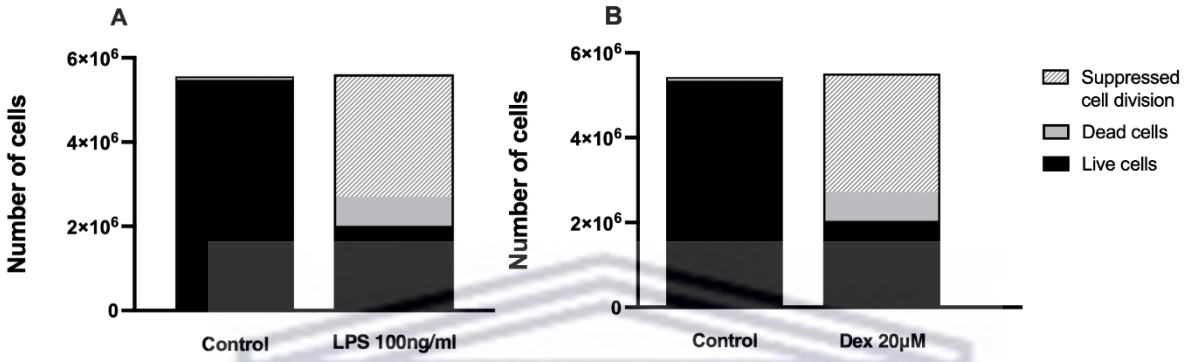

Figure 10: The above graphs demonstrate the effect of LPS (graph A) and Dex (graph B) on suppressing both cell division as well as increasing the number of dead cells, thus also having a toxic effect on bEnd.5 cells.

The glucocorticoid, Dex, is generally used as an anti-inflammatory treatment (32). Although low doses of Dex, but not high doses, were effectively used during the early stages of COVID-19 to decrease mortality in patients that were not yet critical (8), there were no reports in the literature on the effects of Dex on brain vascular inflammation. To ascertain the appropriate Dex concentration to treat the bEnd.5 cells, which were induced to inflammation by LPS, we treated bEnd.5 cells with selected doses, which would represent either a low concentration or a high concentration, and assessed the viability, toxicity, live, and dead cells using a known concentration range of Dex ($0.1\mu M$ represented low doses and 5 to $20\mu M$, represented the high doses). Figure 2 A-D reflects that low doses of Dex $0.1\mu M$ have little to no statistically negative effect on viability. Higher doses suppressed bEnd.5 cell viability in a dose-related manner. Conversely, Dex showed a dose-related effect on cell toxicity and induced high levels of cell death at higher concentrations of Dex, while having no statistical effect at low concentrations (figure 4E-H). These findings could be due to the high binding affinity properties of Dex for glucocorticoid receptors, which means that it can bind to and activate these receptors effectively. This could be the reasoning to why low concentrations do not have a negative effect on cells (33). We also observed that the percentage of dead cells does not account for the decrease in cell number compared to the controls (figure 8B). It is therefore also evident that

higher concentrations of Dex (5-20 μM), in addition to being toxic to cells, are also suppressing cell division via proinflammatory mechanisms (figure 6), which also increases trans endothelial permeability (figure 7).

Anti-inflammatory and pro-inflammatory effects of Dex: To investigate Dex's anti-inflammatory capacity, bEnd.5 cells were exposed to LPS-induced inflammation for 12 hrs, and thereafter co-exposure to both LPS (100ng/ml) and Dex (0.1μM (low dose) and 5-20μM (high dose). Low concentrations of Dex (0.1μM) with and without co-exposure of LPS maintained a toxicity level relatively close to that of control throughout the study ($P>0.05$). Furthermore, the 0.1μM Dex concentration was able to reverse the LPS-induced effects on cell viability (figure 4A-D), toxicity (figure 4E-H), live and dead cell numbers (figure 5A-H). In contrast to this, high concentrations of Dex (5μM-20μM) significantly increased toxicity to a maximum of almost 20%, while co-exposure with LPS was not able to effectively reverse the effects of LPS on cell viability (figure 4A-D), toxicity (figure 4E-H), live and dead cell numbers. Our experiments indicated that high concentrations of Dex are proinflammatory to BECs, causing high levels of toxicity, while also not being able to reverse the inflammatory effects.

