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INYUVESI YAKWAZULU-NATALI

An investigation into the inflammatory, oxidative stress and DNA methylation status of Fumonisin B_1 in the human U87MG glioblastoma cell line

By

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DECLARATION

I, **Ashmika Foolchand**, declare as follows:

1. That the work described in this thesis has not been submitted to UKZN or other tertiary institution for purposes of obtaining an academic qualification, whether by myself or any other party. Where a colleague has indeed prepared a thesis based on related work essentially derived from the same project, this must be stated here, accompanied by the name, the degree for which submitted, the University, the year submitted (or in preparation) and a concise description of the work covered by that thesis such that the examiner can be assured that a single body of work is not being used to justify more than one degree.

2. That my contribution to the project was as follows: This is followed by a concise description of the candidate's personal involvement in and contribution to the project, in sufficient detail that the examiner is in no doubt as to the extent of their contribution.

3. That the contributions of others to the project were as follows: This is followed by a list of all others who contributed intellectually to the project, each accompanied by a concise description of their contribution. This does not include people who ordinarily would be "acknowledged" as opposed to considered for authorship.

Signed: _____ Date:_____

Miss A Foolchand

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TABLE OF CONTENTS

PRESENTATIONS

1. Fumonisin B_1 Induces DNA Hypermethylation in Human Glioblastoma U87MG Cells, Ashmika Foolchand, Terisha Ghazi, Anil A. Chuturgoon. College of Health Sciences Research Symposium, University of KwaZulu-Natal, Durban, South Africa (27 August 2024). Poster presentation.

2. Fumonisin B_1 Induces DNA Hypermethylation in Human Glioblastoma U87MG Cells, Ashmika Foolchand, Terisha Ghazi, Anil A. Chuturgoon. School of Laboratory Medicine and Medical Sciences Research Day (27 September 2024). Oral presentation.

LIST OF FIGURES

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Appendix

LIST OF TABLES

Chapter 2

Chapter 3

Chapter 4

Appendix

ABBREVIATIONS

5-Aza-2-DC	5-Aza-2-deoxycytidine
8-oxoG	8-oxoguanine
APE1	Apurinic/apyrimidinic endonuclease 1
ATP	Adenosine triphosphate
BBB	Blood-brain barrier
BCA	Bicinchoninic acid
BDNF	Brain-derived neurotrophic factor
CAT	Catalase
CNS	Central nervous system
COX-2	Cyclooxygenase-2
CREB	Cyclic AMP responsive element binding
DAMPs	Damage-associated molecular patterns
DMEM	Dulbecco's Minimum Essentials Medium
DMSO	Dimethyl sulfoxide
DNMTs	DNA methyltransferases
EDTA	Ethylenediaminetetraacetic acid
ELEM	Equine leukoencephalomalacia
ETC	Electron transport chain
FADH	Flavin adenine dinucleotide
FB_1	Fumonisin B_1
GBM	Glioblastoma multiforme
GPx	Glutathione peroxidase
GSH	Glutathione
H_2O_2	Hydrogen peroxide

HSP	Heat shock protein
IDH1	Isocitrate dehydrogenase 1
IDH2	Isocitrate dehydrogenase 2
IL	Interleukin
LonP1	Lon protease 1
MBDs	Methyl-CpG binding domain
MDA	Malondialdehyde
mtDNA	Mitochondrial DNA
MTT	Methyl thiazol tetrazolium
NAD^+	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide hydrogen
NADPH	Nicotinamide adenine dinucleotide phosphate hydrogen
NF-κB	Nuclear factor-kappa B
NRF-2	Nuclear factor erythroid 2-related factor 2
OGG1	8-Oxoguanine glycosylase 1
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline
PRR	Pattern recognition receptor
PUA	Polyunsaturated aldehyde
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RT	Room temperature
SAM	S-adenosyl methionine
SIRTs	Sirtuins
SOD	Superoxide dismutase
TBARS	Thiobarbituric acid reactive substances

TET	Ten-eleven translocation
TFAM	Mitochondrial transcription factor A
TH	T-helper
TLRs	Toll-like receptors
TNF-α	Tumour necrosis factor-alpha
TrkB	Tropomyosin-related kinase B
TTBS	Tris-buffer saline with Tween 20

ABSTRACT

A frequently overlooked global health issue includes fungal and mycotoxin contamination, which infects many staple foods across the globe. Owing to its ubiquitous nature, long-term exposures to varying doses of mycotoxins pose major health concerns in humans and animals. Fumonisin B_1 (FB_1) is a toxic secondary metabolite commonly found in contaminated maize, known to induce various toxicities. This study investigated the effects of FB_1 on inflammatory responses, oxidative stress, and global DNA methylation in human glioblastoma U87MG cells after a 24-hour exposure.

The MTT assay was performed to determine the FB_1 IC_{50} value in U87MG cells, which was used for all subsequent experiments. Following FB_1 treatment, DNA, RNA and protein were extracted from U87MG cells and standardized. An ELISA was then conducted using the standardized DNA samples to assess DNA methylation status. cDNA, synthesized from standardized RNA, was used to measure gene expressions of *DNA methyltransferases* (*DNMTs)*, *methyl-CpG binding domain 2* (*MBD2)*, *superoxide dismutase 2* (*SOD2)*, *catalase* (*CAT)*, *glutathione peroxidase* (*GPx)*, *nuclear factor erythroid 2-related factor 2* (*NRF-2)*, *8-oxoguanine glycosylase 1* (*OGG1)*, *interleukin* (*IL)-6*, *IL-10* and *nuclear factor-kappa B (NF-kB)*, via qPCR. Standardized protein samples were diluted in sample buffer and analysed by western blot to evaluate protein expressions of DNMTs, MBD2, SOD2, CAT, mitochondrial transcription factor A (TFAM), IL-1β, tumour necrosis factor-alpha (TNF-α), brain-derived neurotrophic factor (BDNF) and cyclooxygenase-2 (COX-2).

Inflammation is a key mechanism of host immune defense, which is triggered by pro-inflammatory cytokines and maintained by anti-inflammatory cytokines. In U87MG cells, FB_1 significantly reduced the expression of pro-inflammatory cytokines such as IL-1β, IL-6, TNF-α, and NF-κB while enhancing the expression of the anti-inflammatory cytokine IL-10. Additionally, it decreased the pro-inflammatory marker COX-2 and increased the anti-inflammatory brain marker BDNF.

Oxidative stress, caused by the imbalance between free radicals and antioxidants, is a known cellular toxicity mechanism of FB_1 which results in DNA damage, protein degradation and neurodegenerative diseases. Here, FB_1 decreased lipid peroxidation and antioxidant responses of SOD2, CAT, GPx, and NRF-2, while also inhibiting oxidative DNA damage through regulation of the *OGG1* gene. Mitochondria are susceptible to reactive oxygen species (ROS) attack due to oxidative stress, which leads to mitochondrial dysfunction and mutations in mitochondrial DNA. Although FB_1 prompted the

upregulation of Sirtuin 3 (SIRT3), lon protease 1 (LonP1), and heat shock protein (HSP60) to support mitochondrial health in U87MG cells, it simultaneously caused alterations in mitochondrial DNA by downregulating mitochondrial transcription factor A (TFAM).

The regulation of gene expression by DNA methylation is suggested to influence biological processes including neurogenesis and the pathogenesis of brain disorders. FB_1-treated U87MG cells displayed global DNA hypermethylation, evidenced by increased levels of 5-methylcytosine and elevated expressions of DNA methyltransferases (DNMT1, DNMT3A, and DNMT3B), alongside a significant decrease in demethylase expression (MBD2), providing an alternative mechanism for FB_1 toxicity.

Taken together, this data suggests that FB_1, over a short period of time, may initially act as a selective neuroprotective agent by modulating inflammation, oxidative stress and mitochondrial responses, while also highlighting its potential neurotoxic effects through mitochondrial dysregulation and global DNA methylation.

Keywords: Fumonisin B_1, inflammation, oxidative stress, mitochondrial stress, global DNA methylation, human glioblastoma cells.

CHAPTER 1

INTRODUCTION

1.1 Background

Mycotoxins are low-molecular weight natural products produced by fungi that are toxic to humans and animals at low concentrations. These toxins not only pose risks to health but also account for huge economical losses worldwide (Zain 2011). Storage conditions, environmental factors and ecological conditions are all contributing features of mycotoxin production and contamination in food, which are sometimes beyond human control (Hussein and Brasel 2001). Exposure to mycotoxins commonly occurs through ingestion of plant-based food products, with dermal and inhalation also being possible exposure routes (Zain 2011). Some of the mycotoxins that pose major health and agro-economic insults include aflatoxins, zearalenone, trichothecenes, fumonisins and ochratoxins.

Globally, *Fusarium verticillioides* is among the most prevalent fungal contaminants of maize. This fungal strain was isolated in 1970 from mouldy maize, which is believed to have caused horse leukoencephalomalacia in South Africa (Marasas 2001). In 1988, the Fumonisin mycotoxin was isolated from the *Fusarium verticillioides* MRC 826 strain (Gelderblom et al. 1988a) and to date 15 different Fumonisins have been documented (Dutton 1996). Of these, Fumonisin B_1 (FB_1) is the most copious and noxious, posing major health hazards to humans and animals. FB_1 is a heat-stable and water-soluble mycotoxin, with inadequate agricultural resources and poor post-harvest storage procedures contributing to the risk of FB_1 contamination (Gao et al. 2023). In Algeria, the rate of FB_1 contamination in maize samples was reported to be 96.6% with high concentrations of 42.1 mg/kg in feed (Mahdjoubi et al. 2020). This concentration far exceeds the maximum tolerable daily intake of 2 µg/kg body weight of FB_1, which was set by the European Union and the US Food and Drug Administration (Gao et al. 2023). FB_1 ingestion in humans has been associated with oesophageal cancer, neural tube defects and kidney and liver disease, while in animals it is known to cause pulmonary oedema, equine leukoencephalomalacia (ELEM) and carcinogenesis (Stockmann-Juvala and Savolainen 2008). Several potential mechanisms of FB_1 toxicities have also been proposed, which include oxidative stress, cytotoxicity, apoptosis and immunotoxicity (Stockmann-Juvala and Savolainen 2008).

Human and animal diets exposed to Fumonisins produce chronic mycotoxicosis which may alter immunological pathways (Theumer et al. 2002). Inflammation is a response mechanism of the innate immune system that shields the body from harmful stimuli. Inflammatory responses are triggered by

tissue damage due to mechanical injury, and by the invasion of bacteria and viruses, while excessive inflammation contributes to disease pathology (Lyman et al. 2014b). The human immune response is governed by a highly intricate network of regulatory elements. Under physiological conditions, cytokine inhibitors act as immunomodulators, mitigating the potentially harmful effects of prolonged or excessive inflammatory reactions (Shaikh 2011). However, in pathological situations, the anti-inflammatory mediators may fail to adequately control pro-inflammatory activities in immune diseases, or may excessively inhibit the immune response, increasing the host's vulnerability to systemic infections (Shaikh 2011). A dynamic and constantly fluctuating balance exists between pro- and anti-inflammatory cytokines within the human immune system. Pro-inflammatory cytokines such as interleukin (IL)-1, IL-6, IL-11, IL-8 and tumour necrosis factor-α (TNF-α), along with chemokines, are known to mediate the acute and chronic inflammatory responses (Shaikh 2011), while the transcription factor NF-κB is recognized as the regulator of innate immunity (Baltimore 2009). Additionally, prostaglandins are key mediators of inflammation and immune responses. Inflammatory markers and cytokines trigger cyclooxygenase-2 (COX-2) activation which results in prostaglandin production (Arias-Negrete, Keller, and Chadee 1995). In contrast, IL-10 is an essential anti-inflammatory cytokine for preventing inflammation and autoimmune disorders, as its deficiency can result in the development of autoimmune diseases and increased tumorigenicity (Iyer and Cheng 2012a). Peripheral inflammation initiates a neuroinflammatory response that describes the immune responses of the central nervous system (CNS), associated with the blood-brain barrier (BBB), glia and neurons (Lyman et al. 2014b). The BBB is a specialized form of endothelium that is permeable to pro-inflammatory markers and can be triggered by the release of pro-inflammatory mediators, allowing the migration of leukocytes into the brain (De Vries et al. 1996). Neuroinflammation has been implicated in the progression of several brain diseases, neuronal death and synaptic impairment. In addition, brain-derived neurotrophic factor (BDNF) has become a key mediator of neuronal plasticity (Calabrese et al. 2014). It negatively regulates neuroinflammation, while elevated levels of inflammation can decrease BDNF expression (Porter and O'Connor 2022).

The toxic effects of mycotoxins are primarily linked to oxidative stress. Oxidative stress occurs when cells are exposed to reactive oxygen intermediates, including superoxide anion, hydrogen peroxide, and hydroxyl radicals, which damage proteins, nucleic acids, and cell membranes (Storz and Imlayt 1999). Growing evidence indicates that the cumulative damage from reactive oxygen species (ROS) plays a role in various diseases. Damage caused to DNA by ROS also presents a major risk to genetic integrity (Klungland and Bjelland 2007). To combat oxidative stress, cells continuously produce antioxidant enzymes that neutralize these reactive species and repair the resulting damage (Storz and Imlayt 1999). Nuclear factor erythroid 2-related factor 2 (NRF-2) promotes the expression of several antioxidant proteins and enzymes and is regarded as a key regulator of redox metabolism, while also contributing

to mitochondrial functions (Murakami et al. 2023). Under physiological conditions, superoxide anion is the most common free radical produced, which is primarily sourced from the mitochondria (Cadenas and Davies 2000). Within the mitochondria during respiration, the electron transport chain leaks unpaired electrons into the mitochondrial matrix (Turrens 2003), which interacts with molecular oxygen to produce ROS (Sena and Chandel 2012). Recent evidence suggests that mitochondrial sirtuins (SIRTs) play a crucial role in regulating gene expression and the activity of various enzymes, thereby coordinating oxidative metabolism and stress responses (Wang and Wei 2020). The mitochondrial protease, LonP1 plays a vital role in maintaining mitochondrial function via degradation of misfolded and oxidatively damaged proteins, and mitochondrial DNA conservation (Ngo and Davies 2007a). FB_1 is known to interrupt mitochondrial respiration by inhibiting complex I of the electron transport chain, leading to increased production of ROS (Domijan and Abramov 2011). While previous studies have recognized oxidative stress as a consequence of FB_1 exposure, the role of the subsequent antioxidant response in the context of FB_1 toxicology has not been thoroughly established (Mary et al. 2012).

While almost all cells in an organism carry the same genetic information, not every gene is expressed simultaneously across different cell types. Epigenetic mechanisms play a key role in creating diverse gene expression profiles in various cells and tissues within multicellular organisms (Moore, Le, and Fan 2013). Epigenetic modifications, such as DNA methylation and histone modifications, alter DNA accessibility and chromatin structure, thus regulating gene expression patterns. These processes are essential for normal development and the differentiation of specific cell lineages in adult organisms (Handy, Castro, and Loscalzo 2011). The mammalian DNA methylation machinery consists of two main components: DNA methyltransferases (DNMTs), which establish and maintain DNA methylation patterns, and methyl-CpG binding domain (MBD) proteins which modify and remove these methylation marks (Robertson 2005). DNA methylation directly represses transcription by blocking the binding of specific transcription factors, and indirectly, by attracting MBD proteins and their related chromatin remodelling functions (Robertson 2005). Optimal establishment and maintenance of DNA methylation patterns are crucial for mammalian development and for the proper functioning of adult organisms. This process serves as a powerful mechanism for silencing gene expression and preserving genome stability (Robertson 2005). While FB_1 is considered a potent human carcinogen, DNA methylation anomalies are a defining characteristic of cancer and it significantly contributes to neurological disorders (Ladd-Acosta et al. 2007).

The brain is a vital organ that serves as the central hub of the nervous system in mammals. Its primary role is to control and coordinate a diverse range of actions and responses, particularly in the areas of thought processing, movement, memory, and emotion (Xie et al. 2023a). The processes of brain

development and aging are complex and rely on multiple layers of precise regulation. Owing to its high polarity and small size, FB_1 can cross the BBB (Gelderblom et al. 1988a). Although numerous animal and neural cell culture studies have identified the potential of FB_1 as a neurodegenerative agent, the mechanisms of FB_1-induced neurotoxicity remain vague.

1.1.1 Aim and research questions

This study aimed to determine the neurotoxic characteristics of FB_1 in human glioblastoma U87MG cells by investigating its effects on inflammation, oxidative stress and global DNA methylation. It was hypothesized that FB_1 altered brain oxidative homeostasis influencing inflammatory responses and aberrant global DNA methylation patterns. The following research questions were posed:

- Does FB_1 alter the cytokine and NF-κB inflammatory response?
- Does FB_1 predispose U87MG cells to a pro-oxidant state?
- Can FB_1 disrupt mitochondrial homeostasis and maintenance?
- Does FB_1 affect global DNA methylation levels?

1.1.2 Objectives

- To determine the cytotoxic effect of FB_1 in U87MG cells post 24 h exposure
- To investigate the inflammatory response of FB_1 via the gene and protein expressions of inflammatory cytokines and brain-specific inflammatory markers, BDNF and COX-2.
- To evaluate the impact of FB_1 on oxidative stress via antioxidant expressions, and oxidative DNA damage.
- To analyse the mitochondrial stress response and maintenance of mitochondrial integrity via expressions of SIRT3, LonP1 and heat shock protein HSP60.
- To measure the effect of FB_1 on global DNA methylation status, by ELISA, and the subsequent expressions of DNMTs and MBD2, via western blot and qPCR.

1.1.3 UKZN examination guidelines

This thesis has been submitted in the thesis by manuscript format in accordance with the UKZN examination guidelines (Appendix 6.1).

1.2 Literature Review

1.2.1 Mycotoxins

The consumption of fungal infected food products poses major health hazards to humans and animals, including cancer initiation and progression. Globally food security is threatened as mycotoxins pollute almost 25% of feed annually (Omotayo et al. 2019). Mycotoxins are secondary metabolites of filamentous fungi which parasitize several crops throughout the food chain. Hundreds of fungal toxins have been identified; however, not all play key roles in food safety (Shephard 2008). The most concerning mycotoxins are produced by the *Aspergillus*, *Fusarium*, and *Penicillium* species which contaminate crops in the field and during storage. Among the mycotoxins produced by these fungal species, FB_1, aflatoxin B_1 and ochratoxin A are reported to be the most detrimental to mammals, inducing a range of toxic effects including hepatotoxicity, teratogenicity, immunosuppression, mutagenicity and carcinomas (Reddy et al. 2010). Several mycotoxicosis disease outbreaks have been reported in humans and animals upon consumption of food spoilt by mycotoxins (Peraica and Domijan 2001; Reddy and Raghavender 2007), with regulations on tolerable mycotoxin concentration consumptions established in at least 77 countries (Van Egmond, Schothorst, and Jonker 2007). Despite the health risks posed by mycotoxins, it is impossible to completely mitigate its contamination from feed as mycotoxins occur naturally thus it is important to regulate the consumption of contaminated commodities and understand their toxicities to establish and implement appropriate management strategies.

1.2.1.1 FB_1 structure and its role in sphingolipid metabolism

The chemical structure of FB_1 was discovered in 1988 and it was classified as a sphingosine analogue since it shares structural similarities with the sphingolipid components sphingosine and sphinganine (Chen, Wei, et al. 2021a; Gelderblom et al. 1988a). Structurally, FB_1 consists of a tricarballylic acid, a methyl and an amino group attached to a long-chain hydroxylated hydrocarbon backbone (Figure 1.1) (Stockmann-Juvala and Savolainen 2008). FB_1 can inhibit ceramide synthase thus altering sphingolipid metabolism leading to cytotoxicity and apoptosis (Chuturgoon, Phulukdaree, and Moodley 2015). Sphingolipids are present in all eukaryotic cells and mediate numerous cell functions (Stockmann-Juvala and Savolainen 2008). Sphingolipids are synthesized through *de novo* mechanisms, with the main step being the formation of sphinganine. The sphinganine N-acyltransferase enzyme then acylates sphinganine to ceramide and dihydroceramide. The sphingolipid turnover encompasses the hydrolysis of sphingolipids to ceramide and further to sphingosine (Stockmann-Juvala and Savolainen 2008). Inhibition of ceramide synthase by FB_1 results in decreased ceramide biosynthesis, accumulation of free sphingosine and sphinganine and decreased re-acylation of sphingosines liberated during sphingolipid turnover (Domijan 2012).

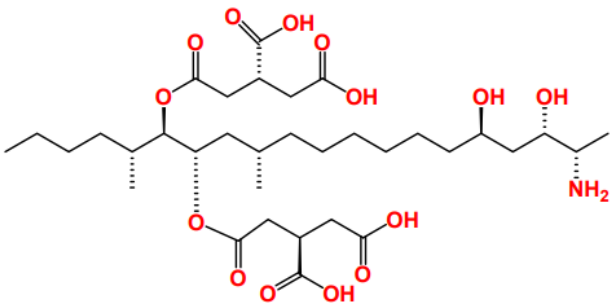

Figure 1.1: Chemical structure of FB_1 (Chen et al. 2023). FB_1: Fumonisin B_1

Animal, neuronal and glial cell studies have documented that FB_1 alters sphingolipid metabolism. In rats subjected to several subcutaneous FB_1 injections, the brain sphinganine and sphinganine to sphingosine ratio was enhanced with the highest FB_1 dose (Kwon, Schmued, and Slikker 1997). Another rat study showed that impaired sphingolipid metabolism from a single subcutaneous FB_1 dose was a result of direct action of FB_1 on the brain alternatively to sphingoid base transfer from the blood (Kwon, Sandberg, and Slikker 1997). FB_1 exposure to cultured hippocampal neurons resulted in decreased complex sphingolipid levels and ceramide synthase inhibition (Harel and Futerman 1993). In hippocampal neurons treated for 66 h with FB_1, a reduction in complex sphingolipid ganglioside, sphingomyelin, and glucosylceramide were observed (Schwarz et al. 1995). In primary rat cerebrum, sphingosine and sphinganine to sphingosine ratio was increased following 5 days of FB_1 exposure, while after 10 days, FB_1 decreased sphingosine levels (Kwon, Slikker, and Davies 2000). Following FB_1 treatment for 4 days in murine BV-2 microglial and murine N2A neuroblastoma and 8 days in primary astrocytes and primary cortical neurons, Osuchowski and Sharma (2005a) observed an increase in sphinganine along with a decrease in sphingosine levels. In cultured rat hippocampal neurons, treatment with FB_1 led to decreased synthesis and complex sphingolipid ganglioside levels and impaired axonal growth, suggesting that ceramide and sphingolipids are essential for optimal neuron development (Harel and Futerman 1993). In Caco-2 cells, FB_1 halted cellular folate uptake by its receptors by diminishing complex sphingolipids (Stevens and Tang 1997a). Additionally, disrupted folate intake by FB_1 was associated with mouse embryotoxicity and neural tube defects (Sadler et al. 2002).

1.2.1.2 FB_1 occurrence and its global impact

Unlike other mycotoxins, FB_1 is mainly produced by fungi in growing crops, prior to harvesting (Stoev 2013). Maize and maize products are more prone to FB_1 contamination, compared to crops such as rice, rye, wheat, oats and barley which are contaminated by FB_1 at lower levels (Chen, Wei, et al. 2021a). Post harvest, damp maize falls prey to FB_1 contamination due to poor storage and management

conditions (Carbas et al. 2021). FB_1 is also heat tolerant, therefore, humid storage environments contribute to the risk of FB_1 contamination. In maize kept at 27.29 to 32.14% humidity with temperatures of 28.97 to 32.14°C and pH 5.5, Fumonisins have shown great stability (Bryła et al. 2017). Therefore, the contamination of FB_1 in agricultural crops is more prevalent in temperate tropical regions of countries. FB_1 has been detected in maize globally with the mean daily FB_1 intake being estimated to range from 12 to 140 μg per person, depending on the geographical region. However, some individuals consume as much as 2,500 μg FB_1 per day (FAO/WHO. 2001).

In Hungary and Sardinia, high FB_1 concentrations of up to 300 mg/kg have been recorded in maize-based feed (Bottalico et al. 1995; Fazekas, Kis, and Hajdu 1996). The rate of FB_1 contamination in feeds from Nigeria, Brazil and Korea were documented as 93.3%, 99.1% and 97.1%, respectively (Vargas et al. 2001; Kim et al. 2013; Akinmusire et al. 2019). In developed countries, the maximum and mean FB_1 concentration was reported as 2.5 and 0.4 mg/kg feed in Italy and 0.4 and 0.2 mg/kg feed in Portugal (Gutleb et al. 2015; Almeida et al. 2011). Contrastingly in developing countries, the FB_1 maximum and mean concentrations were 53.9 and 8.2 mg/kg feed in South Africa, 42.1 and 14.8 mg/kg feed in Algeria and 143.0 and 9.1 mg/kg feed in Thailand (Mahdjoubi et al. 2020; Tansakul et al. 2013; Phoku et al. 2012b). In hot and humid provinces of South China, the rate of FB_1 contamination was up to 100% with the maximum contamination concentration resulting from the Guangxi Province of 71.1 mg/kg feed (Gong et al. 2009). Conversely, the dry northern areas of China had remarkably lower FB_1 contamination rates such as 19.2% in Ningxia, 24.5% in Gansu and 38.1% in Heilongjiang, with conforming maximum contamination concentrations of 2.2, 2.9 and 0.3 mg/kg feed respectively (Liu, Jiang, et al. 2017; Wei et al. 2013). In Iran, 50% of maize and 40.9% of rice samples presented FB_1 contamination with a mean level of 223.64 μg/g in maize and 21.59 μg/g in rice (Alizadeh et al. 2012).

In Bulgaria, the mean FB_1 quantity in pig/chicken farm feeds amounted to 5564.1 ± 584.4 μg/kg and 3254.5 ± 480.6 μg/kg in 2006 and 2007 (Stoev, Dutton, et al. 2010). In South Africa, the detected mean FB_1 content was 5289 ± 1034 μg/kg and 5021 ± 844 μg/kg in feed samples from pig farms in 2007 and 2008 (Stoev, Denev, et al. 2010). These farms were plagued with kidney injuries, which were likely caused by the exposure to high FB_1 concentrations. In Spanish pigs, a maximum of 3959 μg/kg of FB_1 was detected in feed samples (Arroyo-Manzanares et al. 2019). In 12 feed components of European fish, 3576 μg/kg of FB_1 was reported (Pietsch 2020). Chinese feed tests revealed that approximately 96% of feed was contaminated with a maximum of 6568 μg/kg FB_1 (Wang et al. 2013). These findings highlight the seriousness of herd health and economic development. Additionally, maize and maize products are major components of pet feeds, making them vulnerable to FB_1 contamination which could lead to chronic damage in pets (Witaszak et al. 2020).

In humans, the influence of FB_1 is mostly dependent on the dietary habits in the area they reside. In 3 Tanzanian villages, where the food of children between 12 and 22 months is predominantly maize-based, FB_1 was recorded in 96% of urine samples, with the village of Kigwa showing the highest level of urinary FB_1, followed by the Nyabula and Kikelelwa villages (Shirima et al. 2013). The differences in FB_1 contamination in these 3 Tanzanian villages were attributed to the different maize intakes in the respective areas. Maize is inexpensive in Brazil therefore, it is primarily used in industrial beer; however, beer in which maize is the main constituent is also contaminated with FB_1 (Peters et al. 2017). During the 1995 unseasonal rains in 27 villages of the Indian Deccan plateau, ingestion of damaged sorghum and maize resulted in an outbreak characterized by diarrhoea, borborygmi and abdominal pain (Bhat et al. 1997). Epidemiological investigation of the food from affected households contained high contents of FB_1 and *Fusarium* spp. compared to feed from unaffected households. Maize forms part of the staple diets of regions such as the Transkei in South Africa and Linxian in China. These regions show higher prevalences of primary liver and oesophageal cancers which are associated with exposure to dietary FB_1 (Domijan 2012). Similar trends were also seen in populations of Brazil, the United States, Kenya, Zimbabwe and Italy, where high maize intake contributed to the risk of oesophageal cancer development (Stockmann-Juvala and Savolainen 2008). Additionally, a prospective study revealed no significant association between the risk of oesophageal squamous cell carcinoma and serum sphingolipid levels, even though increased sphingolipid levels serve as biomarkers of FB_1 exposure (Abnet et al. 2001). A link between neural tube defects and FB_1-contaminated food has also been established. In parts of South Africa, Mexico, Texas and Guatemala, FB_1 has been implicated in the development of neural tube defects (Domijan 2012). Exposure to FB_1 from maize tortillas, consumed by Mexican American women in Texas before pregnancy and during the first trimester, was associated with increased occurrence of neural tube defects in children (Missmer et al. 2006). Epidemiological and clinical studies have reported folate deficiency as a major contributor to neural tube defects (Werler, Louik, and Mitchell 1999; Blom et al. 2006). Therefore, FB_1-induced sphingolipid metabolism disruption may impact folate uptake and subsequently increase the risk of neural tube defects (Marasas et al. 2004). The aforementioned data highlights the global crisis of FB_1 contamination which poses health risks to humans and animals.