Effects of cytokine secretion

Inflammation of bEnd.5 cells was confirmed using ELISA kits, whereby the expression and quantification of proinflammatory cytokines of IL-1β and TNF-α were established. The ELISA-based experiments showed that LPS does indeed induce inflammatory effects on bEnd.5 cells, and furthermore, that Dex not only attenuated both cytokine expression at low concentrations (0.1μM), but increased cytokine expression at high concentrations of Dex (figure 6). These novel findings once again support the ability of LPS to induce the inflammatory effects on bEnd.5-BECs throughout this experiment and confirm that lower concentrations of Dex (0.1μM) are more favourable in attenuating LPS-induced inflammatory effects on bEnd.5 endothelial cells without altering the physiological effects of the *in vitro* BBB. Higher doses of Dex were proinflammatory, inducing increased secretion of proinflammatory cytokines (figure 6).

Effects on monolayer permeability

The primary role of the capillary endothelial cells of the BBB is to regulate the permeability to all substances crossing into and out of the brain. TEER inversely reflects the permeability across monolayers of confluent bEnd.5 cells, the *in vitro* BBB model. We showed that LPS (100ng/ml) increased proinflammatory cytokine secretion and in support of this observation, it

also increased permeability (figure 7). This is a well-known physiological response of most endothelial cells experiencing inflammation (34) and the expression of matrix metalloproteinase 9 (MMP-9), which breaks down tight junction (TJ) proteins including ZO-1, occludin, and claudin-5, is increased indirectly by IL-1β (35,36). Co-treatment of LPS with low doses of Dex ($0.1\mu M$ and to a lesser extent $5\mu M$) once again showed to have the most favourable effect on permeability recovery across the time frame of our TEER experiments, thus, improving monolayer impermeability to control levels. In contrast, as established already in the co-exposure of Dex experiments of viability, toxicity, and cell division, higher concentrations of Dex were not able to reverse the proinflammatory effects induced by LPS, and were found to be detrimental by increasing the permeability across our *in vitro* BBB model (figure 7).

Limitations and strengths of the study:

The capillaries of the brain are regulated via numerous cellular systems, collectively called BBB or the neuro-vascular unit. The BBB is comprised of centrally the capillary endothelial cells, and are largely surround by functional inputs from the astrocytes and the foot-processes which encircle the outer surface of the capillary endothelium, only separated from the brain endothelial cells by a basement membrane. Secondary inputs are via the pericytes who are central to brain capillary blood flow. Therefore, one of the limitations of this study is that we have investigated inflammatory physiology of brain endothelial cells in isolation from their normal regulatory cellular components and we have to interpret our results keeping this in mind. However, our experimental design has purposefully use the isolated BECs. Our rationale is that we wanted to understand the physiology of BEC without the complexing regulatory inputs from either the astrocytes or pericytes. Given this theoretical position, and that pyrogenic molecules (PAMPs) primarily affect the BBB via the apical surfaces of the capillary endothelium (36), and that compromised endothelium is firstly affected and other neural components are secondarily affected in the inflammatory progression, it makes sense to understand the inflammatory physiology and its treatment in isolation. Furthermore, our data supports our theoretical premise, which is in turn supported by clinical reports regarding the effects of Dex on patients treated for inflammatory pathology (37).

Conclusion

Our study provided extensive support for the verification of the *in vitro* LPS-induced inflammatory BBB model for the study of vascular inflammation of the brain. Using this model, we show for the first time the importance that only low concentrations of Dex were able to reduce the LPS-induced pyrogenic inflammatory effect on brain endothelial cells. In contrast, higher concentrations of Dex were not able to reverse the LPS-induced inflammatory response on brain endothelial cells, and demonstrated proinflammatory effects on the *in vitro* BBB, by increasing the secretion of proinflammatory cytokines, increased toxicity, suppression of endothelial cell division, and increased permeability across monolayers of confluent endothelial monolayers. Most importantly, our data endorse the COVID-19 clinical picture in that only low doses of Dex were able to improve mortality in non-critical hospitalized patients, in that only low concentrations of Dex were able to be physiologically neutral while able to reverse the inflammation of the brain endothelial cells. As reported for the treatment of COVID-19 patients (37), treatment of brain vascular inflammation with Dex seems to be a double-edged sword and careful risk-benefit assessments must be made before it is initiated.