1.2.1.3 FB_1 as a neurotoxin

Over the past few decades, extensive research has been conducted on FB_1 toxicity; however, minimal studies have explored its potential in neurodegeneration therefore, the underlying mechanisms of FB_1 neurotoxicity are not fully understood. ELEM is a neurotoxic condition linked to FB_1-contaminated feed which has a high mortality rate. ELEM is characterized by focal necrosis of cerebral white matter with the development of nervous symptoms such as depression, aimless circling, paresis, ataxia, head pressing and blindness (Wilson et al. 1990). In experimental horses, donkeys and ponies, FB_1 was

reported to be the causative factor of ELEM, upon being fed naturally contaminated Fumonisin feed or administered with pure FB_1 (Wilson and Maronpot 1971; Ross et al. 1993). Horses injected with FB_1 presented characteristic ELEM lesions in the brain (Marasas, Kellerman, et al. 1988), while horses orally fed FB_1 also presented the same effect (Kellerman et al. 1990). In 45 feed samples of the USA, FB_1 levels above 10 ppm were reported in samples associated with ELEM compared to the controls which contained 8 ppm FB_1, suggesting a threshold for FB_1-induced ELEM of 10 mg/kg (Ross et al. 1991). Apart from dietary contamination, factors such as individual susceptibility, previous exposure and length of exposure to FB_1 also contribute to the development of ELEM (Voss, Smith, and Haschek 2007a). Small focal haemorrhages in cerebral white matter, which is similar to the CNS changes in ELEM, were detected in rabbits gavaged with purified FB_1 (Bucci, Hansen, and LaBorde 1996). In adult female Fischer rats, hyperaemia was observed in the brain, however, these rats were fed FB_1 in combination with another maize contaminant (Pepeljnjak, Petrik, and Klarić 2005). Developing rats displayed significantly reduced brain and body weight which led to hypomyelination, following multiple subcutaneous FB_1 dosages (Kwon, Schmued, and Slikker 1997). In developing rats, FB_1 was also reported to cross the BBB upon single subcutaneous FB_1 dosage; however, high-dose FB_1 was only present at low levels in the brain whereas at low-dose treatment no FB_1 was detected in the brain (Kwon, Sandberg, and Slikker 1997). The authors suggested that this observation could be a result of low lipid solubility and the large molecular weight of FB_1. FB_1 also crossed the BBB in carps leading to necrosis of nerve cells, degeneration and brain oedema (Kovacić et al. 2009). In rats fed FB_1 for 42 days (1.0 and 3.0 mg/kg body weight), cell body was significantly reduced in neuronal populations (Sousa et al. 2014). Another rat study showed a significant reduction in nerve conduction velocity after receiving FB_1 for 2 weeks (Banczerowski-Pelyhe et al. 2002). In fish, reduced body-weight gain, brain oedema and increased degenerative cells were observed upon receiving FB_1 for 42 days in fish feed (Kovacić et al. 2009). In adult pigs, exposure to 5.0 mg/kg body weight FB_1 for 6 months resulted in reduced regional brain and hypophyseal acetylcholine hydrolase functions (Gbore 2010). Pigs fed FB_1 also showed a vacuolated cerebral cortex, with some visible glial and neuronal cytolysis under the meninges which also showed signs of congestion and oedema (Stoev et al. 2012). Beef cattle presenting abrupt blindness upon grazing on *Fusarium* contaminated maize displayed optic acute myelin oedema and nerve degeneration (Sandmeyer et al. 2015).

Several *in vitro* cell culture studies have demonstrated that FB_1 disrupts axonal outgrowing and myelination before FB_1-induced cell death. However, it remains unclear which brain cell type or tissue is more sensitive and the likely target of the degenerative effect of FB_1. Although FB_1 inhibited axonal growth in primary rat hippocampal neurons, FB_1 was reported as non-toxic in hippocampal neuron cultures after a 48 h treatment (Harel and Futerman 1993). Continued exposure to FB_1 for 5 days showed no FB_1-induced changes in DNA or protein levels in glial cultures (Kwon, Slikker, and Davies 2000).

This trend was also observed by Galvano, Campisi, et al. (2002) in FB_1 treated primary rat astrocytes which showed no change in cell viability. While cytotoxicity of FB_1 in glial cells may be limited, FB_1-induced cell death seems to be frequent in glial cells. Exposure to 9 $\mu mol/L^{-1}$ FB_1 for 24 h reduced viability by 65% in rat C6 glioblastoma cells (Mobio et al. 2000). In human U118MG glioblastoma cells, 100 $\mu mol/L^{-1}$ FB_1 treatment at 72 h and 144 h reduced cell viability by 73% compared to the lower concentrations which showed no cell death (Stockmann-Juvala et al. 2004b). In human SH-SY5Y neuroblastoma and mouse GT1-7 hypothalamic cells, 48 h exposure to 100 $\mu mol/L^{-1}$ of FB_1 reduced cell viability to approximately 80% while longer exposure times of 72 h and 144 h further decreased viability to 70% (Stockmann-Juvala et al. 2004c). Furthermore, it was suggested that neural cells are more sensitive to FB_1 than glial cells; however, in a study by Osuchowski and Sharma (2005a) murine BV-2 microglial cells showed cytotoxicity within 4 days of treatment with 20 $\mu mol/L^{-1}$ FB_1 compared to primary astrocytes which showed cytotoxicity after 8 days of treatment with 50 $\mu mol/L^{-1}$ FB_1. In human SH-SY5Y neuroblastoma cells and primary rat astrocytes, low cytotoxicity was reported in cells with neural origins upon FB_1 treatment (Domijan and Abramov 2011). Furthermore, treatment of 100 $\mu mol/L^{-1}$ FB_1 for 48 h showed cytotoxicity in human SH-SY5Y neuroblastoma cells (Stockmann-Juvala et al. 2004c), while treatment at 200 $\mu mol/L^{-1}$ FB_1 for 24 h showed no cytotoxicity (Domijan and Abramov 2011). In humans, FB_1 has reportedly caused embryonic neural tube defects which prevent neurodevelopment (Marasas et al. 2004). This phenomenon was also reported in pregnant LM/BC mice which displayed a 79% likelihood of developing neural tube defects, while the neural tube defect was avertible by folic acid supplementation (Gelineau-van Waes et al. 2005b). Cell death was also observed in mouse primary astrocytes following 8 days of incubation with 50 $\mu mol/L^{-1}$ FB_1 (Osuchowski and Sharma 2005a), whereas incubation for 6 days showed no viability changes (Galvano, Campisi, et al. 2002). FB_1 cytotoxicity is distinctly dependent on various factors such as length of exposure, concentration, cell type and species, therefore, it is difficult to compare *in vitro* results of FB_1 cytotoxicity.

1.2.2 Inflammation

The inflammatory response involves the synchronized activation of signalling pathways that regulate the expressions of inflammatory mediators in both resident tissue cells and inflammatory cells from the bloodstream (Lawrence 2009). Inflammation is a common underlying factor in numerous chronic diseases, including cardiovascular disorders, bowel diseases, diabetes, arthritis, and cancer (Libby 2007). While the processes of the inflammatory response depends on the specific nature and location of the initial stimulus, they all share a common mechanism, which can be summarized as follows: 1) recognition of harmful stimuli by cell surface pattern receptors; 2) activation of inflammatory pathways; 3) release of inflammatory markers; and 4) recruitment of inflammatory cells (Chen et al. 2018).

The ancient Roman encyclopaedist Celsus defined inflammation by the presence of "rubor, calor, dolor, tumor," meaning redness, heat, pain, and swelling. Today, scientists understand inflammation as the activation of the innate immune system in response to irritants or a loss of homeostasis due to factors like stress, obesity, and aging (Porter and O'Connor 2022). Acute inflammation arises when tissue injury, pathogens, or harmful stimuli are detected, prompting leukocytes to migrate to the affected area to eliminate the stimuli and to repair damage. In contrast, chronic inflammation is a prolonged and maladaptive response caused by various factors, including chronic diseases, aging, smoking, high-fat diets and obesity (Porter and O'Connor 2022). Chronic inflammation also contributes to chronic conditions like allergies, arthritis, and autoimmune diseases, which frequently coexist with depression (Porter and O'Connor 2022).

Toll-like receptors (TLRs) on the plasma membrane of innate immune cells detect invading pathogens or signals from damaged cells. TLRs are a type of pattern recognition receptor (PRR) that recognize and bind to pathogen-associated molecular patterns (PAMPs) (Jiménez-Dalmaroni, Gerswhin, and Adamopoulos 2016), such as lipopolysaccharides found in the cell walls of gram-negative bacteria as well as damage-associated molecular patterns (DAMPs) in a process known as "sterile inflammation." Activation of TLRs triggers an intracellular signalling cascade, activating the transcription factor NF-κB, which leads to the upregulation of pro-inflammatory mediators, including cytokines, chemokines, and cellular adhesion molecules (Shih, Wang, and Yang 2015), along with the induction of ROS (Lingappan 2018). Among these mediators, macrophage-derived TNF-α, IL-1β, IL-6, and IL-10 are particularly notable for their roles in regulating the immune system and their broader effects on the body (Abdulkhaleq et al. 2018).

1.2.2.1 Pro-inflammatory cytokines

The IL-1 family comprises 11 distinct ligands and antagonistic receptors, which trigger local and systemic inflammation or promote anti-inflammatory responses, independently (Dinarello 2011). Their primary sources are T and B lymphocytes, mononuclear phagocytes, fibroblasts, and keratinocytes (Shaikh 2011). IL-1β is a key pro-inflammatory cytokine that regulates the host's innate immune response. Its inflammatory function has evolved to combat microbial infections and facilitate tissue repair (Dinarello 2011) (Figure 1.3). The recognition of homeostatic disturbances prompts the processing of IL-1β, enabling it to initiate inflammatory activities. However, chronic overproduction or sustained exposure to IL-1β is known to contribute to the pathogenesis of diseases such as inflammatory bowel disease, gout, rheumatoid arthritis, and type 2 diabetes. Pharmacological inhibition of IL-1β signalling can directly or indirectly limit the progression of these diseases (Dinarello, Simon, and van

der Meer 2012). IL-1β also plays a central role in neuroinflammation, being implicated in various CNS disorders such as traumatic brain injury, stroke, Alzheimer's disease, and multiple sclerosis (Mendiola and Cardona 2018). As a pleiotropic cytokine, IL-1β activates microglia and astrocytes, leading to the synthesis of additional pro-inflammatory and chemotactic mediators in the CNS (Shaftel, Griffin, and O'Banion 2008).

IL-6 is produced by various cell types, including mononuclear phagocytes, T cells, and fibroblasts (Toi, Harris, and Bicknell 1991). In addition to stimulating the liver to synthesize acute phase proteins, IL-6 serves as a growth factor for mature B cells and promotes their final maturation into antibody-secreting plasma cells. It also plays a role in T cell activation and differentiation and is involved in the induction of IL-2 and IL-2 receptor expression (Shaikh 2011). Elevated levels of IL-6 have been observed in numerous chronic inflammatory and autoimmune conditions, such as thyroiditis, type I diabetes, rheumatoid arthritis, systemic sclerosis, mesangial proliferative glomerulonephritis, and psoriasis, as well as in neoplasms like cardiac myxoma, renal cell carcinoma, multiple myeloma, lymphoma, and leukaemia (Hirano et al. 1990; Shaikh 2011). IL-6 is frequently used as a marker for the systemic activation of pro-inflammatory cytokines (Hirano 1992) (Figure 1.3). While it is a strong inducer of the acute-phase protein response, IL-6 also exhibits anti-inflammatory properties (Tan et al. 1990).

TNF-α and β are cytokines that bind to shared receptors on the surface of target cells, exhibiting several common biological functions (Dalton et al. 1993) (Figure 1.3). TNF-α is produced by activated macrophages, monocytes, fibroblasts, mast cells, and certain T and natural killer cells (Hart et al. 1989; Jain-Vora et al. 1998). TNF-α and IL-1 share many pro-inflammatory characteristics. Like IL-1, TNF-α can induce fever either directly by stimulating the synthesis of prostaglandin E2 in the vascular endothelium of the hypothalamus or indirectly by promoting the release of IL-1 (Shaikh 2011). Additionally, TNF-α shares an important inflammatory role with IL-6 and IL-11, specifically in inducing the liver to produce acute phase reactant proteins. TNF-α and IL-1 also have secondary inflammatory effects by stimulating IL-6 synthesis in various cell types (Shaikh 2011). Thereafter, IL-6 mediates its own effects and those of TNF-α and IL-1 in inducing fever and the acute phase response, thus preserving the inflammatory response via a cascade of cytokines with overlapping functions (Shaikh 2011). During sepsis caused by gram-negative bacteria, lipopolysaccharides (endotoxins) released from these bacteria trigger widespread production of TNF-α (followed by IL-1 and IL-6) by macrophages. The systemic release of these cytokines is responsible for the fever and hypotension characteristic of septic shock (Shaikh 2011). Furthermore, chronic production of TNF is thought to

contribute to metabolic changes leading to cachexia associated with chronic parasitic infections and certain cancers (Shaikh 2011).

NF-κB is a key regulator of pro-inflammatory gene expression, promoting the transcription of various pro-inflammatory cytokines, chemokines, adhesion molecules, matrix metalloproteinases, COX-2, and inducible nitric oxide synthase (Baeuerle and Baichwal 1997; Tak and Firestein 2001) (Figure 1.3). NF-κB is highly activated in inflammatory sites across various diseases, including inflammatory bowel disease, rheumatoid arthritis, multiple sclerosis, psoriasis, and asthma. This NF-κB activation is linked to the recruitment of inflammatory cells and the production of pro-inflammatory mediators such as IL-1, IL-6, IL-8, and TNF (Li and Verma 2002). It remains unclear whether the rise in pro-inflammatory cytokines is a cause or a consequence of NF-κB activation. While genetic alterations in NF-κB have not been directly associated with these diseases, persistent NF-κB activity may result from defects in the regulatory mechanisms governing its activation (Li and Verma 2002). Targeted inhibition of NF-κB activity has proven effective in managing inflammatory diseases in various animal models. For instance, blocking NF-κB activity through the overexpression of inhibitor kappa B-α reduces both the inflammatory response and tissue damage in rheumatoid synovium (Bondeson et al. 1999). Additionally, the use of NF-κB decoys has shown effectiveness in animal models of rheumatoid arthritis (Miagkov et al. 1998).

1.2.2.2 The anti-inflammatory cytokine IL-10

IL-10 was first identified by Fiorentino, Bond, and Mosmann (1989) as a novel immune mediator produced by T helper 2 (TH2) cells, which inhibit IL-2 and IFN-γ synthesis in TH1 cells. Peripheral IL-10 is secreted by various innate immune cells, such as natural killer cells, mast cells, dendritic cells, macrophages, eosinophils, and neutrophils, including adaptive immune cells like TH1, TH2, TH17, regulatory T cells, and B cell subsets (Moore et al. 2001; Nouël et al. 2014; Saraiva and O'Garra 2010a). IL-10 is expressed by microglia, astrocytes, and neurons within the CNS. Upon activation, IL-10 receptors suppress the release of pro-inflammatory cytokines such as TNF, IL-1β, IL-6, IL-8, IL-12, and IL-23, while increasing the release of anti-inflammatory mediators like the IL-1 receptor antagonist and soluble TNF receptors from innate immune cells (Kwilasz et al. 2015) (Figure 1.3). Moreover, activated IL-10 receptors inhibit both the proliferation and cytokine production of CD4+ T cells, affecting TH1 synthesis of IL-2 and IFN-γ as well as TH2 synthesis of IL-4 and IL-5 (Sabat et al. 2010).

IL-10 is found in concentrations sufficient to have a physiological effect on the host's response to systemic inflammation. Studies have shown that patients who express high IL-10 and lower TNF-α levels are more likely to succumb to meningococcaemia and other community-acquired infections (Dickensheets et al. 1997; Joyce et al. 1994; Marchant et al. 1994). Inadequate IL-10 responses following systemic injury can also have negative outcomes. For example, low lung IL-10 levels in patients with acute lung injury increase the likelihood of developing acute respiratory distress syndrome. In animal models, administering IL-10 during endotoxemia improves survival rates (Shaikh 2011). Humans given IL-10 after endotoxin exposure experience fewer systemic symptoms, reduced neutrophil activity, and decreased cytokine production compared to those treated with a placebo (van Der Poll et al. 1997). Additionally, mice with a genetic deletion of the IL-10 gene are more prone to endotoxin-induced shock than normal mice (Lehmann et al. 1995). IL-10 generally protects against systemic inflammation after toxin-induced injury but increases vulnerability to fatal infections in various experimental studies (Westendorp et al. 1997; van Dissel et al. 1998). This should be considered when using anti-inflammatory cytokines in clinical settings. IL-10 knockout mice also spontaneously develop chronic inflammatory enteritis resembling human inflammatory bowel disease, suggesting that endogenous IL-10 levels play a crucial role in controlling inflammation in response to gut-associated bacteria (Pajkrt et al. 1997; Dai, Köhler, and Brombacher 1997; Greenberger et al. 1995). Consequently, IL-10 is being explored in clinical trials as a potential anti-inflammatory treatment for inflammatory bowel disease and other conditions.

IL-10 has the potential to act as a neuroprotective agent. By inhibiting the production of proinflammatory mediators in microglia, IL-10 prevents the activation of astrocytes and directly suppresses the binding of p65 NF-κB (Balasingam and Yong 1996). This reduction in NF-κB activity leads to increased expression of excitatory amino acid transporter-2, which helps prevent the harmful accumulation of synaptic glutamate (Bachis et al. 2001; Kim et al. 2011). Additionally, certain neuronal populations express functional IL-10 receptors which enhance neuronal survival, when activated (Boyd et al. 2003). For instance, IL-10 activates the PI3K-AKT pathway downstream of IL-10R1, which protects neurons from glutamate-induced excitotoxicity as well as hypoxic and ischemic injury by stimulating the transcription of survival genes and controlling intracellular Ca^{2+} levels (Sharma et al. 2011; Tukhovskaya et al. 2014; Zhou et al. 2009). Moreover, IL-10 prevents glutamate-induced neuronal apoptosis via the restoration of suppressed anti-apoptotic factors like Bcl-2 and Bcl-xl and by reducing the expression of caspase-3 (Zhou et al. 2009; Bachis et al. 2001).

1.2.2.3 Neuroinflammation

The CNS is undoubtedly the most complex system we encounter in daily life. CNS disorders disrupt and often deteriorate the structure and function of this intricate organ (Ransohoff 2016). Neurodegeneration is a frequent feature of these disorders and involves the gradual loss of previously established CNS functions such as learning, judgment, mobility, memory, and coordination (Ransohoff 2016). Neurodegenerative diseases typically manifest later in life, making age a crucial factor in the development of major neurodegenerative disorders.

The endothelial layer, known as the BBB, and the transport of molecules across it are critical to understanding how peripheral inflammation can lead to prolonged and harmful neuroinflammation. Initially, it was believed that inflammatory cytokines and other proteins were too large to cross the blood into the brain. However, over the past two decades, various transport mechanisms have been identified. Active transport systems within the BBB have been found to facilitate the entry of cytokines like TNF and IL into the brain (Gutierrez, Banks, and Kastin 1993). Certain regions, such as the circumventricular organs, which have an incomplete barrier at the blood–brain interface, are areas where cytokine transport is concentrated (Quan et al. 1999). Cytokines such as TNF-α, IL-6, and IL-1β also negatively affect the integrity of the BBB, increasing its permeability and allowing leukocytes to enter the brain (Laflamme, Lacroix, and Rivest 1999; Terrando et al. 2011). Cytokine levels are known to influence BBB permeability by altering the resistance of tight junctions in endothelial cells within brain vasculature (Wong, Dorovini-Zis, and Vincent 2004). Elevated cytokine levels upregulate inflammatory cytokines and COX-2 transcription in the endothelium (De Vries et al. 1996). Damage to key tight junction proteins, such as occludin, can further increase permeability, possibly by disrupting its interaction with the cytoskeleton.

Microglial cells are key players in neuroinflammation. When exposed to cytokines and other signalling molecules during acute inflammation, microglia transition from an inactive, ramified state to an activated, phagocytic form, releasing pro-inflammatory mediators. During chronic neuroinflammation, these cells can stay activated for prolonged periods, continuously releasing large amounts of cytokines and neurotoxic molecules, which contribute to ongoing neurodegeneration (Liu and Hong 2003). Astrocytes, another type of glial cell, release pro-inflammatory signalling molecules such as TNF-α when activated in the cortex and midbrain (Kipp et al. 2008). These cells also play crucial roles in synaptic function and regulation. While microglia are responsible for a much larger release of inflammatory cytokines (Liu et al. 2012), the combined response from both types of glial cells may

significantly contribute to the neurodegeneration observed in dementia (Cagnin et al. 2001; Xing, Bachstetter, and Van Eldik 2011). It is well-established that there is dynamic communication between BBB endothelial cells, glia, and neurons (Abbott, Rönnbäck, and Hansson 2006), suggesting that a neuroinflammatory response in one cell type will likely affect others.

The COX enzyme converts arachidonic acid into eicosanoid groups, including prostaglandins and thromboxane's, which play various roles in inflammation (Hamberg and Samuelsson 1973) (Figure 1.3). The pathways of its two primary isoforms, COX-1 and COX-2, are increasingly linked to neuroinflammation and neurodegeneration, with COX inhibitors, such as non-steroidal anti-inflammatory drugs, showing several therapeutic potentials. These isoforms have distinct functions in normal physiology and disease states. COX-1 is expressed in microglia, where it contributes to prostaglandin synthesis (Hoozemans et al. 2001). Activation of these cells may result in an overproduction of prostaglandins. Several elements of the COX-1 pathway promote inflammation, leading to harmful neuroinflammation and cognitive decline (Matousek et al. 2010). Additionally, pathological connections have been identified in conditions such as traumatic brain injury (Schwab et al. 2002) and neurodegenerative diseases like Alzheimer's disease (Sung et al. 2004). COX-2 expression is mostly seen in neurons and is linked to synaptic function and memory formation (Wang et al. 2005; Cowley, Fahey, and O'Mara 2008). Notably, COX-2 demonstrates anti-inflammatory properties (Gilroy et al. 1999; Aid, Langenbach, and Bosetti 2008), underscoring the complexities of eicosanoid signalling in both neuroprotection and neurodegeneration, as well as the challenges in developing therapeutic approaches. Cytokine signalling also plays a role in these pathways; for instance, IL-1β-induced activation of MAPK enhances *COX-2* gene expression (Lacroix and Rivest 1998). Additionally, the COX pathway itself promotes the production of IL-6, illustrating one of the many positive feedback loops in the systemic inflammatory response (Anderson et al. 1996).

1.2.2.4 FB_1 immunotoxicity

Generally, mycotoxin immunotoxicity is described as the detrimental effects imposed on systemic and local immunity caused from exposure to mycotoxins. Mycotoxins can repress the immune response resulting in reduced resistance to tumours and infections by the host (Sun et al. 2022). Alternatively, mycotoxins can trigger the immune response prompting autoimmune effector cells and autoantibody production. This could induce a pathological immune response against tissues, further damaging tissue structures (Sun et al. 2022). The damage caused to the body by FB_1 exposure, leads to the accumulation of toxic products which perturb optimal immune functioning, resulting in immunotoxicity (Zhu and Wang 2022). In chicks fed FB_1 for 4 weeks, the response of the secondary antibody to the vaccine was significantly reduced suggesting that FB_1 caused immunosuppression in chicks (Li et al. 1999). Stoev et al. (2012) also reported a significant decrease in antibody titre, when pig feed contained 10 mg/kg

FB_1, which altered humoral immune responses during vaccination. In avian lymphocyte cells, FB_1 hindered differentiation and proliferation ultimately affecting immune function (Keck and Bodine 2006). In porcine lymphocytes FB_1 decreased cellular activity to 50% of its original level upon exposure for 72 h at a concentration of 101.15 µg/mL (Kachlek et al. 2017), while in human lymphocytes, survival dropped by 3.5% and 11.3% upon 24 h exposure to 5 and 20 µg/mL FB_1, respectively (Mwanza et al. 2009). Broiler chickens displayed reduced macrophage chemotaxis and phagocytosis when administered 15 mg/kg of FB_1 (Cheng, Ding, and Chang 2006). Decreased macrophage capacity in birds results in metabolic and immune system disorders and aggravates chlamydia severity (Grenier et al. 2016). Reduced macrophage capacity also exacerbated pathogen infection in Mycoplasma pneumoniae infected swine fed FB_1 (Pósa et al. 2013). IL-1, IL-2, IFN-α and IFN-γ expressions were increased in FB_1 (15 mg/kg) fed rats whereas mRNA levels of *IL-4* and *IL-10* were also increased in rat spleen mononuclear cells after being fed 100 ppm (parts per million) FB_1 (Wang, Wu, et al. 2016). Studies in FB_1 gavaged pigs revealed an increase in *IL-8*, *IL-18* and *IFN-γ* expressions in bronchoalveolar lavage fluid (Halloy et al. 2005). In human dendritic cells, IFN-γ expression was also enhanced along with its related chemokine CXCL9, when exposed to FB_1 (Stockmann-Juvala, Alenius, and Savolainen 2008). *In vitro,* lipopolysaccharide activation in the mouse macrophage cell line and mice peritoneal macrophage treated with FB_1 promoted the expression of TNF-α (Dugyala et al. 1998). A dose-dependent increase in TNF-α and IL-1β expression in human colon adenocarcinoma and gastric epithelial cells suggests that FB_1 can stimulate cytokine release in gastrointestinal cells and subsequently trigger inflammation (Mahmoodi et al. 2012). In porcine kidney cells, *TNF-α* mRNA was elevated, establishing TNF-α as a key toxicity marker (Chen et al. 2020). In porcine liver and jejunum, significantly enhanced NF-κB and IL-8 expressions led to apoptosis (Régnier et al. 2017).

Contrastingly, FB_1 also reduced relative bursal weight and cytokine expressions of IL-1β, IL-2, IFN-α and IFN-γ (Cheng, Ding, and Chang 2006), whereas continuous exposure of FB_1 in the spleen significantly downregulated *IL-4* but increased IFN-γ after 28 days (Taranu et al. 2005). 7 days of FB_1 exposure in piglets also led to reduced levels of IL-8 protein and gene expressions (Bouhet et al. 2006). Additionally, researchers have also established a link between cellular immunity and sphingolipid metabolism destruction and its products which can alter the expression of immune-related receptors (Dresden-Osborne and Noblet 2002). For instance, FB_1 hinders the immune response by altering CD3, CD4 and CD8 expressions on cell surfaces via sphingolipid metabolism (Blank et al. 2005). In BALB/c mice thymus and spleen cells, $CD4^+$ and $CD8^+$ contents were reduced, and primary lymphocytes were affected by exogenous sphingosine (Johnson and Sharma 2001). *In vitro* exposure of FB_1 to thymic and splenic cells induced cytotoxicity and brought significant changes to cell surface receptors CD3, CD4 and CD8 (Johnson and Sharma 2001; Blank et al. 2005).

1.2.3 Brain-derived neurotrophic factor (BDNF) expression

Mature BDNF is recognized as the biologically active neurotrophin form, exhibiting a strong affinity for the tropomyosin-related kinase B (TrkB) receptor (Klein et al. 1991; Mizui et al. 2014). Both BDNF and TrkB are found in presynaptic axon terminals and postsynaptic dendritic compartments of neurons, allowing for bidirectional release and activity. When mature BDNF, or other neurotrophins with a lower affinity for TrkB, such as neurotrophin-4 and neurotrophin-3, bind to the extracellular domain of the TrkB receptor, the intracellular domains of the receptor dimerize and auto-phosphorylate at one of three tyrosine residues (Porter and O'Connor 2022). Phosphorylation at each of these residues triggers a unique signalling cascade: Ras-PI3K-Akt, Ras-MAP kinase-Erk, or phospholipase Cγ. These pathways activate transcription factors like CREB, leading to cell proliferation, survival, synaptogenesis, and memory formation (Porter and O'Connor 2022) (Figure 1.2). As is typical for neurotrophic factors, BDNF plays a crucial role in the development and differentiation of new neurons (Alderson et al. 1990; Knüsel and Hefti 1991), promotes long-term potentiation (Korte et al. 1995), and supports neuron survival (Grothe and Unsicker 1987; Bathina and Das 2015). BDNF is widely expressed in both the developing and mature CNS as well as in various peripheral tissues, including muscle, liver, and adipose tissue (Cassiman et al. 2001; Mousavi and Jasmin 2006; Ukropec et al. 2008). Reports often indicate regional variations in BDNF mRNA levels and protein concentrations within the CNS, which may be influenced by regulatory mechanisms, mRNA decay (Malter 2001), or the anterograde transport of BDNF (Altar et al. 1997).

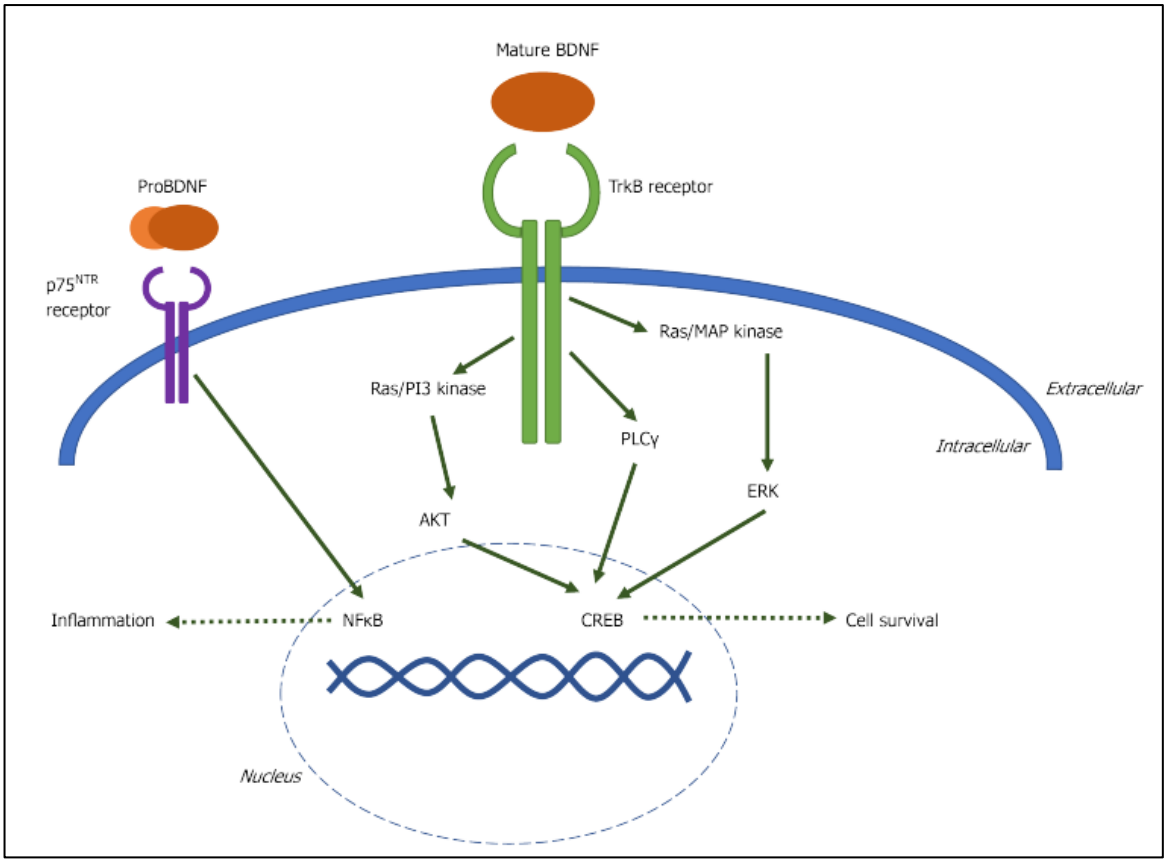

Figure 1.2: BDNF signalling cascade. Upon activation of TrkB by mature BDNF, receptor intracellular domains dimerize and auto-phosphorylate 1 of the 3 tyrosine residues. This triggers a signalling cascade that activates CREB. Contrastingly, proBDNF binds to P75NTR, activating NF-κB, resulting in inflammation and apoptosis (Porter and O'Connor 2022). BDNF: brain-derived neurotrophic factor; TrkB: Tropomyosin-related kinase B; CREB: cAMP response element-binding protein; P75NTR: p75 neurotrophin receptor; NF-κB: Nuclear factor-kappa B

In previous years, studies on BDNF focused mainly on its role in energy homeostasis. An *in vivo* study of BDNF and neuronal plasticity by Lapchak and Hefti (1992) revealed that chronic intraventricular administration of BDNF prevented weight gain. Since then, numerous studies have demonstrated that central administration of BDNF suppresses appetite, promotes weight loss, increases locomotor activity, and raises resting metabolic rate (Pelleymounter, Cullen, and Wellman 1995; Naert et al. 2006). An obese phenotype is seen in BDNF-conditional knockout mice, where BDNF is deleted after birth and restricted to the brain (Rios et al. 2001). In addition to its central effects, BDNF also has peripheral actions that influence glucose metabolism, food intake (Yamanaka et al. 2008) and energy expenditure (Yamanaka et al. 2007). Both centrally and peripherally administered BDNF lowers blood glucose and enhances energy expenditure in animal models of type 2 diabetes (Nakagawa et al. 2000). The combined effects of central and peripheral BDNF are evident in cases where BDNF is globally reduced, as seen

in rodents and humans with gene haploinsufficiency, leading to obesity and hyperphagia (Gray et al. 2006).

1.2.3.1 Neurogenesis and neuroprotection of BDNF

Energy balance has been shown to influence BDNF levels and neurogenesis in the hippocampus. Dietary restriction increases neurogenesis in the adult mouse hippocampus, a process linked to elevated BDNF expression, although this effect is absent in BDNF heterozygous mice (Lee, Seroogy, and Mattson 2002). In Huntington mutant mice, where BDNF expression is reduced, dietary restriction normalizes BDNF levels in the hippocampus, striatum, and cerebral cortex to those found in wild-type, *ad libitum*-fed mice, and helps reverse obesity, abnormal locomotor activity, and hyperphagia (Duan et al. 2003). Conversely, a diet high in saturated fats reduces BDNF levels and impairs cognitive function (Molteni et al. 2002). Environments rich in stimulation and exercise boost BDNF expression in the hippocampus and enhance learning (Olson et al. 2006). On the other hand, studies in rats have shown that consuming a high-fat diet, particularly one rich in saturated fats, decreases BDNF levels and adult hippocampal neurogenesis (Park et al. 2010). This inhibition of neurogenesis was linked to elevated malondialdehyde (MDA) levels, a marker of lipid peroxidation, and direct administration of MDA was found to suppress neurogenesis (Park et al. 2010). Additionally, maternal consumption of a high-fat diet was shown to reduce hippocampal BDNF levels and impair dendritic arborization in the hippocampal neurons of offspring (Park et al. 2010).

During development, neurotrophic factors are essential for neuronal survival as they inhibit apoptosis in developing neurons (Wyllie, Kerr, and Currie 1980; Bathina and Das 2015). BDNF is particularly neuroprotective in the hippocampus, especially against ischemic damage (Beck et al. 1994; Chen et al. 2013). BDNF reduces glutamate-induced apoptotic cell death by acting upstream of caspase-3-like enzymes and increasing the expression of the antiapoptotic protein Bcl-2 (Almeida et al. 2005). BDNF is not always protective; in some cultured hippocampal and cortical neurons, it can be toxic (Friedman 2000). While TrkB receptor activation enhances long-term potentiation and neuronal survival, activation of the p75NTR receptor can lead to apoptosis and long-term depression (Woo et al. 2005; Barrett 2000). BDNF has been shown to protect against oxidative stress by preventing the buildup of peroxides and increasing antioxidant enzymes in hippocampal neurons (Mattson et al. 1995). In rats, local application of BDNF to the spinal cord reduced lipid peroxidation and decreased oxidative stress, possibly due to reduced microglial activation (Joosten and Houweling 2004). In neurodegenerative diseases like Huntington's and Alzheimer's, low BDNF levels contribute to neuronal degeneration

(Hock et al. 2000). Tg2576 mice fed a high-fat diet developed obesity and insulin resistance due to hyperphagia, and this abnormal feeding behaviour was linked to increased amyloid plaque formation and reduced hypothalamic BDNF levels (Kohjima, Sun, and Chan 2010). Oxidative stress plays an early role in Alzheimer's disease (Keller et al. 2005). Vitamin E, an antioxidant, can mitigate oxidative stress, and its supplementation has been shown to counteract high-fat diet-induced reductions in BDNF, suggesting that BDNF levels are responsive to oxidative stress (Wu, Ying, and Gomez-Pinilla 2004).

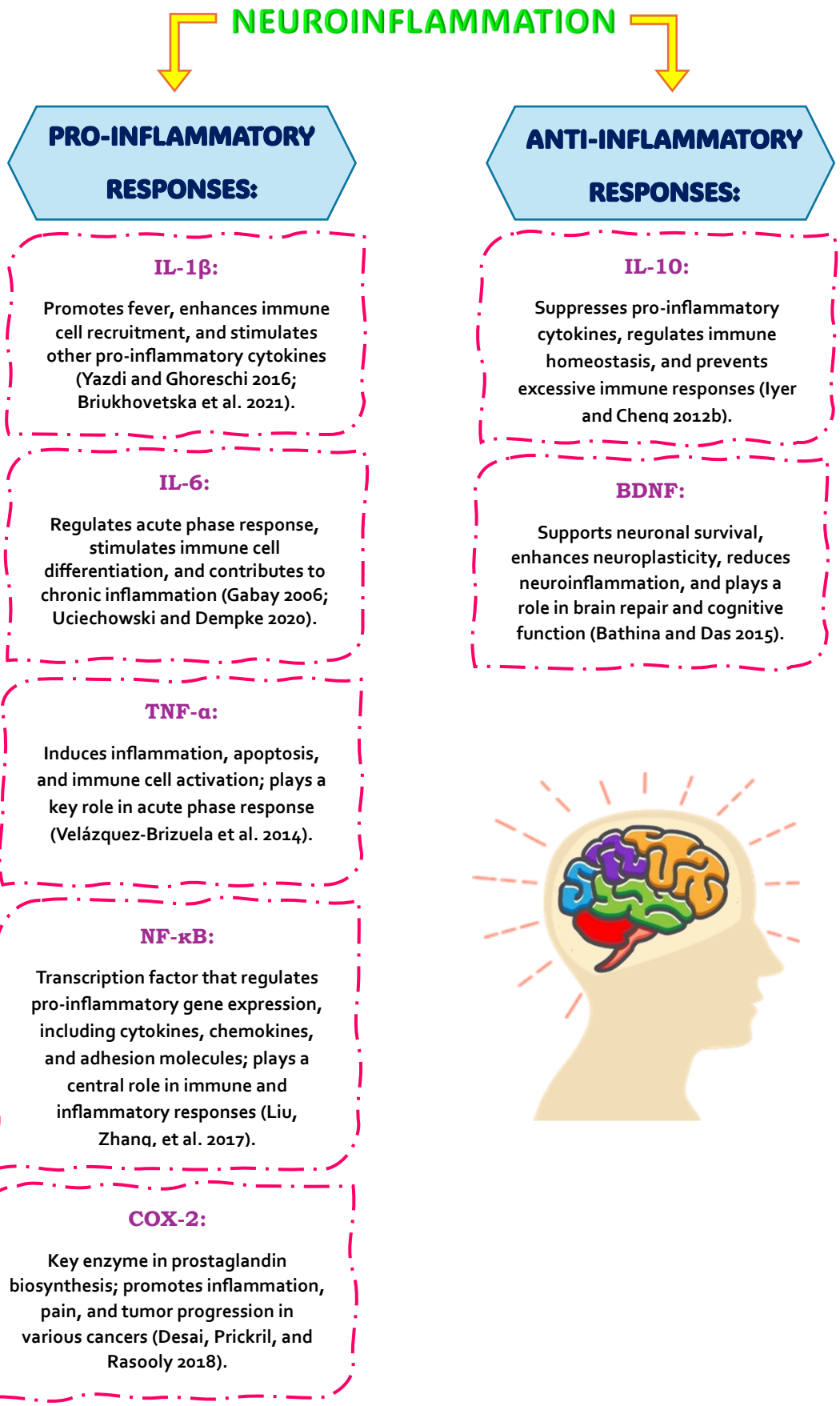

Figure 1.3: An overview of pro-inflammatory and anti-inflammatory markers used in this study, along with their respective functions (prepared by author using Microsoft Word).

1.2.4 Oxidative Stress

Oxidative stress is a significant factor in the development of various diseases, including arthritis, cancer, autoimmune disorders, aging, cardiovascular diseases, and neurodegenerative diseases. As we age, along with genetic and environmental risk factors, the oxidative-redox system becomes imbalanced, leading to increased levels of ROS and reactive nitrogen species (RNS). In this context, mitochondria, which use oxygen for energy production, are considered a primary source of oxidative stress (Islam 2017). Excessive stimulation of nicotinamide adenine dinucleotide phosphate (NADPH) and the electron transport chain results in an overproduction of ROS. While moderate concentrations of ROS/RNS are important for physiological processes, such as signalling pathways, inducing mitogenic responses, and defending against pathogens, excessive production and a failure to balance these with endogenous antioxidant defenses can lead to oxidative damage (Figure 1.4) (Islam 2017). This includes post-translational modifications and the oxidation of proteins, lipids, and DNA/RNA, which are common features of many neurodegenerative diseases, especially since the brain is particularly vulnerable to reactive species (Bhat et al. 2015). The brain is highly metabolically active yet has a limited capacity for cellular regeneration compared to other organs, making the impact of reactive species particularly significant (Bhat et al. 2015). Recent evidence suggests that oxidation products serve as biomarkers for certain neurodegenerative diseases. For instance, markers of lipid peroxidation, such as 4-hydroxynonenal and MDA, have been found in the substantia nigra, while protein nitration markers are observed in the hippocampus and neocortex of patients with Parkinson's disease. Additionally, markers related to protein nitration have been elevated in the same regions, alongside increased levels of 4-hydroxynonenal and MDA in the cortex and hippocampus of Alzheimer's disease patients (Bhat et al. 2015).

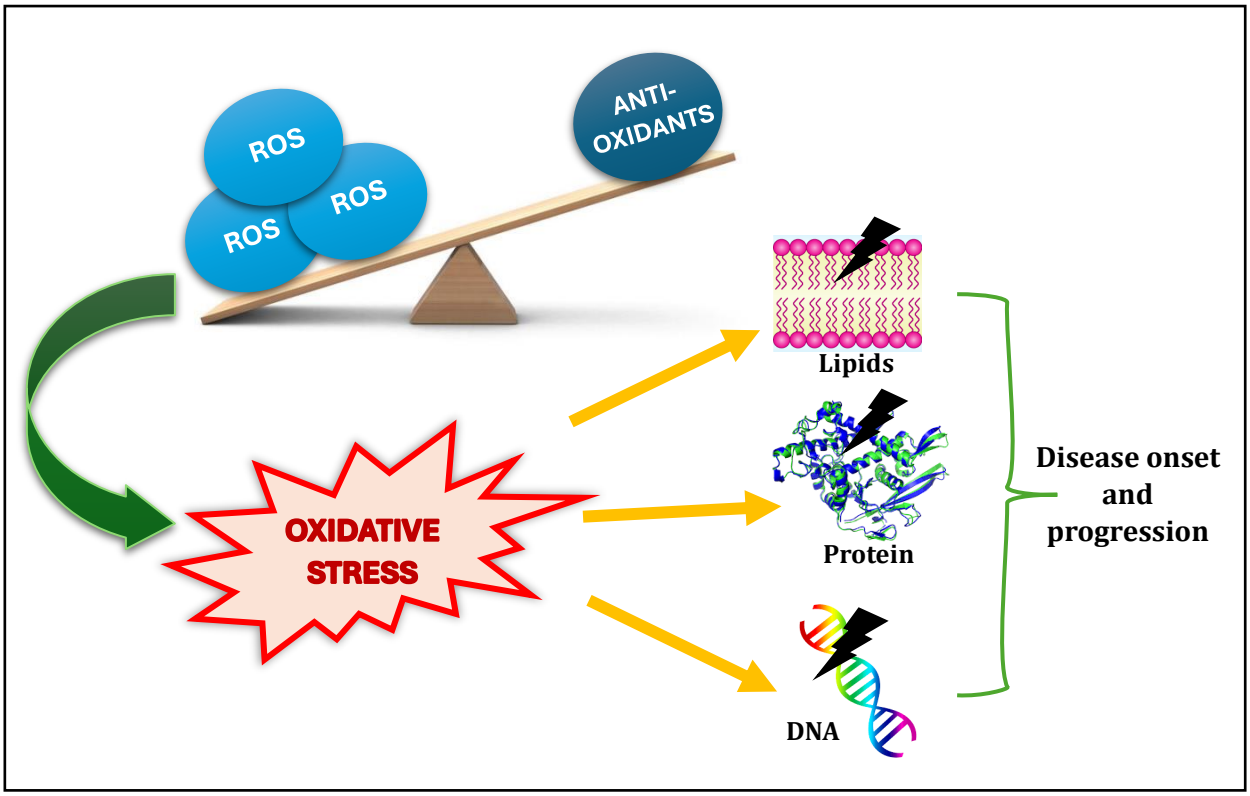

Figure 1.4: The imbalance between ROS and antioxidants promotes oxidative stress, resulting in detrimental health effects due to damageto lipids, protein and nucleic acids (prepared by author) (Akhigbe and Ajayi 2021). ROS: Reactive oxygen species. The lightening shape represent damage to lipids, protein and DNA.

1.2.4.1 Reactive species and antioxidant defense

Adding an electron to molecular oxygen produces a superoxide anion radical. This superoxide anion can arise from metabolic processes or from the activation of molecular oxygen through physical irradiation and subsequent interactions with other molecules to generate primary and secondary reactive species. These species can form directly or through enzyme or metal-catalysed processes (Wu, Cobbina, et al. 2016). Additionally, within the mitochondrial electron transport chain, some electrons can prematurely "leak" to oxygen during energy transduction, resulting in the formation of superoxide anion (Valko et al. 2004; Kovacic et al. 2005). The enzyme inducible nitric oxide synthase facilitates the production of nitric oxide radicals in biological tissues. Notably, water makes up approximately 80% of cells. The radiolysis of water by energetic radiation leads to the generation of hydrogen peroxide, molecular hydrogen, and free radicals, such as hydroxyl and hydrogen radicals (Sterniczuk and Bartels 2016). Superoxide is detoxified by superoxide dismutase enzymes, which convert it into hydrogen peroxide. Although hydrogen peroxide is not a free radical and is, therefore, less reactive than superoxide anion, it falls under the category of ROS because of its close involvement in the generation and detoxification of free radicals (Burton and Jauniaux 2011). Due to its non-polar nature, hydrogen

peroxide can easily diffuse through cell and organelle membranes, functioning widely as a second messenger in signalling pathways. It is then further detoxified into water by the enzymes catalase (CAT) and glutathione peroxidase (GPx) (Burton and Jauniaux 2011). It is crucial for antioxidant enzymes to work together, as an imbalance between superoxide and hydrogen peroxide concentrations can lead to the formation of much more harmful hydroxyl ions. This reaction is catalysed by free ferrous ions in the Fenton reaction (Burton and Jauniaux 2011). The hydroxyl ion has a very short lifespan, approximately 10^{-9} seconds, and reacts with any nearby biological molecules in a diffusion-limited manner. Due to its extreme reactivity, there are no known scavengers for hydroxyl ions (Burton and Jauniaux 2011).

Enzymatic defenses help prevent oxidant damage through transition metals at their core, which can change valence states as they transfer electrons during the detoxification process (Burton and Jauniaux 2011). Two isoforms of superoxide dismutase convert superoxide into hydrogen peroxide: the manganese form, found exclusively in the mitochondria, and the copper-zinc form, located in the cytosol. Subsequently, hydrogen peroxide is broken down into water by CAT or GPx. The activity of GPx depends on the availability of reduced glutathione (GSH) as a hydrogen donor (Burton and Jauniaux 2011). GSH serves as the primary cellular thiol redox buffer and is synthesized in the cytosol from L-glutamate, L-cysteine, and glycine. GSH is involved in numerous detoxification reactions, forming glutathione disulfide, which is then converted back to GSH by glutathione reductase, utilizing NADPH in the process. NADPH is generated through the pentose phosphate pathway, where glucose-6-phosphate dehydrogenase is the initial enzyme (Burton and Jauniaux 2011). This enzyme is subject to common polymorphisms, and reduced activity may compromise GSH levels, potentially leading to embryopathy (Nicol et al. 2000).

1.2.4.2 FB_1-induced oxidative stress

The accumulation of toxic products from sphingolipid metabolism disruption by FB_1 may further contribute to oxidative stress. FB_1-induced oxidative stress can be evaluated via lipid peroxidation and antioxidant response assays. The influence of varied doses of FB_1 on lipid peroxidation has been recognized in animal liver, kidney, glioblastoma and hypothalamic cells as well as in human glioma, neuroblastoma and intestinal cells (Stockmann-Juvala and Savolainen 2008; Joosten and Houweling 2004). Increased lipid peroxidation was seen in rats fed FB_1 over 21 days (Abel and Gelderblom 1998). In egg yolk phosphatidylcholine bilayers, increased oxidation promoted free radical production and enhanced chain reactions linked to lipid peroxidation due to membrane exposure to FB_1 (Yin et al. 1998). The authors suggested that increased oxygen transport in membranes and the subsequent increase in membrane permeability mediated the FB_1-induced cell damage and oxidative stress. In primary

hepatocytes, combined FB_1 and vitamin E treatment for 44 h prevented FB_1-induced lipid peroxidation (Domijan 2012). In C6 rat glioblastoma cells, increased lipid peroxidation due to FB_1 (10 μmol/L^{-1} to 20 μmol/L^{-1} for 24 h) was efficiently reduced by 25 μmol/L^{-1} vitamin E supplementation, while pre-treatment with vitamin E prevented FB_1-induced cell death and inhibited DNA and protein synthesis (Mobio et al. 2000). The same observation was seen in p53 null mouse embryonic fibroblasts and rat C6 glioblastoma, along with increased expression of the oxidative DNA damage marker, 8-hydroxy-2'-deoxyguanosine (Mobio et al. 2003). Mouse GTI-7 hypothalamic cells and C6 glioblastoma displayed increased ROS and lipid peroxidation but decreased glutathione levels after FB_1 exposure (Stockmann-Juvala et al. 2004c). FB_1 in human U-118MG glioblastoma cells also increased ROS production and lipid peroxidation but decreased glutathione levels after 24 – 144 h treatment (Stockmann-Juvala et al. 2004b). Increased ROS has been reported upon exposure to 10–100 μM FB_1 for 48 – 144 h in mouse hypothalamic cells, rat and human glioblastoma cell lines (Stockmann-Juvala et al. 2004c, 2004b). In human SH-SY5Y neuroblastoma cells and primary rat astrocytes, FB_1 increased ROS, but not in a dose-dependent manner (Domijan and Abramov 2011). Pig iliac endothelium cells treated with FB_1 displayed increased MDA levels with decreased glutathione, SOD, CAT, GPx and thioredoxin reductase, which led to reduced cell activity and cell membrane damage (Yuan et al. 2019). Increased ROS has also been reported in HepG2 cells exposed to FB_1 (Singh and Kang 2017). In human fibroblasts subjected to FB_1 for 72 h (Galvano, Russo, et al. 2002b) and in primary cultures of rat astrocytes (Galvano, Campisi, et al. 2002) subjected to 6 days of FB_1, no effects on ROS production were observed.

1.2.4.3 The role of NRF-2 in oxidative stress response

As ROS/RNS are produced, the antioxidant detoxification system is activated to counteract the increased levels of these species (Valko et al. 2006; Obrador et al. 2019). This endogenous antioxidant detoxification system is quite complex and includes: i) enzymatic antioxidants like SODs, GPx, and CAT; ii) non-enzymatic antioxidants such as vitamins E and C, carotenoids, flavonoids, selenium, and thiol antioxidants and iii) various regulatory factors such as NRF-2 and NF-κB, that interact with these antioxidants (Valko et al. 2006; Obrador et al. 2019). SODs are effective at scavenging superoxide and producing hydrogen peroxide (Li et al. 1995; Elchuri et al. 2005), while hydrogen peroxide can be detoxified by CAT, GPx and peroxiredoxins (Kang et al. 2005).

The transcription factor NRF-2 plays a crucial role in the antioxidant response, acting as a sensor for oxidative stress within redox homeostasis. Under normal conditions, NRF-2 is primarily located in the cytoplasm (Li and Kong 2009; Furfaro et al. 2016). When the levels of ROS/RNS increase, leading to oxidative stress, NRF-2 rapidly moves from the cytoplasm to the nucleus to trigger the antioxidant response, thereby protecting cells against oxidative and nitrative damage (Dhakshinamoorthy and Porter

2004; Pi et al. 2008). The NRF-2 signalling regulatory system consists of at least four components: NRF-2, Kelch-like ECH-associated protein 1, small musculoaponeurotic fibrosarcoma, and the antioxidant response element, all of which are essential for the antioxidant response (Furfaro et al. 2016; Kwak and Kensler 2010). NRF-2 signalling pathways regulate several biological processes, including: i) the expression of antioxidant genes, ii) the ubiquitin-proteasome system, iii) molecular chaperone and stress-response systems, and iv) anti-inflammatory responses (Kwak and Kensler 2010; Furfaro et al. 2016). A growing body of evidence indicates that NRF-2 signalling is implicated in the 12 hallmarks of cancer, which include sustained angiogenesis and proliferative signalling, insensitivity to antigrowth signals, resistance to apoptosis, limitless replicative potential, tissue invasion and metastasis, evasion of immune destruction, metabolic reprogramming, altered redox homeostasis, tumour-promoting inflammation, genome instability, and proteotoxic stress (Rojo de la Vega, Chapman, and Zhang 2018). Consequently, any reduction in the antioxidant protective system's efficacy in maintaining redox homeostasis may increase susceptibility to carcinogen toxicity, tumour-related inflammatory responses, oxidative stress, and carcinogenesis (Yates and Kensler 2007).

1.2.4.4 Oxidative damage to DNA

Over 100 different oxidized lesions have been identified in DNA (Lindahl and Barnes 2000), with 8-oxoguanine (8-oxoG) being the most prevalent. These lesions can form in critical regulatory regions of the genome, such as promoters and telomeres, potentially affecting genomic stability and cellular homeostasis (Ba and Boldogh 2018). The accumulation of 8-oxoG is recognized as a biomarker for various diseases, including cancer (Toyokuni et al. 1995; Wang et al. 2021a), Parkinson's disease (Alam et al. 1997; Wang et al. 2021a), systemic lupus erythematosus (Lunec et al. 1994; Wang et al. 2021a), and rheumatoid arthritis (Bashir et al. 1993; Wang et al. 2021a). 8-Oxoguanine glycosylase (OGG1) plays a key role in the base excision repair pathway by effectively recognizing and removing 8-oxoG (Boiteux and Radicella 1999; Zhao et al. 2022). OGG1 binds to the oxidized lesion and catalyses the cleavage of the N-glycosidic bond, releasing the oxidized base and creating an abasic site (Bjørås et al. 1997; Wang et al. 2021a). Additionally, OGG1 can cleave the DNA backbone by forming a Schiff base intermediate, resulting in a DNA nick with 5' phosphate and 3' α, β-polyunsaturated aldehyde (PUA) ends. The resulting DNA intermediate is then processed by apurinic/apyrimidinic endonuclease 1 (APE1), which removes the PUA group, allowing polymerase beta to incorporate a nucleotide. Finally, ligase III/XRCC1 seals the nick (Kim and M Wilson III 2012). Beyond its DNA repair function, OGG1 is also involved in gene transcription (Ba and Boldogh 2018), telomere maintenance (Fouquerel et al. 2019), cell signalling (Boldogh et al. 2012), and the formation of DNA–RNA hybrids (Pan et al. 2019). Specifically, OGG1 binds to 8-oxoG in G-rich promoter regions, influencing the expression of certain oxidative stress response genes. The effects of OGG1 binding in these regions can vary based on whether the interaction leads to enzymatic or non-enzymatic activities (Wang et al. 2021b). Following

OGG1's binding and the formation of an abasic site, DNA is rearranged into a G-quadruplex structure to expose the abasic site. APE1 is then recruited but binds non-enzymatically, leading to the transcriptional activation of genes (Wang et al. 2021b).

1.2.5 Mitochondrial dysfunction

Mitochondria serve as the cellular power plants, with their primary function being the synthesis of ATP through oxidative phosphorylation. This process involves the oxidation of nutrients, such as free fatty acids, to create an electrochemical gradient across the mitochondrial inner membrane, which is then utilized as a potential energy source for ATP generation, substrate or ion transport, and heat production (Wallace 2005; Zong et al. 2024). However, this process also generates ROS as toxic by-products, which can damage mitochondrial and cellular components like DNA, proteins, and lipids, leading to oxidative stress and mitochondrial dysfunction. In skeletal muscle, reduced mitochondrial respiration capacity, lower ATP production rates, and elevated ROS levels have been associated with the development of insulin resistance and type 2 diabetes (Kelley et al. 2002; Short et al. 2005), although it remains unclear whether mitochondrial dysfunction is a cause or a consequence of insulin resistance (Dumas et al. 2009; Pagel-Langenickel et al. 2010). Impaired mitochondrial β-oxidation has been observed in patients with non-alcoholic fatty liver disease, potentially contributing to hepatic steatosis and liver damage (Dabravolski, Bezsonov, and Orekhov 2021). Recent evidence suggests that mitochondrial dysfunction in hepatocytes plays a significant role in the early stages of liver fibrosis. In adipose tissue, mitochondria are essential for producing key intermediates for triglyceride synthesis and are critical for lipogenesis (De Pauw et al. 2009; Pessayre and Fromenty 2005; Dabravolski, Bezsonov, and Orekhov 2021). They also facilitate lipolysis through the β-oxidation of fatty acids, providing an important energy source for ATP production to support normal cellular functions. Mitochondrial dysfunction is associated with decreased fatty acid oxidation and increased levels of cytosolic free fatty acids, which can lead to insulin resistance and conditions like obesity and diabetes (De Pauw et al. 2009; Lowell and Shulman 2005; Sears and Perry 2015).