Author Contributions

Conceptualization, D.F. and T.J.; methodology, D.F.; software, D.F.; validation, D.F., K.M. and T.J.; formal analysis, T.J.,D.F. and K.M; investigation, T.J.; resources, D.F.; data curation, T.J. and D.F.; writing—original draft preparation, T.J.; writing—review and editing, D.F.; visualization, D.F.; supervision, D.F. and K.M.; project administration, D.F.; funding acquisition, D.F. All authors have read and agreed to the published version of the manuscript.

Funding

Funding was received from the National Research Foundation (NRF)-Postgraduate Scholarship. UWC Senate funds were received for the consumables of the research project.

Informed Consent Statement

Not applicable.

Data Availability Statement

All experimental data collected are archived within the University of the Western Cape (UWC) archives and are available as per UWC data and intellectual property policy guidelines and their associated copyright protection.

Acknowledgments

This work was academically supported by the Department of Medical Biosciences at UWC and the Neuromolecular Unit at the UWC, Cape Town, South Africa.In this regard, we especially want to thank Dr Kim Lategan for her insightful suggestions regarding our ELISA experiments. We also thank Morne Swannepoel for his assistance in acquiring the drugs used in this research project.

Conflict of Interest

The authors declare no conflict of interest.

References

1. Abbott NJ, Rönnbäck L, Hansson E. Astrocyte–endothelial interactions at the blood–brain barrier. Nature Reviews Neuroscience 2006 7:1 [Internet]. 2006;7(1):41–53. Available from: https://www.nature.com/articles/nrn1824
2. Markowicz-Piasecka M, Markiewicz A, Darłak P, Sikora J, Adla SK, Bagina S, et al. Current Chemical, Biological, and Physiological Views in the Development of Successful Brain-Targeted Pharmaceutics. Neurotherapeutics [Internet]. 2022;19(3):942–76. Available from: https://pubmed.ncbi.nlm.nih.gov/35391662/
3. Kelleher RJ, Soiza RL. Evidence of endothelial dysfunction in the development of Alzheimer's disease: Is Alzheimer's a vascular disorder? Am J Cardiovasc Dis [Internet]. 2013;3(4):197. Available from: /pmc/articles/PMC3819581/
4. Pohl D, Benseler S. Systemic inflammatory and autoimmune disorders. Handb Clin Neurol. 2013;112:1243–52.
5. Thomas J, Thomas CJ, Radcliffe J, Itsiopoulos C. Omega-3 Fatty Acids in Early Prevention of Inflammatory Neurodegenerative Disease: A Focus on Alzheimer's Disease. Biomed Res Int. 2015;2015.

6. Baeten KM, Akassoglou K. Extracellular matrix and matrix receptors in blood-brain barrier formation and stroke. Dev Neurobiol [Internet]. 2011;71(11):1018–39. Available from: https://pubmed.ncbi.nlm.nih.gov/21780303/
7. Straub RH. The brain and immune system prompt energy shortage in chronic inflammation and ageing. Nat Rev Rheumatol [Internet]. 2017;13(12):743–51. Available from: https://pubmed.ncbi.nlm.nih.gov/29021568/
8. Lukiw WJ, Pogue A, Hill JM. SARS-CoV-2 Infectivity and Neurological Targets in the Brain. Cell Mol Neurobiol [Internet]. 2022;42(1):217–24. Available from: https://pubmed.ncbi.nlm.nih.gov/32840758/
9. Giles AJ, Hutchinson MKND, Sonnemann HM, Jung J, Fecci PE, Ratnam NM, et al. Dexamethasone-induced immunosuppression: mechanisms and implications for immunotherapy. J Immunother Cancer [Internet]. 2018;6(1). Available from: /pmc/articles/PMC5996496/
10. Theoharides TC, Conti P. Dexamethasone for COVID-19? Not so fast. J Biol Regul Homeost Agents [Internet]. 2020;34(3):1241–3. Available from: https://pubmed.ncbi.nlm.nih.gov/32551464/
11. Lammers T, Sofias AM, van der Meel R, Schiffelers R, Storm G, Tacke F, et al. Dexamethasone nanomedicines for COVID-19. Nature Nanotechnology 2020 15:8 [Internet]. 2020;15(8):622–4. Available from: https://www.nature.com/articles/s41565-020-0752-z
12. McMahon D, Oakden W, Hynynen K. Investigating the effects of dexamethasone on blood-brain barrier permeability and inflammatory response following focused ultrasound and microbubble exposure. Theranostics [Internet]. 2020;10(4):1604. Available from: /pmc/articles/PMC6993222/
13. qian Zhou H, mei Zhang L, Li X, hua Huang Z. Crosstalk Between Autophagy and Inflammation in Chronic Cerebral Ischaemia. Cellular and Molecular Neurobiology 2023 [Internet]. 2023 May;1–10. Available from: https://link.springer.com/article/10.1007/s10571-023-01336-6
14. Badshah H, Ali T, Rehman S ur, Amin F ul, Ullah F, Kim TH, et al. Protective Effect of Lupeol Against Lipopolysaccharide-Induced Neuroinflammation via the p38/c-Jun N-Terminal Kinase Pathway in the Adult Mouse Brain. J Neuroimmune Pharmacol [Internet]. 2016;11(1):48–60. Available from: https://pubmed.ncbi.nlm.nih.gov/26139594/