1.2.5.1 Mitochondrial-induced injury

Mitochondrial damage is primarily caused by ROS produced by the mitochondria themselves (Duchen 2004). It is suggested that most ROS originate from complexes I and III (Harper et al. 2004), due to the electron release from NADH and FADH into the electron transport chain. Mitochondria account for about 85% of the oxygen consumed by the cell during ATP production (Shigenaga, Hagen, and Ames 1994; Wilson, Harrison, and Vinogradov 2012). Under normal oxidative phosphorylation, 0.4–4.0% of the oxygen consumed is converted into the superoxide radical within the mitochondria. However, if

these enzymes fail to rapidly convert ROS, such as superoxide radicals, to water, oxidative damage can occur and accumulate in the mitochondria (James and Murphy 2002; Kowalczyk et al. 2021).

Within the mitochondria, components particularly susceptible to free radicals include lipids, proteins, oxidative phosphorylation enzymes, and mitochondrial DNA (mtDNA) (Shigenaga, Hagen, and Ames 1994; Bhatti, Bhatti, and Reddy 2017). Direct damage to mitochondrial proteins reduces their affinity for substrates or coenzymes, subsequently impairing their function (Liu, Killilea, and Ames 2002). Additionally, once a mitochondrion is compromised, its function may be further hindered by increased cellular demands for energy-related repair processes (Aw and Jones 1989; Zong et al. 2024). This can create a feedback loop where mitochondrial damage leads to even more damage.

Complex I is particularly vulnerable to damage from nitric oxide, and studies show that animals treated with both natural and synthetic antagonists of complex I have experienced neuronal death (Dauer and Przedborski 2003; Qi et al. 2003). Dysfunction of complex I has been linked to conditions such as Leber hereditary optic neuropathy, Parkinson's disease, and other neurodegenerative disorders (Schon and Manfredi 2003; Richardson et al. 2019). From a medical perspective, hyperglycaemia prompts endothelial cells to produce mitochondrial superoxide, a key factor in diabetic complications, including cardiovascular disease (Green, Brand, and Murphy 2004). This endothelial superoxide production also plays a role in atherosclerosis, hypertension, heart failure, aging, sepsis, ischemia–reperfusion injury, and hypercholesterolemia (Li and Shah 2004).

Inflammatory mediators such as TNF-α have been linked to mitochondrial dysfunction and increased ROS production *in vitro* (Moe et al. 2004; Desai et al. 2022). In a congestive heart failure model, the addition of TNF-α to cultured cardiac myocytes resulted in heightened ROS generation and myocyte hypertrophy (Nakamura et al. 1998; Suematsu et al. 2003b). TNF-α induces mitochondrial dysfunction by decreasing complex III activity in the electron transport chain, which in turn raises ROS production and damages mtDNA (Suematsu et al. 2003a; Chen et al. 2008).

1.2.5.2 LonP1 maintains mitochondrial homeostasis

LonP1 serves multiple functions in regulating mitochondrial proteostasis, metabolism, and cellular stress responses. As a protease responsible for protein quality control, it breaks down misfolded, misassembled, and oxidatively damaged proteins (Gibellini et al. 2020). Additionally, LonP1 plays a role in mitochondrial metabolism by degrading crucial rate-limiting proteins involved in processes such as cholesterol metabolism, heme biosynthesis, and mitochondrial transcription. It also interacts with mitochondrial transcription factor A (TFAM), which is essential for activating mitochondrial

transcription and maintaining mtDNA (Lu et al. 2013b). TFAM binds to mtDNA both specifically and non-specifically (Kang, Chu, and Kaufman 2018). Its sequence-specific binding to mtDNA promoter regions is essential for initiating mitochondrial transcription, which may also act as the RNA primer for replication initiation. Additionally, non-specific binding of TFAM to mtDNA aids in compacting the genome. Both these types of mtDNA binding contribute to TFAM's effect on mtDNA copy number (Kang, Chu, and Kaufman 2018).

Beyond its proteolytic functions, LonP1 acts as an ATP-dependent chaperone, facilitating protein folding and assembly while preventing aggregation. This chaperone activity is important for maintaining the solubility of newly synthesized polypeptides entering the mitochondrial matrix and supporting their proper folding (Bezawork-Geleta et al. 2015). Furthermore, LonP1's chaperone role is thought to aid in the assembly and regulation of oxidative phosphorylation complexes (Hori et al. 2002). In response to oxidative stress, LonP1 degrades oxidatively damaged proteins and utilizes both its protease and chaperone activities to counteract harmful processes that jeopardize cell survival. Research involving neuroblastoma and HeLa cell lines has demonstrated that LonP1 works alongside ClpXP to reduce ROS in mitochondria by degrading a portion of complex I of the electron transport chain, which is responsible for ROS generation (Pryde, Taanman, and Schapira 2016). Another study with colon cancer cells and mouse embryonic fibroblasts indicated that elevated ROS levels can trigger the translocation of p53 from the cytosol to the mitochondrial matrix, leading to necrosis (Vaseva et al. 2012).

1.2.5.3 The sirtuin family

Metabolism ensures that essential molecules are available at the right time and place to fulfil the needs of the organism and maintain physiological balance. Two primary processes, anabolism and catabolism, work together to regulate this balance. The intricate interplay between these processes is tightly controlled by various pathways, among which sirtuins, a family of proteins, play a significant role in cellular regulation, including metabolic control and epigenetic modification (Bi et al. 2020; Diao et al. 2021). Importantly, sirtuins are also crucial for lifespan extension, highlighting their important link between metabolism and aging (Giblin, Skinner, and Lombard 2014). The sirtuin family consists of seven members, from SIRT1 to SIRT7, each localized to specific subcellular compartments and influencing a wide range of cellular functions, such as metabolism and epigenetic changes (Figure 1.5). SIRT1, SIRT6, and SIRT7 primarily reside in the nucleus and act as deacetylases that modify histones and regulate gene expression, although SIRT1 and SIRT6 are also found in the cytosol. Besides deacetylation, SIRT6 and SIRT7 exhibit ADP-ribosyl transferase activity (Liszt et al. 2005). SIRT2 is mainly located in the cytoplasm but can also be found in the nucleus to help regulate the cell cycle.

SIRT3, SIRT4, and SIRT5 are predominantly associated with mitochondria and are commonly referred to as mitochondrial sirtuins (Kumar and Lombard 2015). Notably, SIRT3 has also been identified in the nucleus, where it stabilizes heterochromatin and helps counteract cellular senescence in human mesenchymal stem cells (Diao et al. 2021), while a portion of SIRT5 is present in both the cytosol and the nucleus (Park et al. 2013; Matsushita et al. 2011).

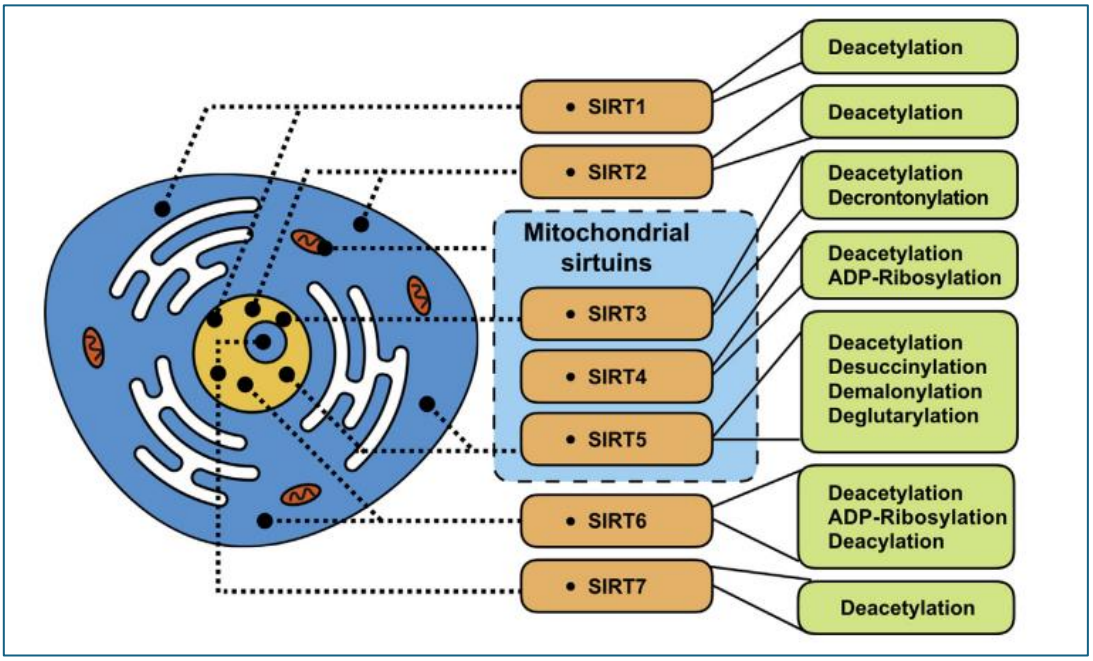

Figure 1.5: Localization and functions of mammalian sirtuins. SIRT3, SIRT4 and SIRT5 are mainly situated in the mitochondria, with a fraction of SIRT3 and SIRT5 present in the nucleus. SIRT1, SIRT2 and SIRT6 are present in both the cytosol and nucleus, whereas SIRT3 is exclusively located in the mitochondria (Ji, Liu, and Qu 2022). SIRT: Sirtuin.

1.2.5.4 Sirtuins and oxidative stress

Increasing evidence highlights the role of sirtuins in regulating cellular homeostasis, particularly in relation to metabolism and inflammation (Haigis and Sinclair 2010). In conditions of metabolic stress, such as obesity and metabolic syndrome, an environment of oxidative stress arises, primarily due to chronic inflammation. Given the critical function of sirtuins in managing metabolic responses (Houtkooper, Pirinen, and Auwerx 2012), it is important to explore how changes in the redox status of cells influence sirtuin activity and the biological implications of these changes. Oxidative stress can impact sirtuin activity in various ways such as: i) inducing or repressing the expression of SIRT genes, ii) causing posttranslational oxidative modifications of sirtuins, iii) altering sirtuin-protein interactions, and iv) modifying NAD levels.

Mild oxidative stress conditions trigger the expression of SIRT1, which alters its activity and subsequently affects its targets involved in the cellular response to changes in redox status (Prozorovski et al. 2008). The first major substrate identified for SIRT1 was p53, a transcription factor that activates antioxidant genes such as *SOD2* and *GPx* (Sablina et al. 2005) Another redox-related transcription factor modified by SIRT1, SIRT2 and SIRT3 is FOXO3a, which promotes an antioxidant response through the expression of SOD2 and CAT (Brunet et al. 2004; Hasegawa et al. 2008). PGC1α, another known substrate of SIRT1, is reported to regulate the expression of mitochondrial antioxidants like SOD2. Additionally, SIRT1 can deacetylate the p65 subunit of NF-κB, thereby reducing its activity and the (Lu et al. 2010; St-Pierre et al. 2006; Tseng, Shieh, and Wang 2013) subsequent production of pro-inflammatory cytokines (Lee et al. 2009). Increased ROS production in the mitochondria has also been linked to the induction of SIRT3 (Chen et al. 2011), which deacetylates and activates SOD2, helping to reduce oxidative stress within mitochondria (Qiu et al. 2010). Additionally, SIRT3 plays a critical role in regulating the key TCA cycle enzyme isocitrate dehydrogenase 2 (IDH2). Its deacetylation of IDH2 helps maintain the mitochondrial pool of NADPH, an essential reducing agent that impacts glutathione reductase, a vital component of the antioxidant defense system against cellular oxidative stress (Someya et al. 2010). SIRT3 also protects cardiomyocytes and microglia from oxidative stress through its interaction with the transcription factor FOXO3a, enhancing the transcription of important antioxidant genes, including SOD2 and catalase (Sundaresan et al. 2008; Rangarajan et al. 2015). Further research has demonstrated that hydrogen peroxide promotes the deacetylation of FOXO3a by SIRT3, facilitating transcriptional responses that help safeguard mitochondria from additional oxidative stress (Tseng, Shieh, and Wang 2013). In adult mouse hearts, SIRT1 expression was significantly upregulated by four-fold in response to oxidative stress induced by paraquat injection, and a similar three-fold increase in SIRT1 levels was noted in older monkey hearts compared to the younger hearts (Alcendor et al. 2007). Furthermore, modest overexpression of SIRT1 was found to slow age-related changes in the hearts of transgenic mice (Alcendor et al. 2007). Low levels of hydrogen peroxide were shown to promote the deacetylation of the tumour suppressor protein promyelocytic leukaemia in HeLa cells via SIRT1 and SIRT5 (Guan et al. 2014).

In contrast, exposure to high levels of hydrogen peroxide or severe oxidative stress leads to increased proteasomal degradation of SIRT1, resulting in deSUMOylation and enzyme inactivation that can trigger apoptosis (Yang et al. 2007). Human monocytes treated with a high dose of hydrogen peroxide (250 μM for 24 h) exhibited a significant reduction in SIRT1 activity as indicated by levels of acetylated p53 and a decrease in both SIRT1 gene and protein expression (de Kreutzenberg et al. 2010). Similarly, human lung epithelial cells exposed to various oxidants showed reduced levels of SIRT1 along with diminished SIRT1 activity (Caito et al. 2010). A recent study on human endothelial cells found that while low doses of hydrogen peroxide had no effect, exposure to 100 μM hydrogen peroxide for 30

minutes resulted in a drastic 50% reduction in SIRT1 activity and a decrease in free thiol content of SIRT1 (Jung et al. 2013).

1.2.6 DNA methylation

Historically, DNA methylation was first identified in mammals around the same time that DNA was recognized as the genetic material (Avery, MacLeod, and McCarty 1995; Moore, Le, and Fan 2013). In 1948, using paper chromatography, Hotchkiss (1948) discovered modified cytosine in calf thymus. He hypothesized that this modification was 5-methylcytosine due to its separation pattern, which resembled that of thymine from uracil, and suggested that this modified cytosine naturally occurs in DNA. While many researchers speculated that DNA methylation could regulate gene expression, it wasn't until the 1980s that various studies confirmed its role in gene regulation and cell differentiation (Holliday and Pugh 1975; Taryma-Lesniak, Sokolowska, and Wojdacz 2021). It is now widely accepted that DNA methylation, along with other regulatory mechanisms, is a key epigenetic factor influencing gene activity.

DNA methylation is catalysed by a family of enzymes known as DNMTs which transfer a methyl group from S-adenosyl methionine (SAM) to the fifth carbon of a cytosine residue, resulting in the formation of 5-methylcytosine (Figure 1.6a) (Moore, Le, and Fan 2013). DNMT3A and DNMT3B induce new methylation patterns on unmodified DNA, earning them the designation of *de novo* DNMTs, while DNMT1 operates during DNA replication to transfer the methylation pattern from the parental DNA strand to the newly synthesized daughter strand (Moore, Le, and Fan 2013). DNA methylation determines the expression of genes via hypomethylation, which results in the activation of gene expression, and hypermethylation, which leads to silenced gene expression (Figure 1.6b) (Holmes et al. 2019).

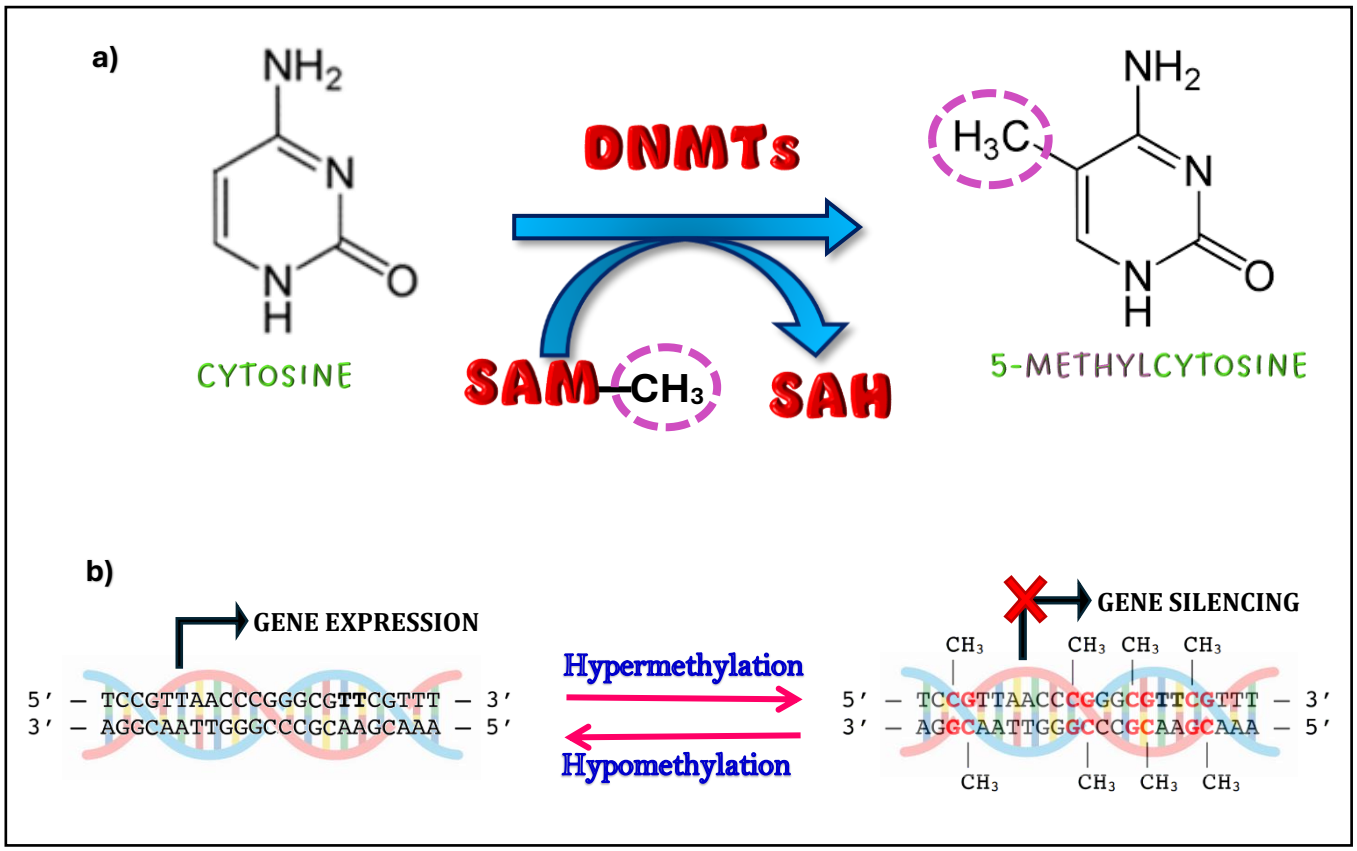

Figure 1.6: a) DNA methylation is facilitated by DNMTs which transfer a methyl group from the universal methyl donor, S-adenosylmethionine (SAM), to the 5th carbon of the cytosine residue to yield 5-methylcytosine. b) DNA methylation is characterized by hypomethylation which induces gene expression, or by hypermethylation which promotes gene silencing (prepared by author) (Ghazi et al. 2020). DNA: Deoxyribonucleic acid; DNMTs: DNA methyltransferases; SAM: S-adenosylmethionine.

DNMTs play crucial roles in embryonic development, but their expression diminishes significantly by the time cells reach terminal differentiation, suggesting that DNA methylation patterns in postmitotic cells are stable. However, postmitotic neurons in the mature mammalian brain still express considerable levels of DNMTs, indicating that these enzymes and DNA methylation might have novel functions in the brain (Goto et al. 1994; Feng et al. 2005). Neurons respond to environmental stimuli through depolarization patterns that transmit information and encode responses. Recently, it has become increasingly clear that alterations in gene expression following depolarization are accompanied by modifications to the epigenetic landscape, including shifts in DNA methylation patterns (Martinowich et al. 2003; Guo et al. 2011a). Despite the brain having some of the highest levels of DNA methylation among all tissues, 5-methylcytosine represents only about 1% of the nucleic acids in the human genome (Ehrlich et al. 1982; Jones 2002). Most DNA methylation occurs on cytosines that precede a guanine nucleotide known as CpG sites. Overall, mammalian genomes show a depletion of CpG sites, likely

due to the mutagenic potential of 5-methylcytosine, which can deaminate to form thymine (Bird 1980). The remaining CpG sites are widely distributed throughout the genome, where they are heavily methylated, except at CpG islands (Bird et al. 1985; Wu and Zhang 2014).

1.2.6.1 DNA methylation writers and erasers

The members of the DNMT family directly catalyse the addition of methyl groups to DNA. While these enzymes share a similar structure, they have distinct functions and expression patterns (Xie et al. 1999; Kim 2025). The most extensively studied DNMT, particularly in the nervous system, is DNMT1, which is highly expressed in various mammalian tissues, including the brain (Goto et al. 1994; Larsen, Kristensen, and Callesen 2018). DNMT1 preferentially methylates hemi-methylated DNA (Ramsahoye et al. 2000; Takeshita et al. 2011). During DNA replication, DNMT1 localizes to the replication fork, where it acts on newly synthesized hemi-methylated DNA (Leonhardt et al. 1992; Al-Yozbaki et al. 2022). It binds to this newly synthesized DNA and methylates it, precisely replicating the original methylation pattern before DNA replication (Hermann, Goyal, and Jeltsch 2004; Larsen, Kristensen, and Callesen 2018). Additionally, DNMT1 can repair DNA methylation (Mortusewicz et al. 2005), and is thus referred to as the maintenance DNMT, as it preserves the original pattern of DNA methylation in a cell lineage. In contrast, both DNMT3A and DNMT3B methylate both native and synthetic DNA without showing any preference for hemi-methylated DNA (Okano et al. 1999; Larsen, Kristensen, and Callesen 2018). Therefore, they are known as *de novo* DNMTs as they introduce new methylation patterns into unmethylated DNA. The primary distinction between DNMT3A and DNMT3B lies in their gene expression patterns, where DNMT3A is expressed more broadly, while DNMT3B has low expression in most differentiated tissues, except in the thyroid, testes, and bone marrow (Xie et al. 1999).

As DNMT1 actively maintains DNA methylation during cell replication, its inhibition or dysfunction can result in newly incorporated cytosine remaining unmethylated, thereby decreasing the overall methylation level with each cell division. Active DNA demethylation can occur in both dividing and nondividing cells, but it requires enzymatic processes to convert 5-methylcytosine back to unmethylated cytosine (Mayer et al. 2000; Zhang et al. 2007). Currently, there is no known mechanism in mammalian cells that can cleave the strong covalent bond between cytosine and its attached methyl group. Instead, demethylation occurs through a series of chemical modifications, including deamination and/or oxidation reactions, producing a form that is recognized by the base excision repair pathway, which replaces the modified base with unmethylated cytosine (Moore, Le, and Fan 2013).

DNA methylation is recognized by three distinct families of proteins: the MBD proteins, the UHRF proteins, and the zinc-finger proteins. Among these, the MBD family was the first to be identified. MBD proteins possess a conserved methyl-CpG-binding domain, which gives them a higher affinity for single methylated CpG sites (Nan, Meehan, and Bird 1993; Leighton et al. 2022). MBDs are expressed at higher levels in the brain than in any other tissue, and many are crucial for normal neuronal development and function (Amir et al. 1999; Yushko et al. 2024). Repressor complexes attach to methylated DNA through MBDs (Razin and Szyf 1984; Nicholson, Veland, and Chen 2015). MBD2 has been shown to demethylate DNA both *in vitro* (Bhattacharya et al. 1999) and *in vivo* (Cervoni and Szyf 2001). Demethylases promote gene activation by removing repressive methyl groups (Detich, Theberge, and Szyf 2002). While methylation typically silences genes, it is anticipated that a demethylase would enhance transcription. However, MBD2 binds to methylated CpG sites and inhibits transcription by recruiting inactive chromatin complexes that contain histone deacetylase (Liu et al. 2011). Thus, MBD2 can activate the expression of certain genes while simultaneously silencing others (Liu et al. 2011).

1.2.6.2 Aberrant DNA methylation alters brain development

Common neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease, and Huntington's disease result in a progressive decline in neuronal function and cognitive impairment. Epigenetic mechanisms are crucial for brain function, as they regulate gene expression (Robertson 2005). Among these mechanisms, DNA methylation is a significant modification that influences transcriptional activity by either promoting or inhibiting the binding of transcription factors to DNA. Numerous brain disorders are characterized by altered gene expression, and growing evidence suggests that dynamic DNA methylation is involved in these changes and related pathological processes (MacArthur and Dawlaty 2021). Additionally, dysregulation of the epigenome has been observed in both neurodevelopmental and neurodegenerative diseases (Younesian et al. 2022).

During early developmental stages, neural stem cells give rise to all cortical neurons. Alterations in 5-methylcytosine at the promoters of genes which are crucial for brain development and neural differentiation, can lead to transcriptional changes that affect neural differentiation (Santiago et al. 2020). There is a strong correlation between gene silencing and non-CpG methylation (Guo et al. 2014). Notably, non-CpG methylation is a conserved DNA modification in the human neuronal genome, playing a role in regulating genes associated with neuronal differentiation, synaptogenesis, and function (Lister et al. 2013). In both humans and mice, non-CpG methylation is established after neurons mature. In humans, 5-methylcytosine levels at specific loci responsible for CNS growth and development

increase significantly after birth, contributing to neuronal differentiation which may persist throughout life (Siegmund et al. 2007). Interestingly, mature neurons do not undergo mitosis, so while 5-methylcytosine cannot be passively removed during cell division, it can still be actively eliminated by TET (Ten-eleven translocation) enzymes (He et al. 2020). Thus, 5-methylcytosine is dynamic and influences neurogenesis. Similarly, DNA methylation is involved in the differentiation and maturation of astrocytes. Additionally, it has been suggested that 5-methylcytosine plays a role in sexual differentiation in the developing mammalian brain, being necessary for the masculinization or feminization of brain structures (Nugent et al. 2015).

DNMT1 is a highly conserved DNA methyltransferase in both mice and humans, ensuring the faithful preservation of DNA methylation patterns during cell division (Ye et al. 2018). In mice, DNMT3A is highly expressed in neural stem and progenitor cells within the developing cerebral cortex, as well as in neurons and oligodendrocytes, but is expressed at very low levels in astrocytes (Feng et al. 2005). DNMT3B is only expressed in the ventricular zone during early embryonic development (Wang, Tang, et al. 2016). Mice lacking DNMT3A can survive for four weeks, while homozygous deletions of either DNMT1 or DNMT3B result in embryonic or postnatal lethality (Okano et al. 1999). Conditional mutant mice lacking DNMT1 or DNMT3A in forebrain excitatory neurons display impairments in learning and memory, along with deregulated neuronal gene expression, highlighting the importance of these DNMTs in DNA methylation maintenance and regulation of adult neuronal gene expression (Feng et al. 2010). Additionally, DNMT3B is crucial for *de novo* methylation during development, as its absence leads to abnormal development of the rostral neural tube (Okano et al. 1999).

1.3 References

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CHAPTER 2

Fumonisin B_1 induces global DNA hypermethylation in human glioblastoma U87MG cells

DNA methylation is the most widely studied epigenetic modification which regulates gene expressions. Epigenetic modifications are essential for overall health and normal development; however, environmental exposure to mycotoxins can disrupt the cells epigenome, often resulting in toxicity (Huang et al. 2019). Aberrant DNA methylation has also been associated with neurological disorders. Multiple reports have documented the harmful effects of FB_1 on neuronal tissue, highlighting its potential for direct neurotoxicity. The health impacts and toxicity mechanisms of FB_1 have been widely researched; however, the mechanisms of FB_1 non-genotoxicity remain unclear. FB_1 has been implicated in the inhibition of cellular macromolecule syntheses which may result in damage to the cell and membrane structure (Mobio et al. 2000). This paper determined the effect of FB_1 on cytotoxicity and DNA methylation in human glioblastoma U87MG cells and suggested an alternative mechanism for FB_1 toxicity.

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Fumonisin B_1 induces global DNA hypermethylation in human glioblastoma U87MG cells

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Abstract

Fumonisin B_1 (FB_1) is a common maize contaminant known to induce toxicity and carcinogenesis in humans and animals; however, its epigenetic mechanisms remain poorly understood. DNA methylation is an epigenetic modification that controls gene expression through DNA methyltransferase and demethylase activities. In this study, the effect of FB_1 on DNA methylation in brain glioblastoma U87MG cells was evaluated. FB_1 cytotoxicity was determined by the MTT assay and an IC_{50} value of 880 µM FB_1 was obtained. The ELISA-based global DNA methylation assay displayed an increase in 5-methylcytosine levels. qPCR and western blot revealed a significant increase in DNA methyltransferase expressions (DNMT1, DNMT3A, and DNMT3B) and a significant decrease in demethylase expression (MBD2). This data indicates that FB_1 induces global DNA hypermethylation, through increased DNA methyltransferase expressions and DNA demethylase suppression in U87MG cells, thus suggesting an alternative mechanism of toxicity.

Keywords: Fumonisin B_1, Epigenetics, DNA methylation, 5-methylcytocine, DNMTs, MBD2

Introduction

Food and feed are susceptible to fungal and mycotoxin contamination, which pose major health hazards to humans and animals. Synthesized by the *Fusarium* fungal species, Fumonisins frequently infect maize and maize-based food products (Thiel et al. 1991; Liu, Fan, et al. 2019), with Fumonisin B_1 (FB_1) being the most abundant and toxic member (Shephard et al. 1990; Liu, Fan, et al. 2019). It is widely understood that FB_1 causes a plethora of toxicities in animals, including hepatotoxicity, nephrotoxicity, immunotoxicity, and neurotoxicity (Liu, Fan, et al. 2019), while in humans FB_1 has been classified as a potential human carcinogen (2B) and was associated with high risks of oesophageal cancer (Sydenham et al. 1990) and hepatocellular carcinoma (Chuturgoon, Phulukdaree, and Moodley 2014). It is broadly recognized that the core mechanism of FB_1 toxicity is through the disruption of sphingolipid metabolism (Voss and Riley 2013), which can be coupled to the epigenetic mechanisms of FB_1 (Voss, Smith, and Haschek 2007b; Sugiyama et al. 2021).

Epigenetics is classified as the heritable changes in gene activity independent of the DNA sequence (Moore, Le, and Fan 2013). The most researched epigenetic mechanisms are DNA methylation, post-transcriptional histone modifications and small non-coding RNAs. Of these, DNA methylation is the most dysregulated epigenetic mechanism (Ghazi et al. 2020), hence it is the focus of this study. DNA methylation is a chemical modification that functions in regulating gene expression (Issa et al. 1993). DNA methylation occurs almost entirely at CpG dinucleotides and is catalysed by DNA methyltransferases (DNMTs), namely DNMT1, DNMT3A, and DNMT3B (Ghazi et al. 2020). During this process, DNMTs facilitate the transfer of a methyl group from the universal methyl donor, S-adenosylmethionine to the 5^{th} carbon of cytosine residues to yield 5-methylcytosine (Moore, Le, and Fan 2013). DNMT1 is essential for DNA replication as it plays a role in maintenance by copying DNA methylation patterns from the parent DNA strand to the next generation (Moore, Le, and Fan 2013). The *de novo* methyltransferases, DNMT3A and DNMT3B target un-methylated cytosine bases to initiate new DNA methylation patterns (Ghazi et al. 2020). Furthermore, DNA methylation patterns can be influenced by the recruitment of methyl-CpG-binding domain 2 (MBD2) protein, which acts as a methylation-dependent transcriptional repressor (Berger and Bird 2005b) and holds demethylation characteristics (Detich, Theberge, and Szyf 2002). Alterations in DNA methylation patterns are considered a significant toxicity regulator. While global DNA hypomethylation results in genomic instability and increased genetic mutations, global DNA hypermethylation has been implicated in transcriptional gene silencing and repression of chromatin structure (Ghazi et al. 2020).

The brain is an essential organ of the nervous system, which functions in controlling and coordinating a variety of actions and reactions in the body (Xie et al. 2023b). FB_1 neurotoxicity has been substantially reported (Gao et al. 2023*)*, with the first report being leukoencephalomalacia in horses (Ross et al. 1992). In rats, FB_1-contaminated diets have led to a significant loss of neuronal cell body (Sousa et al. 2014) and nerve conduction velocity (Banczerowski-Pelyhe et al. 2002). In adult pigs, FB_1 impaired activities of the regional brain and hypophyseal acetylcholine hydrolase (Gbore 2010), while in beef cattle FB_1 induced acute myelin oedema and optic nerve degeneration (Sandmeyer et al. 2015). Furthermore, FB_1 was found to penetrate the blood-brain barrier and cause degeneration, brain oedema and nerve cell necrosis in carps (Kovacić et al. 2009). In humans, FB_1 triggers embryonic neural tube defects, hindering neurodevelopment (Marasas et al. 2004). This FB_1 phenomenon was also monitored in pregnant LM/BC mice, which showed a 79% probability of developing neural tube defects (Gelineau-van Waes et al. 2005a).

Glioblastoma is the most frequent and destructive primary brain tumour in adults and children, with a mean survival of approximately one year. These tumours have been extensively characterized at both

the transcriptome and genome levels, and studies suggest that integrating these molecular insights could aid in identifying robust signatures and clinically relevant molecular classifiers (de Tayrac et al. 2009). A key factor in the development and persistence of treatment resistance in GBM cells is their epigenetic profile (Aanya et al. 2025), making them a good model for this FB_1 toxicity study via DNA methylation. GBM cells are also ideal for this study as they undergo metabolic reprogramming which enables them to modify their metabolism to support rapid growth and enhance survival (Cortes Ballen et al. 2024). This adaptability renders them vulnerable to toxins like FB_1, which interfere with sphingolipid metabolism. Given the limited data on FB_1's neurotoxicity in humans and its classification as a carcinogen, studying the effects of FB_1 in GBM cells can offer valuable insights into its neurotoxic mechanisms and its potential role in tumour progression. Emerging research highlights the contribution of epigenetics in brain functions such as learning and memory, synaptic plasticity, circadian rhythm, drug addiction and adult neurogenesis (Guo et al. 2011b). Moreover, DNMTs are significantly expressed in postmitotic neurons of mature mammals, suggesting a role for DNA methylation in neurodegeneration (Goto et al. 1994; Moore, Le, and Fan 2013). Neurons are stimulated by depolarization which relays information and encodes response to the environment. Recent evidence suggests that gene expression modifications, post depolarization, are accompanied by alterations in DNA methylation patterns (Martinowich et al. 2003; Guo et al. 2011b). As reported by Ehrlich et al. (1982), apart from the thymus, the brain has the greatest level of DNA methylation compared to any other human tissue. Additionally, FB_1 may be considered an epigenetic carcinogen in risk assessment processes, while researchers have also documented that FB_1 can alter DNA methylation via its association with folate deficiency (Abdel Nour et al. 2007; Gelineau-van Waes et al. 2005a; Stevens and Tang 1997b). This thus provides a rationale to explore the role of FB_1 toxicity as a mode of epigenetic action (Demirel, Alpertunga, and Ozden 2015). While epigenetic mechanisms play a well-established role in cancer development and tumour aggressiveness, their changes are potentially reversible, thus offering promise as targets for epigenetic-based cancer treatments (Etcheverry et al. 2010). Furthermore, genome-wide DNA methylation analysis is essential to understand the epigenetic changes that make glioblastomas so hostile and to develop better treatment strategies (Etcheverry et al. 2010). In this study, we investigated the effect of FB_1 on global DNA methylation via the expressions of DNMTs and MBD2 in human brain glioblastoma U87MG cells.

Methods and Materials

Reagents

The U87MG cell line was purchased from Separations Scientific (Johannesburg, SA). Reagents for cell culture were purchased from Lonza Biotechnology (Basel, Switzerland). Western Blot reagents were purchased from Bio-Rad (Hercules, CA, USA). Quantitative Polymerase Chain Reaction (qPCR)

primers were obtained from Inqaba Biotech (Johannesburg, SA). All other reagents were purchased from Merck (Darmstadt, Germany), unless otherwise stated.

Cell Culture and Treatment

U87MG cells were cultured in Dulbecco's Minimum Essentials Medium (DMEM) supplemented with 2.5 mM HEPES buffer, 10% foetal bovine serum, 1% penicillin-streptomycin-fungizone, and 1% L-glutamine at 37ºC with 5% CO_2 in a humidified incubator. Cells were allowed to reach approximately 90% confluency before treatment with FB_1. A stock of 5 mM FB_1 was prepared in 0.1 M phosphate buffered saline (PBS). Following cell viability testing, for all subsequent assays conducted, cells were treated with 880 µM FB_1 (IC_{50}) in DMEM for 24 h. U87MG cells containing DMEM only were cultured under the same conditions and used as the control. Additionally, a 50 mM stock solution of the DNA methylation inhibitor, 5-aza-2'-deoxycytidine (5-aza-2-DC) was prepared in 100% dimethyl sulfoxide (DMSO). Cells treated with 5-aza-2-DC (500 µM; 24 h) (Proto et al. 2022) were used as a negative control for the DNA methylation assay.

Cell Viability Assay

The methyl thiazol tetrazolium (MTT) assay was used to determine the cytotoxicity of FB_1 on U87MG cells. U87MG cells were seeded in a 96-well plate and treated for 24 h with FB_1 (0 - 1000 µM in 100 µM increments) in triplicate. 5 mg MTT salt was made up in 1 ml PBS. Following the 24 h incubation, the treatments were discarded, and cells were incubated (4 h, 37ºC) with MTT salt solution (20 µl MTT salt in 100 µl DMEM per well). Following incubation, the MTT salt solution was removed, 100 µl DMSO was added to each well and incubated (1 h, 37ºC). Optical density of the formazan produced was measured using a spectrometer (SPECTROstar Nano, BMG LABTECH, Ortenburgh, Germany) at a wavelength of 570 nm and a reference wavelength of 690 nm. The percentage cell viabilities were calculated relative to the control and used to construct a dose-response curve from which the half maximal inhibitory concentration (IC_{50}) of FB_1 was determined.

DNA Isolation and DNA Methylation Assay

The DNA methylation assay was performed to determine the percentage of 5-methylcytosine in control, FB_1-treated and 5-aza-2-DC-treated U87MG cells. Genomic DNA was isolated from these cells. Briefly, control, FB_1 and 5-aza-2-DC treated U87MG cells were incubated with 600 µl cell lysis solution (0.5 M EDTA, 1 M Tris-Cl, 0.1% SDS; 15 min, RT), scraped and transferred into fresh 1.5 ml microcentrifuge tubes. 600 µl potassium acetate buffer (5 M potassium acetate, glacial acetic acid; 8 min, RT) was added to the cells followed by centrifugation (13,000 rpm, 5 min, 24ºC). 600 µl

isopropanol (100%) was added, DNA was precipitated by centrifugation (13,000 rpm, 5 min, 24ºC) and washed in 300 µl ethanol (100%). Following centrifugation (13,000 rpm, 5 min, 24ºC), DNA pellets were air dried (15 min, RT), resuspended in 40 µl hydration solution (10 mM EDTA (pH 8), 100 mM Tris-Cl (pH 7.4) and heated (15 min, 65ºC). All DNA samples were then quantified using the Nanodrop2000 spectrophotometer (Thermo-Fisher Scientific) and standardized to 100 ng/µl. The A260/A280 absorbance ratio was used to assess DNA purity. Standardized DNA was used to quantify global DNA methylation using the Methylated DNA Quantification Kit (Abcam, ab117128), as per the manufacturer's instructions. The percentage 5-methylcytosine content was determined using the supplied formula (below) and represented as a fold change relative to the control.

Quantification of DNA Methylation Formula:

$$\text{Amount of 5mC (ng)} = \frac{Sample\ OD - Negative\ Control\ OD}{Slope\ x\ 2}$$

$$\text{Percentage of 5mC} = \frac{5mC\ (ng)}{input\ sample\ DNA}\ x\ 100$$

RNA Isolation and qPCR

qPCR was conducted to determine gene expression of DNA methylation markers. A cell scraper was used to lyse cells from control and FB_1-treated U87MG cells upon incubation (RT, 5min) with 500 µl each of Qiazol Reagent (Qiagen, 79306) and PBS. Cell lysates were incubated overnight (-80ºC) after being transferred to fresh 1.5 ml micro-centrifuge tubes. Following the addition of 100 µl chloroform, samples were centrifuged (4ºC, 12 000x*g*, 15 min) and the aqueous phase was transferred to fresh 1.5 ml micro-centrifuge tubes. Thereafter, 100 µl isopropanol was added, incubated overnight (-80ºC) and centrifuged (4ºC, 12000x*g*, 20 mins). Sample supernatant was removed, and RNA pellets were washed with 500 µl ethanol (75%). RNA pellets were air dried (RT, 45 min) following centrifugation (4ºC, 7400x*g*, 15 min), and resuspended in 15 µl nuclease-free water. Isolated RNA was quantified (Nanodrop2000) and standardized to 500 ng/µl. cDNA was synthesized from standardized RNA using the Maxima™ H Minus cDNA synthesis kit (Thermo-Fisher Scientific) with the following Thermocycler conditions: 25ºC for 5 min, 42ºC for 30 min and 85ºC for 5 min. DNMT1, DNMT3A, DNMT3B and MBD2 gene expression was measured using the PowerUp™ SYBR™ Green Master Mix (Thermo-Fisher Scientific). All mRNA expressions were normalized against the housekeeping gene GAPDH. The CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) was used for qPCR, with the following thermal conditions: initial denaturation (8 min, 95°C) followed by 40 cycles of denaturation (15 s, 95°C), annealing (40 s, see Table 2.1 for temperature) and extension (30 s, 72°C). Primer sequences and annealing temperatures for each gene are listed in Table 2.1. The CFX manager software version 3.1 was used to analyse qPCR data following the comparative threshold

(Ct) method (Livak and Schmittgen 2001). Results were represented as mean fold change relative to the control.

Table 2.1: Primer sequences and annealing temperatures used for qPCR.

Gene	**Forward Sequence**	**Reverse Sequence**	**Annealing Temperature**
DNMT1	5' – ACC GCT TCT ACT TCC TCG AGG CCT A– '3	5' – GTT GCA GTC CTC TGT GAA CAC TGT GG– '3	60 ºC
DNMT3A	5' – GGG GAC GTC CGC AGC GTC ACA C – '3	5' – CAG GGT TGG ACT CGA GAA ATC GC – '3	58 ºC
DNMT3B	5' – CCT GCT GAA TTA CTC ACG CCC C – '3	5' – TAA GCC AAA CAG CAG GGT TCT T – '3	58 ºC
MBD2	5' – AGG TAG CAA TGA TGA GAC CCT TTT A– '3	5' –TAA GCC AAA CAG CAG GGT TCT T– '3	56 ºC
GAPDH	5' - TCC ACC ACC CTG TTG CTG TA – '3	5' – ACC ACA GTC CAT GCC ATC AC – '3	---

Protein Isolation and Western Blot

DNMT1, DNMT3A and MBD2 protein expression was determined by western blot. 200 µl cytobuster supplemented with phosphatase and protease inhibitors was used to isolate crude protein from control and FB_1-treated U87MG cells. Cells were incubated on ice (30 min), scraped, and transferred to a fresh 1.5 ml microcentrifuge tube, followed by centrifugation (4ºC, 12000x*g*, 10 min). Crude protein was then quantified by the bicinchoninic acid (BCA) assay (Sheik Abdul, Nagiah, and Chuturgoon 2019b) and standardized to 1 mg/ml. Prior to boiling (100ºC, 5 min), standardized protein samples were diluted in 1x Laemmli buffer [dH_2O, 0.5M Tris-HCl (pH 6.8), glycerol, 10% SDS, 5% β-mercaptoethanol and 1% bromophenol blue]. Standardized proteins were loaded on a sodium dodecyl sulphate polyacrylamide gel (10% resolving gel, 4% stacking gel) and separated by electrophoresis (150V, 1 h). Separated proteins were transferred onto nitrocellulose membranes using the Bio-Rad Trans-Blot® Turbo Transfer System (20 V, 30 min), followed by blocking in 5% BSA (RT, 1 h), made up in Tris-buffer saline (TTBS) [0.05% Tween 20, dH_2O, 3mM KCl, 25mM Tris, 150mM NaCl, pH 7.5]. Membranes were probed overnight (4ºC) with primary antibody (Table 2.2), washed with TTBS (5 times, 10 min), probed with an HRP-conjugated secondary antibody (RT, 1 h) and washed again with TTBS (5 times, 10 min). Clarity Western ECL Substrate (Bio-Rad) detection reagent was used to visualize protein bands on the Thermo-Fisher Scientific iBright™ CL1500 Instrument. Following detection, membranes were quenched in 5% hydrogen peroxide (37ºC, 30 min), washed with TTBS,

blocked, and probed with the housekeeping protein anti-β-actin (RT, 1 h). Protein bands were analysed on the iBright™ analysis software (version 5.2.1) and results were expressed as relative band density.

Table 2.2: Western blot primary and secondary antibodies and their dilutions.

Antibody	**Dilution**	**Company**	**Catalogue number**
DNMT1 (D63A6) Rabbit mAb	2:1000	Cell Signalling	5032 S
DNMT3A (D23G1) Rabbit mAb	2:1000	Cell Signalling	3598 S
Anti-MBD2/3 (D-7) Mouse	1:1000	Santa Cruz	Sc-271562
Anti-Rabbit IgG HRP	2:5000	Cell Signalling	7074S
Anti-mouse IgG HRP	2:5000	Cell Signalling	7076S
Anti-β-Actin	1:5000	Sigma-Aldrich	A3854

Statistical Analysis

All experimental data was analysed on GraphPad Prism version 5.0 (GraphPad Software Inc., California). The one-way analysis of variance (ANOVA) with the Bonferroni multiple comparisons test was used to determine the statistical significance between control, FB_1, and 5-aza-2-DC treatment groups for the DNA methylation assay. The unpaired t-test was conducted to evaluate statistical significance between control and FB_1 treatment groups for qPCR and western blot assays. Data with a *p*-value <0.05 was considered statistically significant, while results were represented as mean ± standard deviation of 3 independent experiments.

Results

FB_1 decreases U87MG cell viability

The MTT assay measures metabolic cellular activity as an indicator of cell viability. U87MG cells were treated with a range of FB_1 concentrations (0 – 1000 μM) for 24 h, from which a dose-response curve was obtained, showing decreased cell viability upon FB_1 treatment (Figure 2.1). An IC_{50} of 880 μM FB_1 in U87MG cells was determined and used for all subsequent experiments.

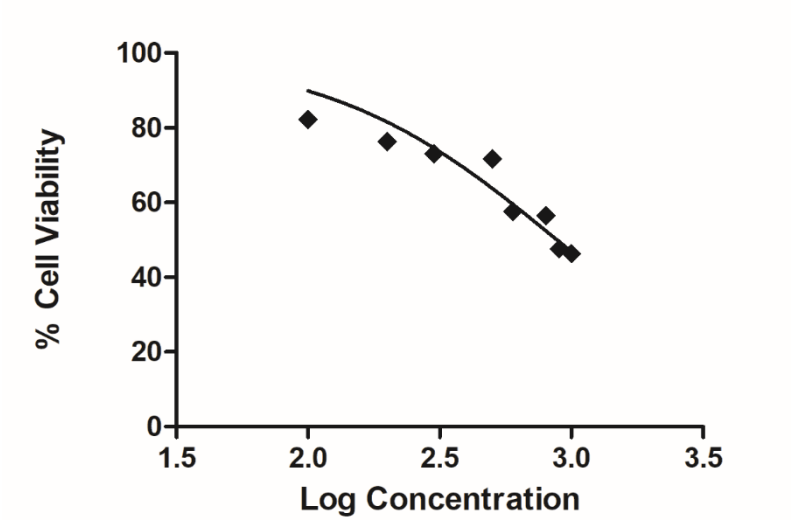

Figure 2.1: The cytotoxic effect of FB_1 on U87MG cells. U87MG cell viability was reduced in a dose-dependent manner following 24 h exposure to FB_1. An IC_{50} of 880 µM FB_1 was determined. FB_1: Fumonisin B_1.

FB_1 increases DNA methylation levels in U87MG cells

The effect of FB_1 on global DNA methylation status in U87MG cells was measured via 5-methylcytosine using the ELISA-based DNA methylation quantification kit. 5-Aza-2-DC was also assessed as a negative control. FB_1 significantly increased 5-methylcytosine content in U87MG cells compared to the control (p=0.0009; Figure 2.2). The DNA hypomethylation agent 5-aza-2-DC significantly reduced 5-methylcytosine content compared to the control (p=0.0009; Figure 2.2). Thus, FB_1 induced global DNA hypermethylation in U87MG cells.

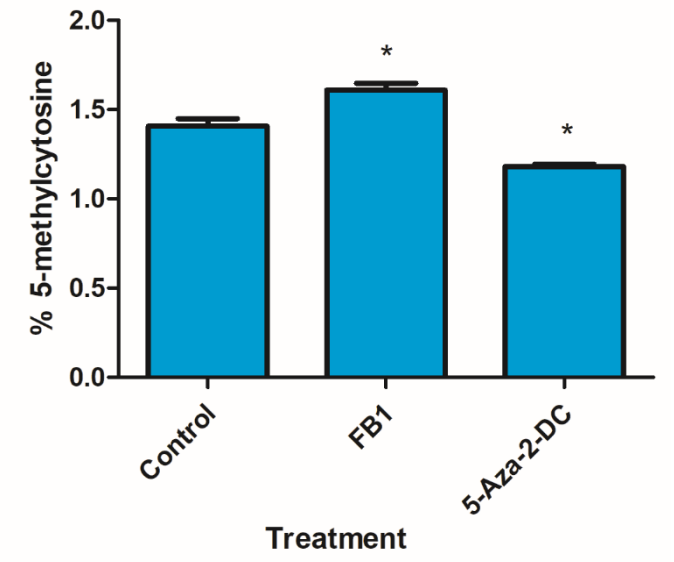

Figure 2.2: FB_1 increased global DNA methylation in U87MG cells. The negative control, 5-aza-2-DC, significantly decreased DNA methylation in U87MG cells. Results are expressed as mean fold change ± SD (n=3) relative to the control. Statistical significance was determined by one way ANOVA with the Bonferroni multiple comparisons test (*p<0.05). FB_1: Fumonisin B_1.

FB_1 upregulates DNA methyltransferases and downregulates DNA demethylase expressions in U87MG cells

The expressions of DNA methyltransferases and DNA demethylase were determined in U87MG cells after treatment with FB_1. mRNA expressions of *DNMT1*, *DNMT3A*, *DNMT3B,* and *MBD2* in FB_1-treated U87MG cells were measured by qPCR. Post FB_1 exposure, *DNMT1* (p=0.0053; Figure 2.3A), *DNMT3A* (p=0.0016; Figure 2.3B) and *DNMT3B* (p=0.0194; Figure 2.3C) expressions were significantly upregulated, while *MBD2* (p=0.0058; Figure 3D) showed a significant downregulation in U87MG cells compared to the control.

Protein expression of DNMT1, DNMT3A and MBD2 were measured by western blot. Consistent with its gene expressions, DNMT1 (p=0.0258; Figure 2.4A) and DNMT3A (p=0.0061; Figure 2.4B) proteins were upregulated while MBD2 protein was downregulated (p= 0.0048; Figure 2.4C) in FB_1-treated U87MG cells compared to the control. This data suggests that FB_1 induced global hypermethylation of DNA in U87MG cells by upregulating DNMTs and downregulating MBD2 expression.

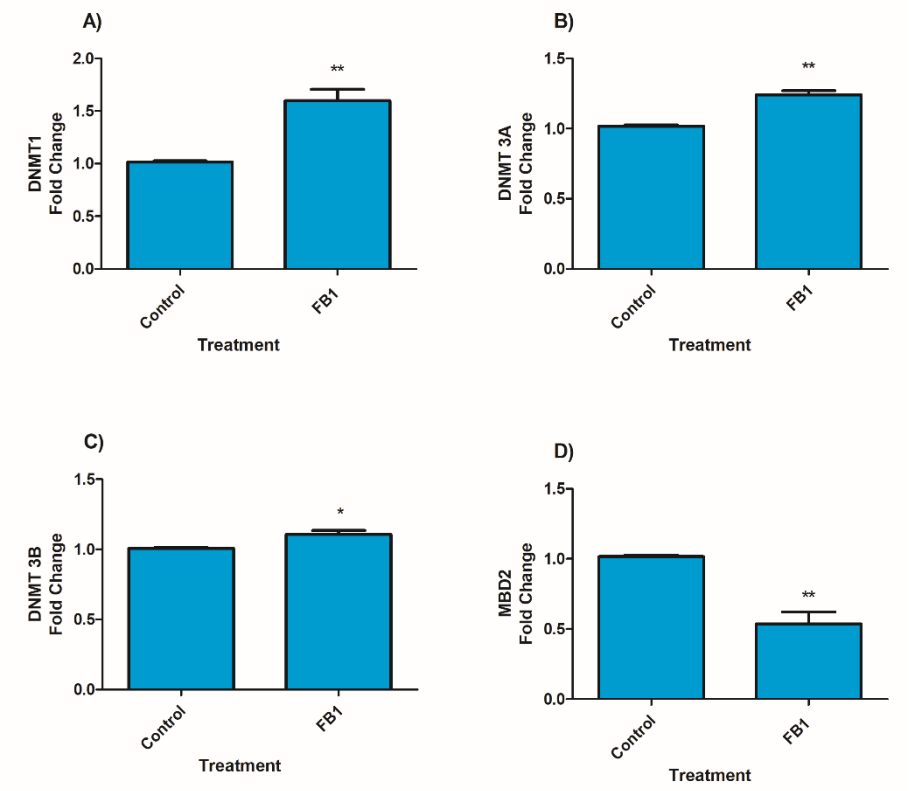

Figure 2.3: FB_1 increased gene expression of *DNMT1* (A), *DNMT3A* (B), *DNMT3B* (C) but decreased *MBD2* (D) expression in U87MG cells. mRNA expression results are expressed as mean fold change ± SD (n=3). Statistical significance for Control vs FB_1 was determined by unpaired t test (*p<0.05, **p<0.01). FB_1: Fumonisin B_1; DNMT: DNA methyltransferase; MBD2: Methyl-CpG binding domain 2.

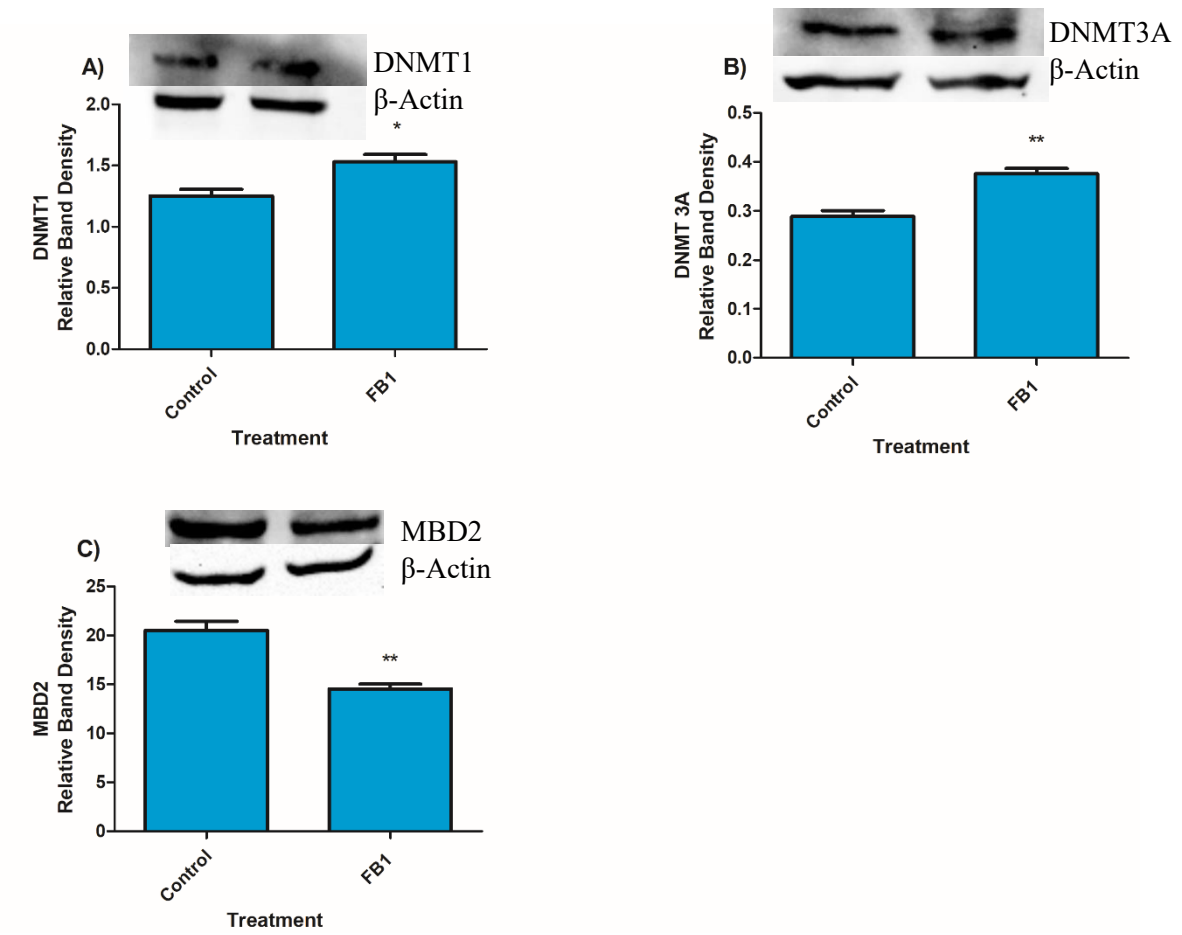

Figure 2.4: FB_1 increased protein expressions of DNMT1 (A) and DNMT3A (B) but decreased MBD2 (C) expression in U87MG cells. Protein results are expressed as relative band density to the control ± SD (n=3). Statistical significance for Control vs FB_1 was determined by unpaired t test (*p<0.05, **p<0.01). FB_1: Fumonisin B_1; DNMT: DNA methyltransferase; MBD2: Methyl-CpG binding domain 2.