15. Okun E, Griffioen KJ, Mattson MP. Toll-like receptor signaling in neural plasticity and disease. Trends Neurosci [Internet]. 2011;34(5):269–81. Available from: https://pubmed.ncbi.nlm.nih.gov/21419501/
16. Gárate I, García-Bueno B, Madrigal JLM, Caso JR, Alou L, Gómez-Lus ML, et al. Toll-like 4 receptor inhibitor TAK-242 decreases neuroinflammation in rat brain frontal cortex after stress. J Neuroinflammation [Internet]. 2014;11(1):1–14. Available from: https://jneuroinflammation.biomedcentral.com/articles/10.1186/1742-2094-11-8
17. Sambamurti K, Kinsey R, Maloney B, Ge YW, Lahiri DK. Gene structure and organization of the human beta-secretase (BACE) promoter. FASEB J [Internet]. 2004;18(9):1034–6. Available from: https://pubmed.ncbi.nlm.nih.gov/15059975/
18. van den Boogaard M, Ramakers BP, van Alfen N, van der Werf SP, Fick WF, Hoedemaekers CW, et al. Endotoxemia-induced inflammation and the effect on the human brain. Crit Care [Internet]. 2010;14(3). Available from: https://pubmed.ncbi.nlm.nih.gov/20444270/
19. Brandenburg LO, Kipp M, Lucius R, Pufe T, Wruck CJ. Sulforaphane suppresses LPS-induced inflammation in primary rat microglia. Inflamm Res [Internet]. 2010;59(6):443–50. Available from: https://pubmed.ncbi.nlm.nih.gov/19924513/
20. Li T, Zheng LN, Han XH. Fenretinide attenuates lipopolysaccharide (LPS)-induced blood-brain barrier (BBB) and depressive-like behavior in mice by targeting Nrf-2 signaling. Biomedicine & Pharmacotherapy. 2020;125:109680.
21. Seemann S, Zohles F, Lupp A. Comprehensive comparison of three different animal models for systemic inflammation. J Biomed Sci [Internet]. 2017;24(1):1–17. Available from: https://jbiomedsci.biomedcentral.com/articles/10.1186/s12929-017-0370-8
22. Young GB. Encephalopathy of infection and systemic inflammation. J Clin Neurophysiol [Internet]. 2013;30(5):454–61. Available from: https://pubmed.ncbi.nlm.nih.gov/24084178/
23. Jaishankar M, Tseten T, Anbalagan N, Mathew BB, Beeregowda KN. Toxicity, mechanism and health effects of some heavy metals. Interdiscip Toxicol [Internet]. 2014;7(2):60. Available from: /pmc/articles/PMC4427717/
24. Wyss-Coray T. Ageing, neurodegeneration and brain rejuvenation. Nature 2016 539:7628 [Internet]. 2016;539(7628):180–6. Available from: https://www.nature.com/articles/nature20411