Discussion

Annually, global food security is threatened as nearly 25% of foodstuffs are contaminated by mycotoxins (Omotayo et al. 2019). Mycotoxins are produced by fungi that target agricultural crops in response to environmental factors including humidity, pest infestation, and inappropriate storage and harvest operations (Omotayo et al. 2019). The ingestion of mycotoxin-contaminated food and feed products proves detrimental to health as it may result in cancer initiation and progression (Ghazi et al. 2020). FB_1 has previously displayed tumour-aggravating characteristics (Gelderblom et al. 1988b), which may be attributed to its ability to disrupt sphingolipid biosynthesis. FB_1 consumption has also been linked to heightened primary liver and oesophageal cancer risks in residents of South Africa and China (Rheeder et al. 1992). The present study was thus designed to investigate the toxic potential of FB_1 in glioblastoma U87MG cells. We determined the cytotoxic effect of FB_1 in U87MG cells via the

MTT assay. U87MG cells incubated with increasing FB_1 concentrations demonstrated high tolerance to FB_1 exposure, yielding a high IC_{50} of 880 μM (Figure 2.1). Our finding is parallel with a study on FB_1 in HK-2 cells (225 to 1100 μM FB_1) which revealed low cell death potential (Pinhão et al. 2020). Similarly, FB_1 toxicity was minimal in porcine kidney and swine jejunal epithelial cells (Wan, Turner, and El-Nezami 2013), while HepG2, Caco-2 and Madine-Darby bovine kidney cells showed no FB_1 cytotoxicity (Clarke et al. 2014; Sobral et al. 2018). While our study is the first to report an IC_{50} for FB_1 in U87MG cells, previous research has reported significantly lower FB_1 IC_{50} values in various cell lines including C6 glioma cells (Mobio et al. 2000), neuroblastoma SH-SY5Y cells (Krupashree and Rachitha 2022), mouse primary neuronal and astroglia cells (Szentgyörgyi et al. 2024), and HepG2 cells (Chen et al. 2022). Interestingly, Chen et al. (2022) observed a relatively high IC_{50} (589.7 ± 23.4 μg/mL**)** in Caco-2 cells upon 24 h exposure to FB_1, suggesting that the differences in FB_1 cytotoxicity may be influenced by the cell line. Additionally, GBM is the most aggressive brain tumour in adults, with several molecular markers identified to aid in its diagnosis, prognosis, and treatment (Nie et al. 2015). Glioblastomas are heterogeneous tumours consisting of several sub clonal driver mutations, rendering them highly adaptable and resistant to treatment, while increasing their survival (Prager et al. 2020). Notably, mutations in isocitrate dehydrogenase 1 (IDH1), particularly the IDH1R132H mutation, are a common feature of a major subset of human gliomas and are commonly found in secondary GBM, which develops from lower-grade gliomas (Hill et al. 2014). Interestingly, patients with IDH1R132H mutations tend to have significantly better survival rates (Yan et al.). Therefore, it may be worth exploring the role of IDH1 mutations in U87MG cells in relation to its high tolerance to FB_1 cytotoxicity, yielding a high IC_{50}. Also, FB_1's key mode of action is via sphingolipid metabolism disruption by inhibiting ceramide synthase. However, sphingolipids help control glioma cell growth and response to chemotherapy. Ceramide acts as a tumour suppressor by slowing cell growth and triggering cell death in tumours like GBM (Bassi et al. 2023). Therefore, altered ceramide synthase metabolism by FB_1, may reduce U87MG cells susceptibility to the toxin's effects.

Advancements in molecular pathology have revealed that abnormal epigenetic mechanisms, including histone modifications, altered non-coding RNA expression, DNA methylation, and chromatin remodelling, are well-documented contributors of glioma progression and development (Uddin et al. 2022; Jones and Baylin 2007). DNA methylation involves the addition of a methyl group to the 5′ cytosine of CpG sites. This modification leads to chromatin condensation, thereby restricting transcription. Mechanistically, DNA methylation can repress transcription by recruiting methyl-CpG-binding proteins like MECP2 to methylated CpG dinucleotides (Fuks et al. 2003). These proteins, in turn, attract histone deacetylases (HDACs), which further reinforce a closed chromatin state (Roos-Araujo et al. 2014). Additionally, this compact structure inhibits the transcriptome complex, including DNA polymerase, from accessing promoter regions and ultimately suppresses gene expression.

Through this coordinated regulation, DNA methylation plays a crucial role in controlling gene activity (Roos-Araujo et al. 2014; Riggs 1975). Furthermore, DNA wraps around histones which undergo various post-translational modifications, including acetylation, methylation, phosphorylation, and ubiquitination (Bhaumik, Smith, and Shilatifard 2007; Kouzarides 2007). These modifications influence DNA-histone interactions and chromatin structure. For instance, histone acetyltransferases (HATs) add acetyl groups to lysine residues, generally leading to a more open chromatin state that promotes transcription, while HDACs remove these groups, resulting in chromatin condensation and transcriptional gene repression (Guil and Esteller 2009). Key histone modifications linked to DNA methylation include the repressive marks trimethylation of lysine 27 in histone 3 (H3K27me3) and lysine 36 in histone 3 (H3K36me3). H3K27me3 is enriched in methylated DNA regions with low transcription and aids in DNMT recruitment (Schlesinger et al. 2007). It is also abundant in aberrantly methylated cancer genes (Schlesinger et al. 2007), especially with DNMT3B overexpression (Zhang, Charlton, et al. 2018). H3K36me3 methylation results in DNA methylation distribution (Wagner and Carpenter 2012), while it also enhances DNMT3A activity and its DNA binding (Dhayalan et al. 2010). Trimethylation of lysine 9 in histone 3 (H3K9me3), established by SUV39H, overlaps with pericentromeric and heterochromatic regions, promoting DNA methylation via DNMT-3A and -3B (Lehnertz et al. 2003). The SUV39H-HP1 complex is crucial for this process, while UHRF (ubiquitin-like containing plant homeodomain and RING finger domain) binds H3K9me2/3 for DNMT1-mediated maintenance methylation (Nishiyama et al. 2020). In contrast, trimethylation of lysine 4 in histone 3 (H3K4me3) is mutually exclusive with DNA methylation and is enriched in active promoters (Loaeza-Loaeza, Beltran, and Hernández-Sotelo 2020). Earlier studies have also shown that FB_1 alters global DNA methylation, histone modifications, and chromatin-regulating enzymes in rat foetuses (Pellanda et al. 2012) and HepG2 cells (Chuturgoon, Phulukdaree, and Moodley 2014). Thus, epigenetic changes in histone modifications may play a key role in FB_1's carcinogenic mechanism.

Alternatively, growing evidence has shown that many microRNA (miRNA) genes also undergo epigenetic modifications. miRNA genes that are actively transcribed produce primary miRNA transcripts, which undergo a two-step process to become mature, functional molecules about 21 nucleotides long. These miRNAs join the RNA-induced silencing complex to regulate target mRNAs (Guil and Esteller 2009). This regulation can directly or indirectly affect DNA and histone-modifying enzymes, as well as chromatin remodelling factors. When standard regulation is disrupted, whether naturally or due to disease, various repressive epigenetic marks work together to silence specific genomic regions. As a result, when miRNA genes are silenced, it can lead to altered expression of multiple downstream target genes (Guil and Esteller 2009). The miR-29 family (miR-29a, miR-29b, miR-29c), a key epigenetic regulator, modulates DNA methylation by targeting DNMTs. In lung cancer, where DNMT3A and DNMT3B are often upregulated and linked to poor prognosis, miR-29 expression

is inversely correlated with these DNMTs and directly suppresses them (Fabbri et al. 2007). Ectopic miR-101 reduces DNMT3A expression and global DNA methylation in the lung, while its loss accelerates tumorigenesis through DNMT3A-dependent methylation (Yan et al. 2014). In laryngeal squamous cell carcinoma, miR-148a-3p targets DNMT1, reducing DNA methylation (Wu, Qu, et al. 2016). In colorectal cancer, miR-124 and miR-506 are downregulated, leading to increased DNMT3B (directly) and DNMT1 (indirectly) (Chen, Liu, et al. 2015). In astrocytoma cells, miR-101 directly targets DNMT3A (Lei et al. 2015). DNMT1 has been identified as a high-scoring target of miR-152. In bladder cancer cells and tissue miR-152 expression was downregulated due to promoter DNA hypermethylation (Zhang, Qi, et al. 2018). This study also revealed that miR-152 expression was inversely proportional to DNMT1 expression, and confirmed that miR-152 directly targets the 3' untranslated region (3′-UTR) of DNMT1, suppressing its expression (Zhang, Qi, et al. 2018). Similarly, in nickel sulphide (NiS)-transformed human bronchial epithelial (16HBE) cells, miR-152 was due to promoter hypermethylation, while its ectopic expression significantly reduced DNMT1 levels by directly targeting its 3′-UTR (Ji et al. 2013). Conversely, miR-152 inhibition in normal 16HBE cells increases DNMT1 expression and DNA methylation, establishing a double-negative feedback loop between miR-152 and DNMT1 (Ji et al. 2013). These findings highlight miRNAs as key regulators of DNA methylation in cancer by targeting DNMTs.

On the other hand, oxidative stress plays a crucial role in the onset and progression of various human diseases, including neurodegenerative disorders. Growing evidence indicates that oxidative stress broadly impacts chromatin structure, DNA methylation, and post-translational modifications of histones and DNA-binding proteins (Kreuz and Fischle 2016). These alterations influence key cellular processes such as gene expression, cell survival, apoptosis, and mutagenesis, all of which contribute to disease development and progression (Kreuz and Fischle 2016). Oxidative stress disrupts redox signalling and homeostasis, leading to molecular damage, with reactive oxygen species (ROS) being the primary oxidants driving this process. Uncontrolled ROS has been implicated in altering DNA methylation levels (Franco et al. 2008). For instance, hydrogen peroxide acts as a nucleophile, deprotonating cytosine at the carbon 5 position, which enhances its reaction with the positively charged intermediate S-adenosylmethionine during DNA methylation (Afanas'ev 2013). DNA methylation may also be directly inhibited via the depletion of S-adenosylmethionine by oxidative stress, through various mechanisms (Kreuz and Fischle 2016). Glutathione (GSH) is a key antioxidant involved in various biological processes. Disruptions in GSH synthesis or depletion can lead to global DNA hypomethylation, likely due to reduced levels of S-adenosylmethionine (Cyr and Domann 2010; García-Giménez et al. 2017). S-adenosylmethionine is synthesized from methionine by methionine-adenosyl transferase (MAT) before being utilized (García-Giménez et al. 2017). Both MAT and methionine synthase are highly sensitive to oxidative stress and GSH balance, which can impair

methyltransferase activity and lower global DNA methylation under conditions of redox imbalance (García-Giménez et al. 2017; Cyr and Domann 2010). ROS can also influence the expression of DNMTs (Rang and Boonstra 2014). *In vitro* studies, in SH-SY5Y human neuroblastoma cells, showed that short-term exposure (1 h) to hydrogen peroxide increased global DNA methylation levels, whereas prolonged exposure (72 h) led to a reduction in DNA methylation, accompanied by decreased expression of DNMT1, DNMT3A, and DNMT3B (Gu et al. 2013). Additionally, peroxides can modify nucleobases, producing 5-chlorocytosine, which mimics 5-methylcytosine and disrupts proper DNMT1 methylation within CpG sequences, leading to gene silencing (Lao et al. 2009). High oxidative stress may hinder the catalytic cycle of iron, leading to inhibition of the ten-eleven translocation (TET) family DNA demethylases and resulting in increased DNA methylation levels (Kreuz and Fischle 2016). Reportedly, oxidative stress-induced ROS production also enhances 8-hydroxydeoxyguanosine (8-OHdG) levels in certain cancer types. 8-OHdG causes conformational changes that shift chromatin from an active to a repressive state, potentially contributing to tumorigenesis by altering the methylation patterns of tumour suppressor genes (Nishida et al. 2013). Furthermore, 8-OHdG restricts DNMTs' ability to bind DNA thus leading to global genome hypomethylation (Udomsinprasert et al. 2016; Ziech et al. 2011). In CpG dinucleotides, cytosine is the primary site for DNA methylation, while guanine is highly susceptible to oxidative damage, with the guanine oxidation product, 8-oxoguanine, being a key marker of oxidative DNA damage (Cheng et al. 1992; Donkena, Young, and Tindall 2010a). Additionally, the methyl group on 5-methylcytosine is vulnerable to oxidation, producing 5-hydroxymethylcytosine (Masuda, Shinohara, and Kondo 1975; Donkena, Young, and Tindall 2010a). This modification disrupts sequence-specific DNA-protein interactions by altering the binding affinity of methyl-binding proteins, potentially leading to heritable epigenetic changes (Valinluck et al. 2004b). Conversely, changes in DNA methylation may regulate the expression of oxidative stress-related genes (Niu et al. 2015b). Despite the biological plausibility, human population-based studies examining the relationship between oxidative stress biomarker levels and DNA methylation of oxidative stress-related genes remain limited, with even less research on how oxidative stress-driven DNA methylation changes may contribute to cancer development (Schöttker et al. 2015).

Research on epigenetic changes in GBM has primarily focused on DNA methylation, highlighting genome-wide hypomethylation, gene-specific hypomethylation, and hypermethylation as significant factors (Kloosterhof et al. 2013; Uddin et al. 2022). Although multiple epigenetic and non-epigenetic pathways contribute to tumourigenesis, this paper specifically examines the impact of FB_1 on DNA methylation to offer a preliminary understanding of the epigenetic characteristics of U87MG cells.

Mounting research has identified DNA methylation as a key regulator of the human brain through development, cell-type differentiation, and disease vulnerability (Jeong et al. 2021). Due to its inactivity in genotoxicity assays (Norred et al. 1992) and mutagenicity (Gelderblom and Snyman 1991) and its inability to bind to DNA, FB_1 is regarded as a non-genotoxic (epigenetic) carcinogen. Herein, we provide evidence that FB_1 alters the epigenetic landscape of U87MG cells. In humans, alterations to genomic 5-methylcytosine quantity and distribution play a key role in development and diseases, with the bulk of 5-methylcytosine being present at CG dinucleotides (Ehrlich 2019). 5-methylcytosine has been classified as the 5th base of the human genome as it functions in repressing transcriptional activities (Wu and Morris 2001), while aberrant 5-methylcytosine patterns may result in developmental disorders including growth abnormalities, global developmental delays, movement disorders and intellectual disability (Lister et al. 2013). In this study, global DNA methylation status in U87MG cells was determined by quantifying the 5-methylcytosine content post 24 h FB_1 exposure (Figure 2.2). We found that FB_1 induced DNA hypermethylation by significantly increasing the 5-methylcytosine percentage in FB_1-treated cells as compared to the control and the negative control 5-aza-2-DC (Figure 2.2). DNA hypermethylation functions in controlling DNA replication and gene expression in cell differentiation and division processes (Doerfler 1983; Mobio et al. 2000). Our findings are similar to Mobio et al. (2000) who obtained significant hypermethylation of DNA in C6 glioma cells following FB_1 treatment (9 – 18 μM). Additionally, FB_1 exposure (100 μM) significantly increased 5-methylcytosine percentage and global DNA methylation in genomic DNA of HEK293 cells (Sugiyama et al. 2021) while 5-methylcytosine percentage increased by approximately 8% in the DNA of Caco-2 cells following FB_1 treatment (Kouadio et al. 2007). Interestingly, in HepG2 cells FB_1 increased global m6A RNA modifications (Arumugam, Ghazi, and Chuturgoon 2021) but induced global DNA hypomethylation (Chuturgoon, Phulukdaree, and Moodley 2014). In epileptic rat hippocampus, global DNA methylation was implicated as the disease phenotype (Williams-Karnesky et al. 2013), whereas Bradley-Whitman and Lovell (2013) observed a significant increase in 5-methylcytosine levels in human hippocampus of Alzheimer's disease brain. Additionally, Semick et al. (2019) reported DNA methylation as an underlying epigenetic mechanism for molecular pathological alterations linked to Alzheimer's disease, while Coppieters et al. (2014) observed global hypermethylation in Alzheimer's disease brain with increased 5-methylcytosine levels in neurons. In an Alzheimer's disease and bipolar disorder brain study, Rao et al. (2012) detected global hypermethylated DNA suggesting an epigenetic association of these disorders with reduced transcriptional activity. Disease-specific hypermethylation was also discovered in the promoter of CREB in the frontal cortex of Alzheimer's brain (Rao et al. 2012). Moreover, Hannon et al. (2016) revealed that CG sites with single nucleotide polymorphisms related to DNA methylation contribute to the risk of schizophrenia in human foetal samples. DNA methylation regulation is also known to influence vital neurobiological and cognitive functions such as memory maintenance and formation (Day and Sweatt 2010), circadian processes (Azzi et al. 2014) and neuronal plasticity (Borrelli et al. 2008). Gliomas, pose major challenges in neuro-oncology while its treatment

outcomes and prognosis are heavily impacted by genetic and epigenetic factors (Chen et al. 2024). Gliomas frequently display aberrant DNA methylation, which can silence tumour suppressor and other key regulatory genes. The interaction between genetic mutations and epigenetic changes plays a vital role in glioma pathogenesis. Glioma CpG island methylator phenotype is a distinctive hypermethylation pattern that is linked to better prognosis and survival (Christensen et al. 2011). This phenotype arises from IDH mutations, resulting in global DNA hypermethylation which impacts gene expression and tumour behaviour (LeBlanc and Marra 2016). Studies have identified specific DNA methylation patterns in gliomas, particularly in promoter regions of genes regulating the cell cycle, apoptosis, and DNA repair (Weng and Salazar 2021). For example, aberrant O6-Methylguanine-DNA methyltransferase (MGMT) promoter methylation is a well-known marker associated with better responses to alkylating agents and increased survival (Aoki and Natsume 2019). DNA methylation profiles are also beneficial for classifying gliomas into prognostic subgroups, with certain methylation markers indicating poorer survival outcomes (Chen et al. 2019). Moreover, DNA methylation functions not only as an epigenetic modification that influences chromatin structure and regulates gene transcription but also increases the mutation rate of methylated DNA sequences by prompting the transition of cytosine to thymine (J. Dabrowski and Wojtas 2019).

The expression of DNMTs is vital to DNA methylation patterns and is closely regulated during various developmental stages (Sugiyama et al. 2021). DNMTs facilitate the addition of a methyl group to carbon 5 of a cytosine residue. DNMT1 is highly expressed in mammalian tissues, including the brain, and preferentially methylates hemi-methylated DNA (Moore, Le, and Fan 2013). During DNA replication, DNMT1 positions at the replication fork, where it attaches to and methylates newly synthesized DNA, reproducing the original methylation pattern that was present prior to replication (Hermann, Goyal, and Jeltsch 2004). DNMT1 also holds DNA methylation repair properties and is thus known as the maintenance DNMT (Moore, Le, and Fan 2013). DNMT3A and DNMT3B share similar structures and both function in methylating DNA with no preference for hemi-methylated DNA (Okano et al. 1999). Known for their ability to introduce new methylation patterns into DNA, DNMT3A and DNMT3B are distinguished by their gene expression patterns, where DNMT3A is ubiquitously expressed while DNMT3B is poorly expressed in most differentiated tissues (Xie et al. 1999). Moreover, the dynamic expression of DNMT3A and DNMT3B during prenatal brain development endorses the significance of DNA methylation in neurodevelopment (Feng et al. 2005). Following the global DNA hypermethylation pattern observed, we examined the expressions of DNMTs in FB_1-treated U87MG cells. qPCR analysis revealed that *DNMT1* (Figure 2.3A), *DNMT3A* (Figure 2.3B) and *DNMT3B* (Figure 2.3C) gene expressions were upregulated by FB_1 in U87MG cells relative to the control. Similarly, DNMT1 and DNMT3A protein expressions were also elevated in FB_1 exposed U87MG cells (Figures 2.4A and 2.4B). The increase in DNMT activities (Figure 3A – C and Figure 4A

– B) correlates to the hypermethylated state of DNA (Figure 2.2) in U87MG cells. In HEK293 cells, Sugiyama et al. (2021) also showed that FB_1 activated DNMT activity, leading to increased global DNA methylation. The overexpression of DNMTs has also been observed in cancer studies. Results from a human hepatocellular carcinoma study suggested that hepatocarcinogenesis is associated with escalated mRNA expression of *DNMT1*, *DNMT3A* and *DNMT3B*, along with a progressive increase in the number of methylated genes (Park, Yu, and Shim 2006). Lin et al. (2007) concluded that increased expression and binding of DNMTs ultimately results in lung tumourigenesis and poor prognosis, while Girault et al. (2003) reported increased DNMTs in breast carcinoma, with DNMT3B showing the highest degree of overexpression. Likewise, increased mRNA expression of DNMT1 was linked to DNA hypermethylation in gastric and colorectal cancer (Kanai et al. 2001). To validate the role of DNMTs in schizophrenia, Zhubi et al. (2009) compared the *DNMT* expressions of nonpsychiatric subjects with schizophrenia patients. They found that *DNMT1* and *DNMT3A* were overexpressed and co-localized in neuron populations, while *DNMT3B* was under expressed (Zhubi et al. 2009). Likewise, Zhu et al. (2012) also observed an increase in DNMT1 and DNMT3A expressions in human temporal lobe epilepsy compared to control patients, suggesting that DNMT expressions may promote epileptogenesis by influencing synaptic plasticity. Additionally, a significant overexpression of DNMT1 and DNMT3B was observed in glioma tumours and the glioblastoma cell lines U87MG and LN18, resulting in aberrant genomic stability and impaired cell cycle progression (Rajendran et al. 2011). These authors suggested that DNMT1 and DNMT3B overexpression in gliomas cause hypermethylation of several tumour suppressor genes, contributing to poor prognosis, and serves as a cancer cell marker and potential target for cancer therapies. Sun et al. (2017) evaluated the expression of DNMT1 to assess its role in the regulation of tumour suppressor gene neurofibromatosis type 2 (NF2) in gliomas. They observed increased *DNMT1* mRNA expression in GBM cell lines (U251, U87, T98-G and A172) compared to normal glial cells, suggesting that NF2 may play a role in GBM development, potentially via DNMT1 regulation (Sun et al. 2017).

In contrast to DNMTs, the DNA demethylase MBD2 regulates the removal of a methyl group from cytosine residues to promote DNA hypomethylation. Following the rise in DNMT expressions observed, we measured the expression of MBD2 and discovered that FB_1 significantly downregulated MBD2 gene (Figure 2.3D) and protein (Figure 2.4B) expressions in U87MG cells compared to the control. This indicates that FB_1 prevents MBD2 from removing methyl groups from the DNA of U87MG cells, thus initiating DNA hypermethylation. MBD proteins have a high affinity for single methylated CG dinucleotides and are vital for neuronal growth and activity as it is highly expressed in the brain (Moore, Le, and Fan 2013). Considering this, the FB_1-induced MBD2 loss in U87MG cells would lead to altered brain development. In human colorectal and stomach cancers, Kanai et al. (1999) observed a decrease in MBD2 mRNA expression and suggested that reduced DNA demethylase

expression may play a role in carcinogenesis. Reduced DNA demethylase expression may influence DNA hypermethylation and promote its maintenance, thus attenuating tumour suppressor gene expressions in human cancers (Kanai et al. 1999). In the hippocampal of rat pups, decreased MBD2 expression was associated with increased stress and repression of the glucocorticoid-receptor (Weaver et al. 2014). In mice, Lax et al. (2023b) discovered that MBD2 deficiency increased methylation and decreased neuronal-gene expression, suggesting that MBD2 is linked to hippocampal genome functions and Autism Spectrum Disorder. Loss of MBD2 in mice has also led to maternal behavioural abnormalities, suggesting that MBD2 contributes to neural processing (Hendrich et al. 2001a). Furthermore, MacDonald et al. (2010) revealed that MBD2 deficit led to dysregulated adult olfactory epithelium progenitor-driven neurogenesis, and impaired survival and synaptic fields of olfactory receptor neurons in mice. Taken together, the present data offers a role for FB_1 as a toxin through DNA methylation modifications in U87MG cells.

Conclusion

FB_1 induced epigenetic modifications by increasing global DNA methylation in the human glioblastoma U87MG cell line. The global DNA hypermethylation was mediated via a DNMT-dependent manner, as FB_1 enhanced expressions of DNMT1, DNMT3A and DNMT3B, while suppressing demethylase (MBD2) transcript and protein expression levels (Figure 2.5). This finding suggests an alternative mechanism for FB_1-induced toxicity via DNA methylation in the human glioblastoma U87MG cell line; however, future work is required to determine this in an *in vivo* model.

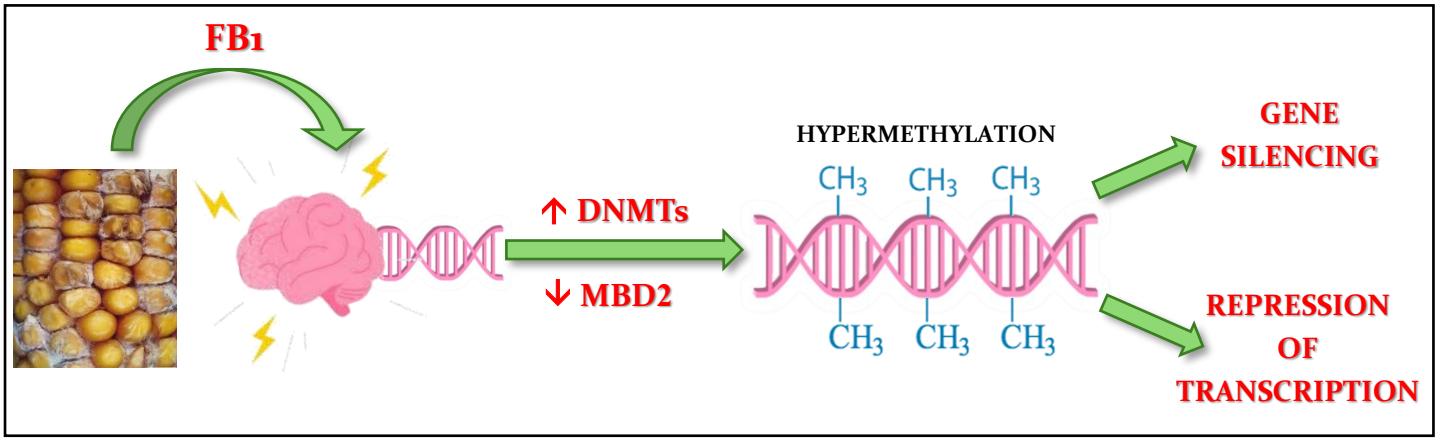

Figure 2.5: FB_1 induces global DNA hypermethylation in U87MG cells. FB_1 enhances 5-methylcytosine content via increased expression of DNMTs and decreased MBD2 expression. DNA hypermethylation is associated with the silencing of genes and transcriptional repression, ultimately resulting in aberrant cellular function. FB_1: Fumonisin B_1; DNA: Deoxyribonucleic acid; DNMTs: DNA methyltransferases; MDB2: Methyl-CpG binding domain 2.

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CHAPTER 3

Fumonisin B_1 ameliorates cellular oxidative stress by reducing antioxidant activity and maintains mitochondrial stress markers but alters TFAM in human glioblastoma U87MG cells

Chapter 2 reports on FB_1-induced global DNA hypermethylation, which is important in regulating DNA replication and gene expression. Changes in DNA methylation patterns may be influenced by excessive ROS levels. Hydrogen peroxide mimics a nucleophile to deprotonate the 5^{th} carbon of the cytosine molecule which is involved in the reaction between DNA and S-adenosylmethionine during DNA methylation (Afanas'ev 2014). Additionally, ROS may also influence the activity of DNA methyltransferase enzymes (DNMTs) (Rang and Boonstra 2014), while DNA methylation can alter oxidative stress-related genes (Niu et al. 2015a). Elevated ROS levels significantly contribute to oxidative damage to DNA as well as altered mitochondrial functions. Therefore, this chapter explored the effects of FB_1 on oxidative stress and mitochondrial stress.

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Fumonisin B_1 ameliorates cellular oxidative stress by reducing antioxidant activity and maintains mitochondrial stress markers but alters TFAM in human glioblastoma U87MG cells

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Abstract

Fumonisin B_1 (FB_1) is among the most toxic mycotoxins that commonly contaminate maize. FB_1 has been implicated in cytotoxicity, hepatotoxicity, nephrotoxicity, and neurotoxicity, while it has also been classified as a carcinogen in humans. Oxidative stress is a cellular mechanism by which FB_1 induces its toxicity. The effect of FB_1 was investigated on oxidative and mitochondrial stress pathways in the human glioblastoma U87MG cell line. The TBARS assay displayed a decrease in lipid peroxidation, followed by decreased antioxidant responses (SOD2, CAT, GPx and NRF2), as assessed by qPCR and western blot. FB_1 also inhibited oxidative DNA damage via *OGG1* gene regulation. Western blot analysis further revealed that mitochondrial well-being was conserved by the FB_1-induced upregulation of SIRT3, LonP1 and HSP60; however, FB_1 altered mitochondrial DNA via TFAM downregulation. Here we suggest a potential role of FB_1 as a selective neuroprotective marker through downregulation of oxidative stress and mitochondrial stress responses and as a neurotoxic marker through mitochondrial DNA dysregulation.

Keywords: Oxidative Stress, Mitochondrial Stress, Antioxidants, Reactive Oxidative Species, DNA Damage, Fumonisin B_1

Introduction

A typical African diet consists of maize which is highly susceptible to fungal contamination by toxic secondary metabolites known as mycotoxins (Fandohan et al. 2003). Fumonisins are mycotoxins produced by *Fusarium verticillioides* and are common fungal maize contaminants of which Fumonisin B_1 (FB_1) is the most noxious and widespread, out of 28 analogues (Li, Zhao, et al. 2021). FB_1 shares structural similarities with sphingoid bases and exhibits its toxicity through *de novo* sphingolipid biosynthesis disruption. Consequently, FB_1 inhibits ceramide synthase by restricting sphingosine acylation to ceramide while also inhibiting sphinganine conversion to dihydroceramide (Chen, Wei, et al. 2021b; Wang et al. 1991). This disruption of sphingolipid metabolism results in an accumulation of

free sphingoid bases and its metabolites, leading to cytotoxicity, carcinogenesis, cell proliferation and apoptosis (Riley et al. 2001). Animal studies suggest that the liver and kidney are key FB_1 toxicity targets, with additional effects reported in porcine pulmonary oedema, equine leukoencephalomalacia and rodent carcinomas (Arumugam et al. 2019). In humans, dietary FB_1 exposure has been linked to elevated occurrences of oesophageal cancer and primary liver cancer (Domijan and Abramov 2011), while FB_1-induced sphingolipid depletion, in mice and humans, is believed to reduce folate uptake, thus inducing neural tube defects (Stevens and Tang 1997b; Stockmann-Juvala et al. 2004a). The FB_1 neurotoxic potential is well established and extensively reviewed by Domijan (2012). Some researchers have documented oxidative stress as a FB_1 neurotoxicity mechanism (Stockmann-Juvala et al. 2004a), while others suggest that oxidative stress is a resultant rather than a cause of FB_1 neurotoxicity (Galvano, Campisi, et al. 2002).

Oxidative stress occurs when the antioxidant defense system is unable to overcome the rise in production of reactive oxygen species (ROS) (Mary et al. 2012). Living organisms produce ROS in response to cellular metabolism and environmental factors. At low to moderate levels, ROS functions in physiological cell processes, while at elevated levels they induce adverse modifications to lipids, proteins and DNA (Birben et al. 2012). To maintain homeostasis, cells recruit antioxidants to scavenge ROS and overcome oxidative insult to macromolecules (Birben et al. 2012). Key enzymes of the antioxidant defense system include superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) (Atika and Naouel 2021). Furthermore, the transcription factor, nuclear factor erythroid 2 p45–related factor 2 (NRF-2) plays a role in cellular resistance to oxidative stress and regulation of the antioxidant defense (Ma 2013). Due to its high affinity towards DNA guanine bases, ROS also induces damage to DNA, resulting in mutations and lesions (Nagiah, Phulukdaree, and Chuturgoon 2015). 8-hydroxyguanine is the oxidized form of guanine and a major lesion generated by ROS in DNA which is implicated in carcinogenesis (Boiteux and Radicella 2000). The base-excision repair pathway defends against ROS-induced oxidative DNA damage, by catalysing the removal of 8-hydroxyguanine from DNA (Bravard et al. 2006). This repair pathway is initiated by lesion-specific DNA glycosylases, of which OGG1 is the principal glycosylase that identifies and erases 8-hydroxyguanine lesions in nuclear and mitochondrial DNA (Jensen et al. 2003).

Mitochondria perform a key role in cellular redox homeostasis as electron leakage through the electron transport chain (ETC) generates ROS (Valera-Alberni and Canto 2018). Studies have highlighted the protective role of sirtuin 3 (SIRT3) against ROS production via activation of antioxidant enzymes (Bause and Haigis 2013). Additionally, Lon protease 1 (LonP1) is a target of SIRT 3 deacetylation (Gibellini et al. 2014), while LonP1 eradicates oxidative stress by eliminating oxidized proteins (Pinti

et al. 2015). The molecular chaperone, heat shock protein 60 (HSP60), also plays a protective role by maintaining protein homeostasis of the mitochondria (Caruso Bavisotto et al. 2020). Optimal cellular functions also depend on mitochondrial DNA integrity. The mitochondrial transcription factor A (TFAM) binds to DNA, thus stabilizing mitochondrial DNA via nucleoid formation (Kang, Kim, and Hamasaki 2007).

It is well known that ROS production and mitochondrial metabolism alterations are implicated in neurodegeneration (Domijan and Abramov 2011). Research has documented the brain as a target of FB_1 toxicity (Domijan 2012), while numerous studies have also investigated the effect of FB_1 on oxidative damage. Moreover, the brain is a major oxygen consumer, thus increasing its susceptibility to ROS-mediated toxicities (Lushchak et al. 2021). Thus, this study further investigates the pathway of FB_1 toxicity, via endogenous and mitochondrial oxidative insults in the human glioblastoma U87MG cell line.

Methods and Materials

Materials

The U87MG cell line (HTB-14) was purchased from the American Type Culture Collection (Johannesburg, SA). Cell culture reagents were purchased from Lonza Biotechnology (Basel, Switzerland). Western Blot reagents were purchased from Bio-Rad (Hercules, CA, USA), while antibodies were obtained from Cell Signalling Technologies (Danvers, MA, USA), unless otherwise stated. qPCR primer sequences were obtained from Inqaba Biotechnical Industries (Johannesburg, SA). All other reagents were purchased from Merck (Darmstadt, Germany), unless otherwise stated.

Cell Culture and Treatments

U87MG cells were cultured (37 ºC, 5% CO_2) in Dulbecco's minimum essentials medium (DMEM) supplemented with 25 mM HEPES, 10% foetal bovine serum, 1% pen-strep-fungizone and 1% L-glutamine. The FB_1 stock of 5 mM was prepared with 0.1 M phosphate buffer saline (PBS). For all subsequent experiments, cells were cultured to 80% confluency in 25 cm^3 flasks prior to treatment of 880 µM FB_1 (IC_{50} as determined in Chapter 2) in DMEM for 24 h. A control, consisting only of DMEM was also prepared for 24 h.

Lipid peroxidation assessment

Oxidative damage was measured by the thiobarbituric acid reactive substances (TBARS) assay (Sheik Abdul, Nagiah, and Chuturgoon 2016) where levels of the lipid peroxidation by-product malondialdehyde (MDA) was quantified. 200 µl supernatant collected from FB_1-treated and control culture flasks were added to separate test tubes. Additionally, a positive control tube containing 1 µl MDA and a negative control containing 400 µl of 3 mM HCl, were set up. 200 µl each of 2% H_3PO_4, 7% H_3PO_4 and 1M HCl were added to each tube, followed by 400 µl thiobarbituric acid butylated hydroxytoluene (TBA/BHT) solution to the FB_1-treated, control and positive control. All tubes were then boiled for 15 min and allowed to cool to RT before 1500 µl butanol was added to each tube. All tubes were vortexed and allowed to settle into 2 phases upon which 100 µl of the upper butanol phase was pipetted in duplicate into a 96-well plate and read at an absorbance of 532 nm with a reference wavelength of 600 nm.

RNA Isolation and quantitative polymerase chain reaction (qPCR)

Total RNA from control and FB_1-treated U87MG cells were isolated using Qiazol Reagent (Qiagen, 79306). Briefly, control and treated U87MG cells were washed with 0.1M PBS. Cells were incubated for 5 mins at RT with 500 µl each of PBS and Qiazol, after which cells were lysed with a cell scraper. These cell lysates were transferred to 1.5 ml micro-centrifuge tubes and incubated at - 80ºC overnight. 100 µl chloroform was added and samples were centrifuged (4ºC, 12 000x*g*, 15 min). The clear aqueous phase was transferred to a new 1.5 ml micro-centrifuge tube, to which 100 µl isopropanol was added and tubes were incubated overnight at – 80ºC. Following incubation, samples were centrifuged (4ºC, 12000x*g*, 20 mins) and the supernatant was discarded. RNA pellets were washed in 500 µl 75% ethanol, centrifuged (4ºC, 7400x*g*, 15 min) and air dried (RT, 45 min). Finally, RNA pellets were resuspended in 15 µl nuclease-free water. Isolated RNA was quantified (Nanodrop 2000) and standardized to 500 ng/µl. Standardized RNA was used to synthesize cDNA, using the Maxima™ H Minus cDNA synthesis kit (Thermo-Fisher Scientific). Thermocycler conditions were 25ºC for 5 min, 42ºC for 30 min and 85ºC for 5 min. Gene expression was assessed for *NRF2*, *Catalase*, *SOD2*, *GPx*, *OGG1* and *SIRT3*, using the PowerUp™ SYBR™ Green Master Mix (Thermo-Fisher Scientific), while GAPDH was used as a housekeeping gene for normalization of the mRNA expressions. qPCR was conducted in the CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA), with the following thermal conditions: initial denaturation (8 min, 95°C) followed by 40 cycles of denaturation (15 s, 95°C), annealing (40 s, see Table 3.1 for temperature) and extension (30 s, 72°C). Primer sequences and annealing temperatures for each gene are listed in Table 3.1. qPCR data was analysed using the CFX manager software version 3.1 followed by the comparative threshold (Ct) method (Livak and Schmittgen 2001) and represented as a mean fold change relative to the control.

Table 3.1: Primer sequences and annealing temperatures used to assess gene expression via qPCR.

Gene	Forward Sequence	Reverse Sequence	Annealing Temperature
SOD2	5' – GAG ATG TTA CAC GCC CAG ATA GC – '3	5' – AAT CCC CAG CAG TGG AAT AAG G – '3	59ºC
Catalase	5' – TCT CAC CAA GGT TTG GCC TC – '3	5' – GCG GTG AGT GTC AGG ATA GG – '3	57.7ºC
GPx	5' – GAC TAC ACC CAG ATG AAC GAG C – '3	5' – CCC ACC AGG AAC TTC TCA AAG – '3	57.4ºC
NRF2	5' – AGT GGA TCT GCC ACC TAC TC – '3	5' – CAT CTA CAA ACG GGA ATG TCT G – '3	52.6ºC
OGG1	5' – GCA TCG TAC TCT AGC CTC CAC – '3	5' – AGG ACT TTG CTC CCT CCA C – '3	55ºC
SIRT3	5' – CAT TCG GCC TGA CGT GAT G – '3	5' –ACC CAC ATG CAG CAA GAA CCT – '3	59.4ºC
GAPDH	5' - TCC ACC ACC CTG TTG CTG TA – '3	5' – ACC ACA GTC CAT GCC ATC AC – '3	---

Protein Isolation and Western Blot

The western blot assay was conducted to further investigate the protein expressions of NRF2, Catalase, SOD2, SIRT3, LONP1, HSP 60 and TFAM. Crude protein was isolated from control and FB_1-treated U87MG cells using 200 µl Cytobuster reagent supplemented with phosphatase and protease inhibitors. Following incubation on ice (30 min), cells were mechanically lysed, transferred to fresh 1.5 ml microcentrifuge tubes and centrifuged (4ºC, 12000x*g*, 10 min). The bicinchoninic acid (BCA) assay was then conducted to quantify protein samples, which were standardized to 0.5 mg/ml, diluted in 1x Laemmli buffer [dH_2O, 0.5M Tris-HCl (pH 6.8), glycerol, 10% SDS, 5% β-mercaptoethanol and 1% bromophenol blue] and boiled (100ºC, 5 min). Thereafter, proteins were separated via electrophoresis (150 V, 1 h) in a sodium dodecyl sulphate polyacrylamide gel (10% resolving gel, 4% stacking gel). The separated proteins were transferred onto nitrocellulose membranes using the Bio-Rad Trans-Blot® Turbo Transfer System (20 V, 30 min). The membranes were then blocked in 5% BSA (RT, 1 h), made up in Tris-buffer saline (TTBS) [0.05% Tween 20, dH_2O, 3mM KCl, 25mM Tris, 150mM NaCl, pH 7.5] and probed overnight (4ºC) with primary antibody. Membranes were then washed with TTBS (5 times, 10 min) and probed with an HRP-conjugated secondary antibody (RT, 1 h). All antibody dilutions used are presented in Table 3.2. Subsequently, membranes were washed with TTBS (5 times, 10 min) and protein bands were visualized using Clarity Western ECL Substrate (Bio-Rad) detection reagent,

while detection images were captured using the Thermo-Fisher Scientific iBright™ CL1500 Instrument. After detection, the membrane was quenched in 5% hydrogen peroxide (37ºC, 30 min), washed once in TTBS, incubated in blocking solution (RT, 1h), and probed with HRP-conjugated anti-β-actin (RT, 1 h). β-actin was the housekeeping protein used to normalise protein expression. Results were analysed using the iBright™ Analysis software (version 5.2.1) and expressed as relative band density.

Table 3.2: Primary and secondary antibody dilutions used for the Western blot assay.

Antibody:	**Dilution:**	**Company:**	**Catalogue Number:**
SOD2 (D9V9C) Rabbit mAb	1:1000	Cell Signalling	13194 S
Catalase (D4P7B) XP® Rabbit mAb	1:1000	Cell Signalling	12980 S
Anti-NRF2 (phospho S40) Rabbit	2:1000	Abcam	ab76026
Anti-NRF2 Rabbit	2:1000	Abcam	ab62352
Anti-SIRT 3 Rabbit	1:1000	Abcam	ab264041
LONP1 (D8W1J) Rabbit mAb	1:1000	Cell Signalling	28020S
Mouse Anti - HSP 60	2:1000	BD Biosciences	BD 611563
TFAM (D5C8) Rabbit mAb	1:1000	Cell Signalling	8076S
Anti-Rabbit IgG HRP	2:5000	Cell Signalling	7074S
Anti-mouse IgG HRP	2:5000	Cell Signalling	7076S
Anti-β-Actin	1:5000	Sigma-Aldrich	A3854

Statistical Analysis

Statistical analysis was performed using GraphPad Prism version 5.0 (GraphPad Prism Software Inc.). All data were analysed using an unpaired t-test, with a p-value <0.05 considered as significant. All data were represented as mean ± standard deviation (SD) of 3 independent experiments (n=3).

Results

Lipid Peroxidation Assessment

The TBARS assay measured oxidative damage due to ROS, where extracellular MDA was quantified following 24 h FB_1 treatment. FB_1 significantly reduced MDA-TBA adduct concentrations in U87MG cells (p=0.0022; Figure 3.1) compared to the control.

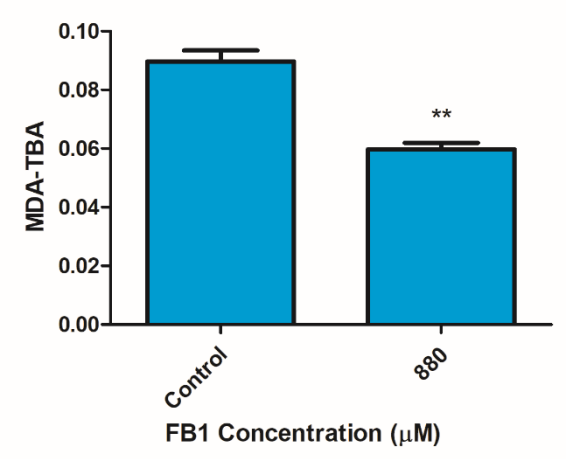

Figure 3.1: Extracellular MDA levels in U87MG cells after 24 h exposure to FB_1. Lipid peroxidation was decreased in FB_1-treated cells (**p<0.01) relative to the control. MDA: Malondialdehyde.

Endogenous antioxidant response

Endogenous antioxidant enzymes are recruited to provide defense against oxidative stress. Thus, enzymes of the antioxidant defense system were analysed to confirm the oxidative stress status in FB_1-treated U87MG cells. Oxidative DNA damage was also assessed through OGG1. Gene expressions of *SOD2*, *CAT*, *GPx*, *NRF-2* and *OGG1* were assessed via qPCR with *GAPDH* being used as the housekeeping gene. FB_1-treated U87MG cells showed significant decreases in *SOD2* (p=0.0063; Figure 3.2A), *CAT* (p<0.0001; Figure 3.2B), *GPx* (p=0.0012; Figure 3.2C), *NRF-2* (p=0.0036; Figure 3.2D) and *OGG1* (p=0.0003; Figure 3.2E) in comparison to the control. Furthermore, the protein expressions of SOD2, catalase, NRF-2 and pNRF-2 were also assessed by Western blot and were normalised against the housekeeping protein, β-Actin. Decreased protein expressions of SOD2 (p=0.0048; Figure 3.3A), catalase (p=0.0420; Figure 3.3B), NRF-2 (p=0.0298; Figure 3.3C) and pNRF-2 (p=0.0106; Figure 3.3D) were also observed in FB_1-treated U87MG cells as compared to the control.

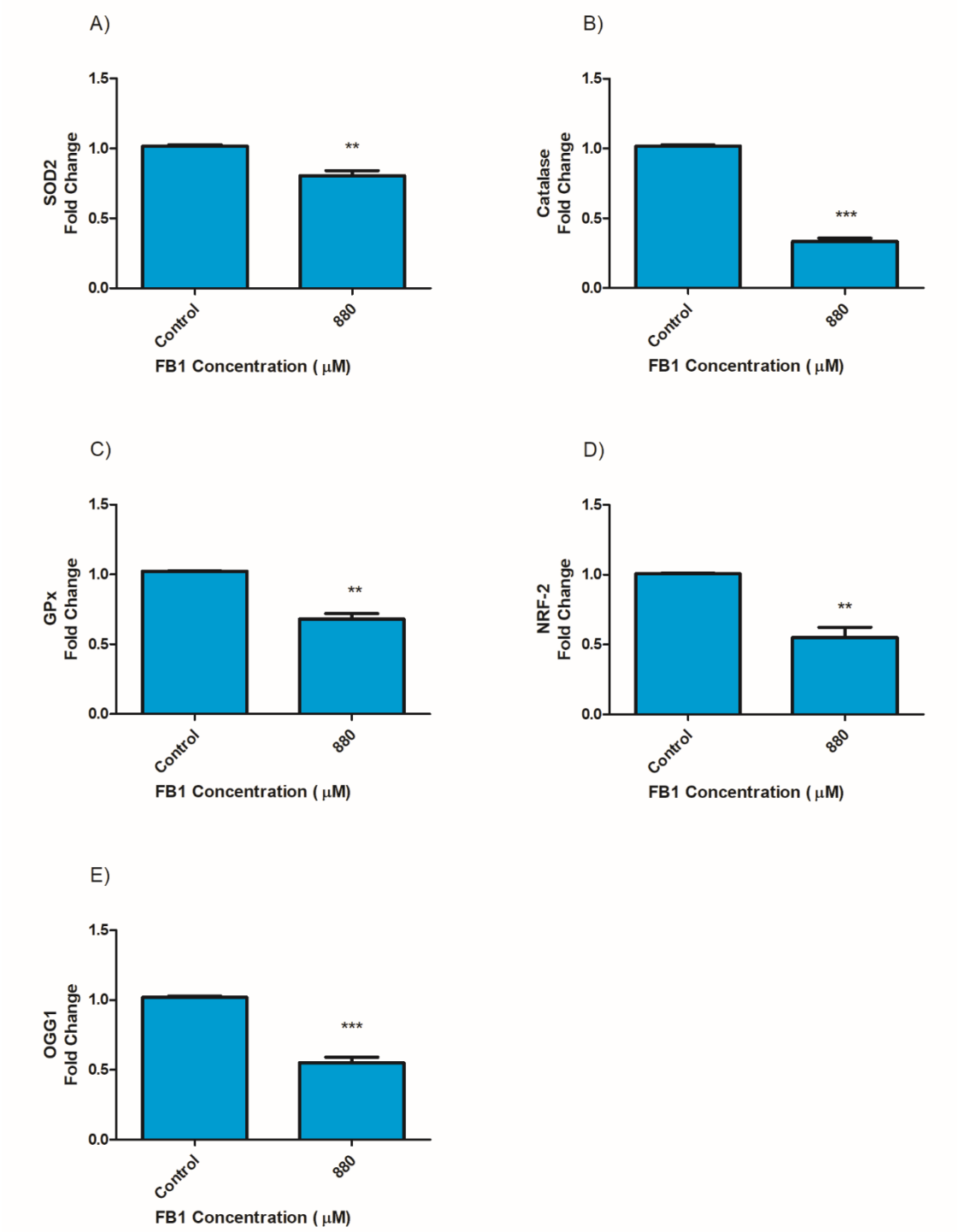

Figure 3.2: Effects of FB_1 on gene expression of selected antioxidants, the oxidative stress transcription factor and DNA repair marker. factor in U87MG cells. FB_1 decreased *SOD2* (A), *CAT* (B), *GPx* (C), *NRF-2* (D) and *OGG1* (E) expressions following 24 h treatment. mRNA expression results are expressed as mean fold change ± SD (n=3). Statistical significance for control vs FB_1 was determined by unpaired t test (**p<0.01, ***p<0.001). SOD2: Superoxide dismutase 2 ; CAT; Catalase; GPx: Glutathione peroxidase; NRF2: Nuclear factor erythroid 2-related factor 2; OGG1: 8-Oxoguanine glycosylase 1.

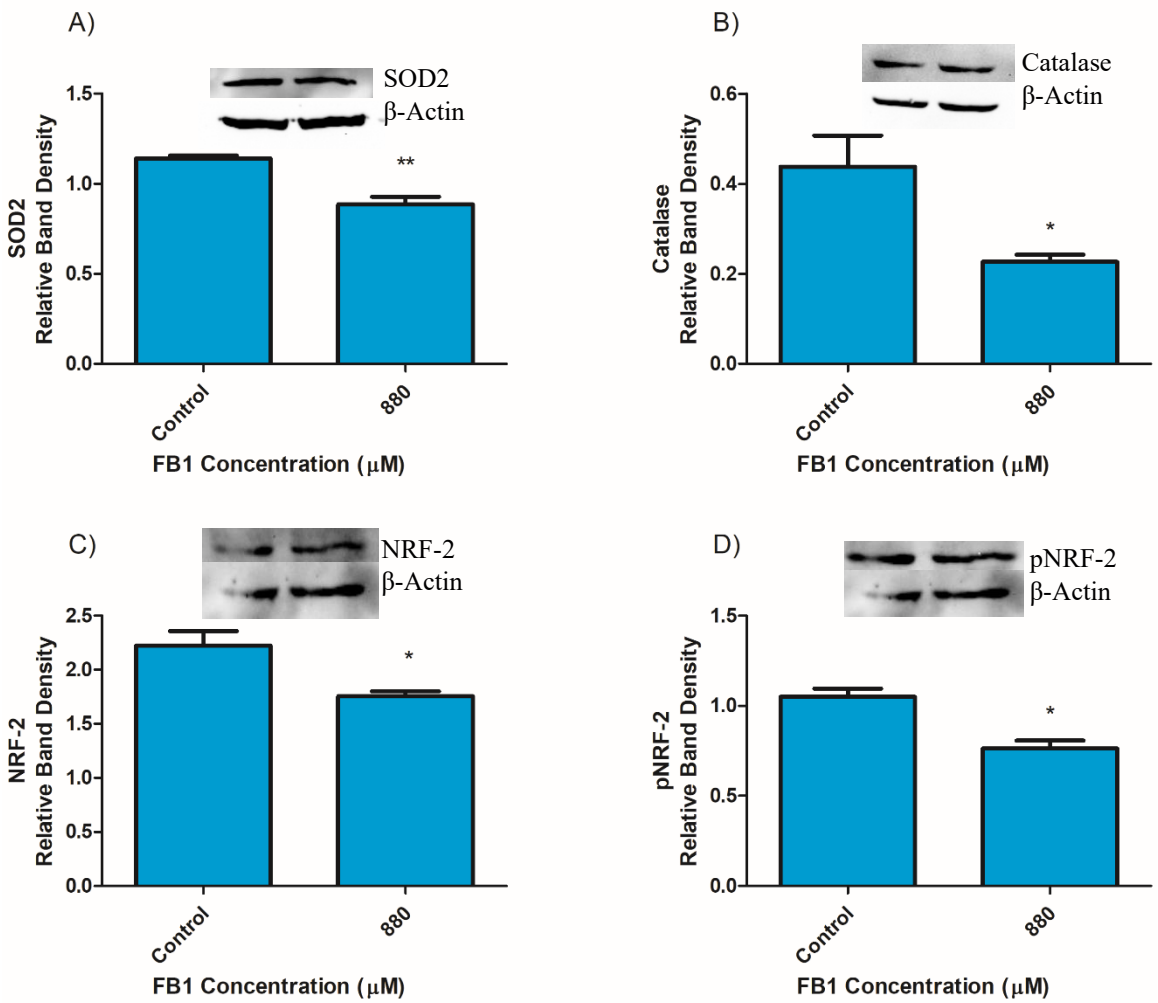

Figure 3.3: Effects of FB_1 on the protein expressions of antioxidants and oxidative stress transcription factor. SOD2 (A), CAT (B), NRF-2 (C) and pNRF-2 (D) expression was decreased in U87MG cells, following 24 h treatment. All protein expression results are expressed as band density relative to the control ± SD (n=3). Statistical significance for control vs FB_1 was determined by unpaired t test (*p<0.05, **p<0.01). SOD2: Superoxide dismutase 2 ; CAT; Catalase; NRF2: Nuclear factor erythroid 2-related factor 2.

Mitochondrial stress response

Mitochondria are the main source of endogenous ROS as electron leakage during the electron transport chain can cause damage to macromolecules and structures within the mitochondria. SIRT3 is primarily located in the mitochondria and has been highlighted for its protective role against ROS production. LonP1 maintains mitochondrial protein homeostasis by degrading misfolded proteins that accumulate in the mitochondria during stress conditions. HSP60 also plays a role in mitochondrial stress alleviation. Furthermore, the transcription factor TFAM is a marker of mitochondrial oxidative DNA damage. These markers were assessed to determine the mitochondrial stress status in FB_1-treated U87MG cells.

Following FB_1 treatment, qPCR and western blot showed increased SIRT3 gene (p=0.0007; Figure 3.4A) and protein (p=0.0069; Figure 3.4B) expressions, respectively. Correspondingly, protein expressions of LonP1 (p=0.0058; Figure 3.4C) and HSP60 (p=0.0013; Figure 3.4D) were also increased, while TFAM protein expression (p=0.0044; Figure 3.4E) was decreased by western blot in FB_1-treated cells compared to the control. GAPDH was used as a housekeeping gene for qPCR while β-Actin was used as a housekeeping protein for western blot.