25. Chesnokova V, Pechnick RN, Wawrowsky K. Chronic peripheral inflammation, hippocampal neurogenesis, and behavior. Brain Behav Immun [Internet]. 2016;58:1–8. Available from: https://pubmed.ncbi.nlm.nih.gov/26802985/
26. Tietz S, Engelhardt B. Brain barriers: Crosstalk between complex tight junctions and adherens junctions. J Cell Biol [Internet]. 2015;209(4):493–506. Available from: https://pubmed.ncbi.nlm.nih.gov/26008742/
27. Rhea EM, Banks WA. Role of the Blood-Brain Barrier in Central Nervous System Insulin Resistance. Front Neurosci. 2019;13(JUN):521.
28. Tucureanu MM, Rebleanu D, Constantinescu CA, Deleanu M, Voicu G, Butoi E, et al. Lipopolysaccharide-induced inflammation in monocytes/macrophages is blocked by liposomal delivery of Gi-protein inhibitor. Int J Nanomedicine [Internet]. 2017;13:63–76. Available from: https://pubmed.ncbi.nlm.nih.gov/29317816/
29. Li JC, Wang S, Wang HC, Zhang Q, Wang Q, Guo MH, et al. Dexamethasone affects the chemotaxis and phagocytic activity of neutrophils for boar spermatozoa and the quality of liquid preserved boar semen in vitro. Theriogenology. 2023 May;195:46–54.
30. Huang Z, Kraus VB. Does lipopolysaccharide-mediated inflammation have a role in OA? Nat Rev Rheumatol [Internet]. 2016;12(2):123. Available from: /pmc/articles/PMC4930555/
31. Dauphinee SM, Karsan A. Lipopolysaccharide signaling in endothelial cells. Laboratory Investigation 2006 86:1 [Internet]. 2005;86(1):9–22. Available from: https://www.nature.com/articles/3700366
32. Yasir M, Goyal A, Sonthalia S. Corticosteroid Adverse Effects. StatPearls [Internet]. 2022; Available from: https://www.ncbi.nlm.nih.gov/books/NBK531462/
33. Hübner M, Hochhaus G, Derendorf H. Comparative pharmacology, bioavailability, pharmacokinetics, and pharmacodynamics of inhaled glucocorticosteroids. Immunol Allergy Clin North Am [Internet]. 2005;25(3):469–88. Available from: https://pubmed.ncbi.nlm.nih.gov/16054538/
34. Brandt M, Gerke V, Betz T. Human endothelial cells display a rapid tensional stress increase in response to tumor necrosis factor-α. PLoS One [Internet]. 2022;17(6):e0270197–e0270197. Available from: https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0270197
35. Suprewicz Ł, Fiedoruk K, Czarnowska A, Sadowski M, Strzelecka A, Galie PA, et al. Blood-brain barrier function in response to SARS-CoV-2 and its spike protein. Neurol

Neurochir Pol [Internet]. 2023;57(1):14–25. Available from: https://pubmed.ncbi.nlm.nih.gov/36810757/

36. Kircheis R, Planz O. The Role of Toll-like Receptors (TLRs) and Their Related Signaling Pathways in Viral Infection and Inflammation. Int J Mol Sci [Internet]. 2023 May;24(7). Available from: /pmc/articles/PMC10095430/

37. Asif S, Frithiof R, Larsson A, Franzén S, Anderberg SB, Kristensen B, et al. Immuno-Modulatory Effects of Dexamethasone in Severe COVID-19—A Swedish Cohort Study. Biomedicines 2023, Vol 11, Page 164 [Internet]. 2023 May;11(1):164. Available from: https://www.mdpi.com/2227-9059/11/1/164/htm

List of Figures

Chapter 3
Conclusion and research questions

3.1 General conclusions

Overall, Dex has demonstrated a significant ability to attenuate LPS-induced inflammation on the *in vitro* BBB. Several significant findings are presented in this study that support the validity of the *in vitro* LPS-induced inflammatory BBB model. Using this model, we demonstrate how imperative it is that only modest doses of Dex may inhibit the pyrogenic inflammatory action of LPS on brain endothelial cells. In contrast, Dex administered at higher doses did not restore the inflammatory response induced by LPS on brain endothelial cells. Additionally, it increased pro-inflammatory cytokine release, toxicity, and inhibited endothelial cell proliferation. Ultimately, this study has demonstrated modest doses of Dex improved mortality in non-critically ill hospitalized patients, supporting COVID-19 clinical assessments.

3.2 Research questions to still be addressed

In spite of the growing body of research regarding the use of Dex in treating conditions affecting the BBB, a number of key questions remain unanswered in order to fully comprehend its effectiveness and potential side effects. Some of the research questions that are still to be addressed include:

- What role does Dex play in affecting the permeability of the BBB in different regions of the brain and in different types of neurological disorders?
- Is it possible to use Dex in combination with other drugs in order to improve its effectiveness and to reduce its potential side effects?
- How does Dex affect the BBB at a molecular level?
- When Dex is administered, how does it affect the astrocytic end-feet and the pericytes of the BBB?

It is important to address these and other research questions to gain a better understanding of how Dex is used in treating conditions that affect the BBB and to optimize its use in clinical practice.