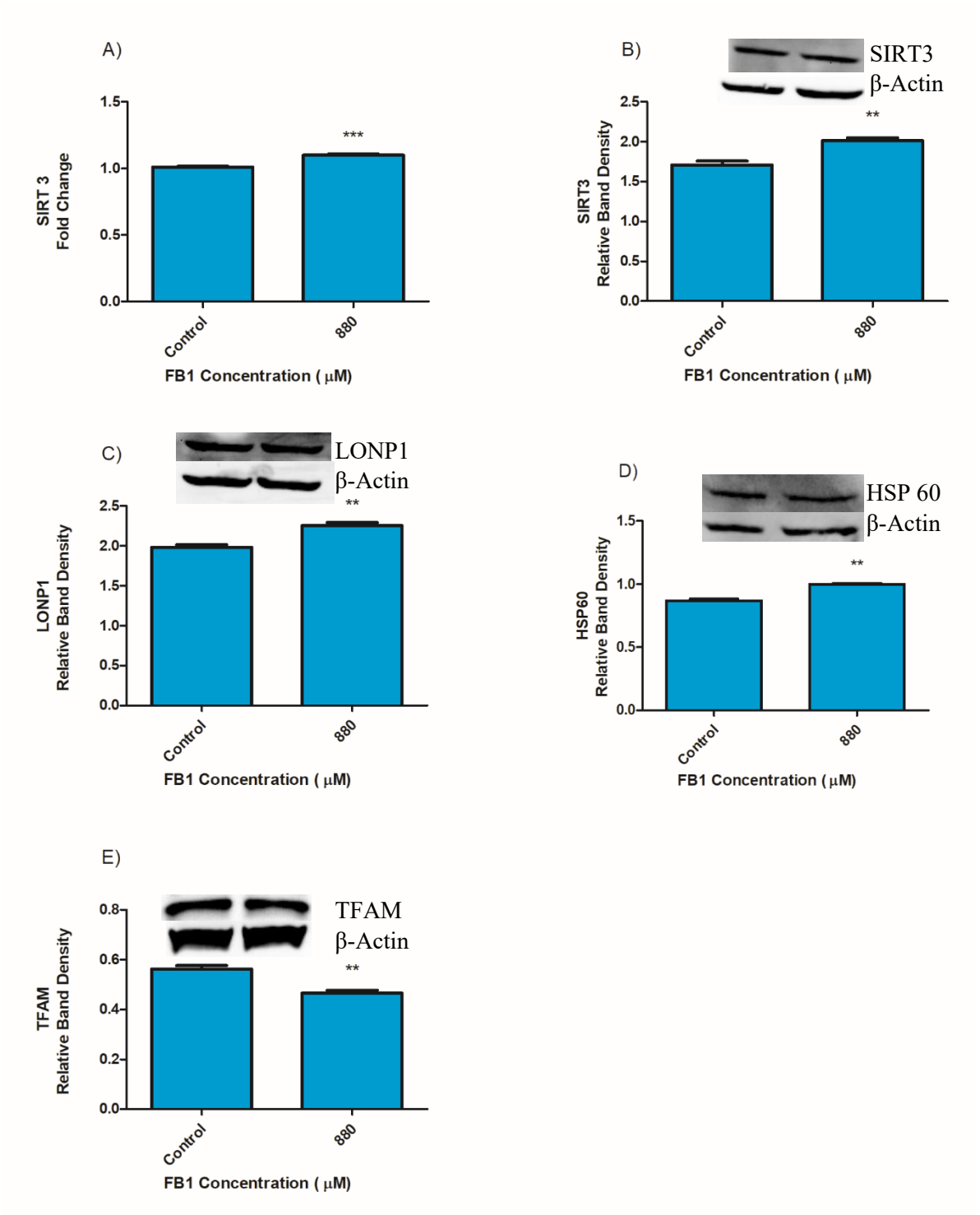

Figure 3.4: Mitochondrial stress response in FB_1-treated U87MG cells. *SIRT3* gene (A) and SIRT3 protein (B) expressions were elevated following FB_1 treatment. LonP1 (C) and HSP60 (D) proteins also showed elevated protein expressions while TFAM (E) protein expression was suppressed by FB_1 treatment. The mRNA expression result is expressed as mean fold change ± SD (n=3), while protein expression results are expressed as band density relative to the control ± SD (n=3). Statistical significance for control vs FB_1 was determined by unpaired t test (**p<0.01, ***p<0.001). SIRT: Sirtuin; LonP1: Lon protease 1; TFAM: Mitochondrial transcription factor A.

Discussion

FB_1 is one of the most abundant mycotoxins which poses a health risk to both animals and humans. FB_1 toxicity is mainly attributed to its ability to alter sphingolipid metabolism via ceramide synthase inhibition. In humans, FB_1 consumption has been linked to oesophageal cancer, kidney and liver diseases, heart failure and high spinal cord or brain defects in children (Chen, Wen, et al. 2021). In humans and animals, FB_1 has induced acute toxicity along with organ, reproductive and immune toxicities, via mechanisms such as apoptosis, cellular autophagy, endoplasmic reticulum stress and oxidative stress (Chen, Wen, et al. 2021). Recent studies have noted oxidative stress and related mitochondrial dysfunction as key regulators in neurodegeneration (Olufunmilayo, Gerke-Duncan, and Holsinger 2023).

Although Domijan (2012) has documented FB_1 as an inducer of ROS, studies have also suggested otherwise. In this study, the TBARS assay was conducted to determine lipid peroxidation in FB_1-treated U87MG cells as an indicator of endogenous ROS production. FB_1 showed a significant decrease of MDA-TBA adducts in U87MG cells (Figure 3.1), implying a reduced oxidative stress state. Stockmann-Juvala et al. (2004a) observed varying effects of ROS in different brain cell lines where high FB_1 doses elevated ROS levels in C6 rat glioblastoma and GT1-7 mouse hypothalamic cells but displayed no significant ROS modifications in human neuroblastoma SH-SY5Y cells. In FB_1-treated rat astrocytes, Galvano, Campisi, et al. (2002) found no significant change in ROS production, while the same trend was seen in FB_1-treated fibroblasts (Galvano, Russo, et al. 2002a). These findings suggest that FB_1 in the brain affects ROS production in a cell-specific manner. It is also noteworthy that although excess ROS production proves detrimental, low levels of ROS are also important for normal physiological functions. Considering this, the low ROS levels observed in FB_1-treated U87MG cells (Figure 3.1) may assist in the optimal functioning of U87MG cells. Additionally, studies have shown that tumour-suppressor proteins play an antioxidant role by activating antioxidant gene expression in response to oxidative stress. These genes regulate various cellular processes, including DNA repair, cell cycle control, proliferation, differentiation, migration, and apoptosis (Asai et al. 2011). PTEN, a tumour-

suppressor gene, has been shown to reduce ROS production in cells by modulating PI3K/AKT signalling when upregulated (Xu et al. 2011). Furthermore, in human kidney tubular epithelial cells, FB_1 treatment was found to upregulate PTEN expression while downregulating PI3K and AKT expression (Song et al. 2021). Given this, the reduction in ROS level observed in FB_1-treated U87MG cells might be due to PTEN activation and regulation of the PI3K/AKT pathway; however, further research is needed to confirm this.

ROS results as a by-product of oxygen metabolism and is classified as superoxide (O_2^-), hydroxyl free radical (OH^-) and hydrogen peroxide (H_2O_2) (Zhou et al. 2016). Eukaryotes guard cells from uncontrolled ROS via a built-in antioxidant defense strategy, primarily facilitated by SOD2, CAT and GPx. SOD2 catalyse the conversion of O_2^- to H_2O_2 and O_2, while CAT and GPx complete SOD detoxification by breaking down H_2O_2 into H_2O and O_2 (Valera-Alberni and Canto 2018). Consequent to the TBARS results, the present study showed a decrease in antioxidant responses (Figures 3.2 and 3.3). Analysis of the key antioxidants *SOD2*, *CAT*, *GPx*, and *NRF-2* (Figure 3.2 A-D), showed decreased expression in U87MG cells following FB_1 treatment. The decreased transcript levels of the key antioxidants were mirrored by reduced SOD2, CAT, NRF-2 and pNRF2 protein expressions in U87MG cells (Figure 3.3 A-D). Antioxidants detoxify and neutralize the effects of excess ROS. Therefore, the lowered oxidative stress level present in FB_1-treated U87MG cells is likely to have relieved the antioxidant defense system of its role to scavenge ROS, thus displaying low expressions of the antioxidants (Figures 3.2 and 3.3). Additionally, excess ROS initiates phosphorylation of NRF-2, which activates its translocation to the nucleus and promotes transcription of the antioxidants SOD2, CAT and GPx (Chen, Lu, et al. 2015). As such, minimized ROS would halt NRF-2 phosphorylation and disable its activation of antioxidant transcription, thus resulting in reduced SOD2, CAT, GPx and NRF-2 levels, as observed in this study (Figures 3.2 and 3.3). In mice testes, Ouyang et al. (2022) discovered a decrease in *NRF-2* mRNA expression following 21 days of FB_1 treatment, while NRF-2 and SOD2 protein expressions were decreased after 42 days of FB_1 treatment. In bovine PBMCs, 2 and 7 days of FB_1 treatments reduced *SOD* and *GPx* expressions (Bernabucci et al. 2011), while in male Wistar rats, FB_1 treatment of 2 and 7 days inhibited catalytic activity of CAT (Domijan et al. 2007). Furthermore, transcription levels of *CAT*, *SOD2* and *GPx* were decreased in FB1-treated porcine oocytes (Li, Zhao, et al. 2021).

Once produced, ROS also readily attacks DNA, establishing mutagenic lesions, such as oxidized DNA bases. Base excision repair pathways are then prompted to limit ROS-induced DNA lesions (Cui, Kong, and Zhang 2012). ROS-induced DNA damage poses a major hazard to the genetic integrity of the cell. Upon damage, protein and lipids can be degraded and resynthesized, whereas DNA must be repaired

prior to cell division and replication. Aberrant DNA bases are repaired by the DNA glycosylase OGG1, by removing oxidised bases from the DNA backbone, via the base excision repair pathway (Klungland and Bjelland 2007). Herein, the effect of FB_1 on oxidative DNA damage was assessed via *OGG1* expression in U87MG cells. qPCR analysis revealed a reduction of *OGG1* expression in FB_1-treated U87MG cells (Figure 3.2E). A reduction in OGG1 activity would prevent the activation of the base excision repair pathway, with minimal detection of aberrant DNA bases in cells. Therefore, our results suggest that FB_1 does not alter DNA in U87MG cells, via mechanisms of ROS production, as evidenced by dampened *OGG1* expression (Figure 3.2E). Interestingly, oesophageal cancer cells treated with FB_1 displayed varied *OGG1* expressions, where *OGG1* was increased at 1.25 µM FB_1, was unaltered at 10 µM FB_1 but was decreased at 20 µM FB_1 (Khan, Phulukdaree, and Chuturgoon 2017).

Optimal mitochondrial function is essential to the brain, due to its function in energy output and redox status. Mitochondria are the main ROS producers, with the ETC producing majority of mitochondrial ROS. Mitochondrial dysfunction is closely associated with ROS production owing to the incomplete reduction of O_2 by the ETC (Sheik Abdul, Nagiah, and Chuturgoon 2019a). SIRT3 is a regulator of mitochondrial function and is considered a mitochondrial fidelity protein, where the loss of SIRT3 can lead to cellular damage (Sheik Abdul, Nagiah, and Chuturgoon 2016). In U87MG cells, FB_1 increased both SIRT3 protein (Figure 3.3B) and gene (Figure 3.3A) expressions, suggesting that U87MG cells were protected from mitochondrial stress. SIRT3 promotes homeostasis via mitochondrial regulatory enzymes involved in the Krebs cycle and oxidative phosphorylation (Sheik Abdul, Nagiah, and Chuturgoon 2016). SIRT3 is also a nicotinamide adenine dinucleotide (NAD^+)-dependent deacetylase (Onyango et al. 2002). NAD^+ is an important molecule that carries electrons during metabolism (Cortés-Rojo et al. 2020). When surplus electrons are supplied, it increases the conversion of NAD^+ to NADH (Cortés-Rojo et al. 2020). A high NAD^+/NADH ratio typically suggests a cellular environment that supports oxidative reactions, which are crucial for meeting increased energy demands (Cuenoud et al. 2020). Recently, Abdul and Marnewick (2023) demonstrated that FB_1 increases the NADH:NAD^+ ratio in oxidatively poised HepG2 cells. Considering this, the effect of FB_1 on NADH:NAD^+ ratio could suggest an energy deprived state in U87MG cells, where FB_1 reduced mitochondrial output and metabolic processes rather than maintaining it. However, additional experiments are required to fully evaluate this phenomenon in FB_1-treated U87MG cells. SIRT3 sustains mitochondrial activity via the ATP-dependent protease LonP1. LonP1 removes oxidatively damaged proteins from the mitochondrial matrix via proteolytic degradation, as a defense mechanism (Sheik Abdul, Nagiah, and Chuturgoon 2016). LonP1 protein expression was upregulated in U87MG cells post FB_1 treatment (Figure 3.3C). Upregulated LonP1 aids in the clearance of oxidized protein, thus LonP1 overexpression in FB_1-induced U87MG cells (Figure 3.3C) suggests that the mitochondrion was protected from protein aggregation, accumulation and cross-linking (Bota and Davies 2016). Previously, LonP1 upregulation was associated

with mitochondrial protein quality maintenance in mice brain tissue (Docrat et al. 2020), while downregulated LonP1 was linked to impaired respiration, loss of mass and cell death in human fibroblast mitochondria (Bota, Ngo, and Davies 2005). Accordingly, elevated LonP1 diminishes mitochondrial stress and sustains mitochondrial health (Ngo, Pomatto, and Davies 2013).

Protein homeostasis of the mitochondria is preserved by a protein chaperone family known as heat shock proteins (HSP), which aid cell survival through hostility to stress (Nagiah, Phulukdaree, and Chuturgoon 2016). HSP60 is a molecular chaperone that prevents mitochondrial dysfunction by facilitating protein folding in the mitochondrial matrix (Cabiscol et al. 2002). Previous studies have documented HSP60's neuroprotective role, where the loss of HSP has been linked to protein oxidation and neurodegeneration (Kleinridders et al. 2013). In this study, FB_1 increased HSP60 protein expression (Figure 3.4D) in U87MG cells, which parallels with the amplified LonP1 expression (Figure 3.4C), suggesting that mitochondria of U87MG cells induce specific mechanisms to minimize oxidative damage to proteins and to maintain their function when exposed to FB_1. This trend was also seen in mice brain tissue where increased HSP60 was attributed to LonP1 overexpression, protecting the tissue from oxidative damage (Docrat et al. 2020). Optimal protein folding is an ATP-dependent process, thus the amplified expression of ATP-dependent protease LonP1 and subsequent rise in HSP60 supports the protective role of FB_1 in mitochondria of U87MG cells.

Increased mitochondrial ROS can impact mitochondrial DNA, which is vulnerable to oxidative stress, due to its close proximity with ROS generation sites and lack of protective histones (Ott et al. 2007). Mitochondrial DNA is therefore organized in a nucleoid structure to avoid its oxidation. Components of the nucleoid are activated by transcription factor TFAM, potentiating it as a key regulator of mitochondrial DNA maintenance (Kang, Kim, and Hamasaki 2007). Human TFAM binds to DNA, in a sequence-independent manner, enhancing its capacity to shield mitochondrial DNA from oxidative insults by directly binding to the whole genome (Kanki et al. 2004). Moreover, TFAM is degraded by LonP1 to facilitate regulation of mitochondrial gene expression and control mitochondrial DNA copy number (Lu et al. 2013a). Here, FB_1 decreased TFAM expression (Figure 3.4E) in U87MG cells which may impair mitochondrial homeostasis and mitochondrial DNA. Furthermore, compromised TFAM expression (Figure 3.4E) may alter DNA copy number, increasing the risk of cancer and/or neurodegeneration (Hu, Yao, and Shen 2016). Together, SIRT3, LonP1, HSP60 and TFAM expressions contribute to mitochondrial health under oxidative stress conditions. Although FB_1 did not induce mitochondrial stress in U87MG cells, its probable interference with mitochondrial DNA provides an alternate mechanism for FB_1 toxicity in U87MG cells.

Conclusion

FB_1 reduced the expression of antioxidants SOD2, CAT, GPx and transcription factor NRF-2 to maintain low oxidative stress levels in U87MG cells (Figure 3.5). Oxidative DNA damage was also limited in U87MG cells via suppressed activity of its repair system (Figure 3.5). U87MG cells avoided mitochondrial oxidative damage due to the increased expression of the mitochondrial maintenance proteins SIRT3, LonP1 and HSP60 (Figure 3.5). However, mitochondrial DNA was vulnerable to FB_1 toxicity (Figure 3.5). Therefore, this strongly suggests that FB_1 induces toxicity by targeting mitochondrial DNA in U87MG cells.

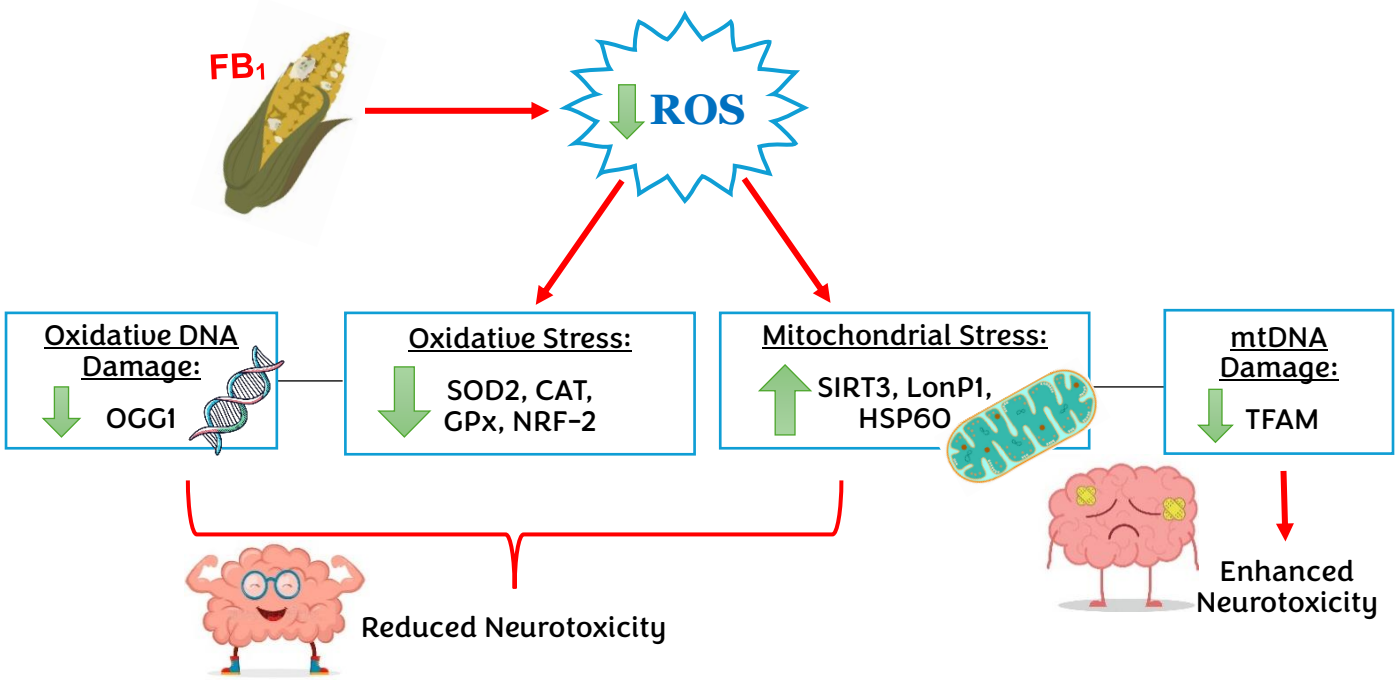

Figure 3.5: FB_1 suppresses antioxidant expressions and limits oxidative DNA damage in U87MG cells. While FB_1 also preserves mitochondrial integrity through upregulated mitochondrial proteins, it induces toxicity by altering TFAM expression in U87MG cells, leading to mtDNA damage. FB_1: Fumonisin B_1; DNA: Deoxyribonucleic acid; TFAM: Mitochondrial transcription factor A; mtDNA: Mitochondrial DNA.

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CHAPTER 4

The anti-inflammatory effect of Fumonisin B_1 in human U87MG glioblastoma cells

Inflammation and oxidative stress are closely interconnected. ROS and its related damage to proteins and DNA can trigger signalling pathways that regulate the onset of inflammatory disorders. Inflammation serves to protect the host against pathogens which may increase ROS production. While ROS generated during the inflammatory response helps eliminate invasive agents, prolonged production can lead to oxidative stress and disorders linked to chronic inflammation (Chatterjee 2016). Furthermore, superoxide and intracellular ROS are key signalling mechanisms in phagocytes and regulate various pro-inflammatory functions of microglia (Hemmati et al. 2020). Generally, the higher the level of intracellular ROS, the more intensified the inflammatory response, which persists until apoptosis or necrosis occurs (Block, Zecca, and Hong 2007). Additionally, FB_1 has been reported as an immunosuppressive. Inhibition of ceramide synthase and the subsequent disruption of sphingolipid metabolism is considered the primary mechanism of FB_1 toxicity. Continued ceramide synthase inhibition leads to detrimental outcomes that alter the functional and structural balances of neuronal tissues. Furthermore, studies have linked cellular immunity with sphingolipid metabolism destructions as the by-products of sphingolipid metabolism can influence the expression of immune-related receptors. Considering the relationship between oxidative stress and inflammation, as well as the data obtained in Chapter 3, we determined the inflammatory response of FB_1 in U87MG cells.

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The anti-inflammatory effect of Fumonisin B_1 in human U87MG glioblastoma cells

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Abstract

Fumonisin B_1 (FB_1) is a toxic secondary metabolite that contaminates maize and induces a range of toxicities including neurotoxicity and immunotoxicity. Upon entry of foreign particles, excess release of pro-inflammatory cytokines in the brain hinders anti-inflammatory cytokines from combating pathogens, thus leading to neuroinflammation and the onset of neurodegenerative diseases. Here, we evaluated the effect of FB_1 on the inflammatory response in human glioblastoma U87MG cells following a 24 h treatment. FB_1 significantly reduced pro-inflammatory cytokine expressions of IL-1β, IL-6, and TNF-α, as well as the transcription factor NF-κB in U87MG cells, while it enhanced IL-10 anti-inflammatory cytokine expression. Consequently, FB_1 reduced the pro-inflammatory marker COX-2 expression and increased the anti-inflammatory marker BDNF expression in U87MG cells. This data suggests a reduced inflammatory response to FB_1 in U87MG cells.

Keywords: Neuroinflammation, Interleukins, NF-κB, BDNF, COX-2, Fumonisin B_1

Introduction

Neuroinflammation is a physiological response that shields the brain from harmful extrinsic and intrinsic factors, as excess secretion of inflammatory mediators is injurious to the central nervous system (Rauf et al. 2022). Neuroinflammation is a consequence of over expressed pro-inflammatory and under expressed anti-inflammatory molecules (Rauf et al. 2022), which lead to the initiation and progression of neurodegenerative diseases such as Parkinson's, Alzheimer's, amyotrophic lateral sclerosis, and multiple sclerosis (Schain and Kreisl 2017; Liu et al. 2020). Inflammation, triggered by the invasion of pathogens or harmful substances, involves the activation of immune cells and the subsequent release of various inflammatory cytokines to combat toxic substances or repair injured tissues (Li et al. 2020). Pro-inflammatory cytokines are primarily produced by activated macrophages and are associated with upregulated inflammatory reactions (Zhang and An 2007). Systemic inflammatory responses produce pro-inflammatory cytokines including interleukin-1β (IL-1β), IL-6,

and tumour necrosis factor-alpha (TNF-α), which circulate in the blood and interact with neurons (Perry 2004).

IL-1β is a host-defense cytokine released by macrophages, monocytes, and non-immune cells during cell injury, invasion, infection, and inflammation (Zhang and An 2007). While IL-1β plays vital roles in host defense and homeostasis, its overproduction can lead to detrimental effects during disease (Ren and Torres 2009). Likewise, IL-6 has been reported to play a role in the activation of microglia and astrocytes and the control of neuropeptide expressions (Klein et al. 1997). IL-6 functions in neuronal differentiation, development, degeneration, and regeneration, with both favourable and harmful potentials (Kummer et al. 2021). TNF-α is one of the best characterized cytokines known to boost inflammation by inducing cell adhesion molecules, stimulating cytokines, and promoting the proliferation of cells (Germolec et al. 2018). Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), a key transcription factor, is crucial for inflammation and is activated by inflammatory cytokines, contributing to neuroinflammation and chronic diseases (Nennig and Schank 2017).

Contrastingly, anti-inflammatory cytokines are immunoregulatory molecules that control the response of pro-inflammatory cytokines (Zhang and An 2007). Among the known anti-inflammatory cytokines, IL-10 is the most studied repressive molecule. IL-10 is known to have suppressive properties against TNF-α, IL-6, and IL-1β expressions, with roles described in neurodegenerative disorders, tumour surveillance, and chronic infection (Dennis et al. 2013; Saraiva and O'Garra 2010a; Kwilasz et al. 2015). Cyclooxygenase-2 (COX-2) is a key component in inflammatory responses in peripheral tissue (Hinz and Brune 2002). In the brain, COX-2 expression has been linked to pro-inflammatory activities which may contribute to neurodegeneration in acute and chronic diseases (Minghetti 2007). The role of COX-2 in chronic inflammation and neural cell death has been highlighted by its overexpression in progressive neurodegenerative disorders such as Down Syndrome, Huntington's, Parkinson's, and Alzheimer's diseases (Strauss 2008).

Neurotrophins and their receptor, produced by immune cells, regulate immunity by prompting cytokine release (Scuri, Samsell, and Piedimonte 2010). Brain-derived neurotrophic factor (BDNF) is a well-studied member of the neurotrophin family possessing effects on the immune system, central nervous system, endocrine system, and neurosensory system (Papathanassoglou, Miltiadous, and Karanikola 2015). BDNF is well expressed in the brain and has vital roles in neuronal development, survival, and differentiation (Huang and Reichardt 2001). Among various brain regions, lowered BDNF levels are associated with psychiatric and neurodegenerative disorders (Balaratnasingam and Janca 2012).

Produced by the fungus *Fusarium verticillioides*, Fumonisin B_1 (FB_1) is a common worldwide maize contaminant, and it is the most noxious and abundant among all Fumonisins. Its adverse effects are well-documented in humans and animals, impacting immune function and antibody levels by reducing macrophage numbers (Antonissen et al. 2014). Previous studies have documented that FB_1 alters cytokine profiles in various cell types and organs, increasing IL-1β and TNF-α in primary hepatic cultures (Stockmann-Juvala and Savolainen 2008) and mouse kidney and liver (Bhandari and Sharma 2002). FB_1 has enhanced TNF-α in mice peritoneal macrophages and J774A.1 cells (Dugyala et al. 1998) but decreased IL-1β and TNF-α in swine alveolar macrophages (Liu et al. 2002), indicating variability across cell types and species. Additionally, FB_1 also attenuated IL-1β-induced suppression of BDNF in primary cerebral cortical neurons (Tong et al. 2008) and increased COX-2 expression in J774A.1 cells (Meli et al. 2000), though it had no significant effect in jejunal explants (da Silva et al. 2014). To further understand FB_1 neurotoxicity, we investigated the inflammatory effects of FB_1 in the human glioblastoma U87MG cell line.

Methods and Materials

Materials

The U87MG cell line was purchased from Separations Scientific (Johannesburg, SA). Cell culture reagents were purchased from Lonza Biotechnology (Basel, Switzerland). Western Blot reagents were purchased from Bio-Rad (Hercules, CA, USA). Primer sequences for quantitative polymerase chain reaction (qPCR) were obtained from Inqaba Biotechnical Industries (Johannesburg, SA). All other reagents were purchased from Merck (Darmstadt, Germany) unless otherwise stated.

Cell Culture and Treatments

U87MG cells were cultured in Dulbecco's Minimum Essentials Medium (DMEM) supplemented with 25 mM HEPES, 10% foetal bovine serum, 1% penicillin-streptomycin-fungizone, and 1% L-glutamine. Cells were cultured in a humidified incubator (37ºC, 5% CO_2) to approximately 90% confluency in 25 cm^3 flasks prior to treatment with FB_1. The FB_1 stock of 5 mM was prepared in 0.1 M phosphate-buffered saline (PBS). For all assays conducted, cells were treated for 24 h with 880 µM FB_1 in DMEM. Cells consisting only of DMEM was prepared for 24 h and used as the control.

RNA Isolation and Quantitative Polymerase Chain Reaction (qPCR)

Total RNA was extracted from control and FB_1-treated U87MG cells using Qiazol Reagent (Qiagen, 79306). Briefly, control and treated U87MG cells were washed with 0.1 M PBS. Following incubation (room temperature (RT), 5 mins) with 500 µl each of 0.1 M PBS and Qiazol reagent, treated and control cells were lysed with a cell scraper, transferred to 1.5 ml micro-centrifuge tubes, and incubated at -80ºC overnight. Samples were centrifuged (4ºC, 12 000 x*g*, 15 min) upon addition of 100 µl chloroform. Samples were incubated overnight at –80ºC after transferring the clear aqueous phase to a new 1.5 ml micro-centrifuge tube and adding 100 µl isopropanol to it. Following centrifugation (4ºC, 12 000 x*g*, 20 mins) and removal of the supernatant, RNA pellets were washed in 500 µl 75% ethanol. Samples were then centrifuged (4ºC, 7400 x*g*, 15 min), allowed to air dry (RT, 45 min), and finally resuspended in 15 µl nuclease-free water. Isolated RNA was quantified (Nanodrop 2000) and standardized to 500 ng/µl with nuclease-free water. The Maxima™ H Minus First Strand cDNA synthesis kit (Thermo-Fisher Scientific, K1652) was used to synthesize cDNA from the standardized RNA samples with the following thermocycler conditions: 25ºC for 5 min, 42ºC for 30 min, and 85ºC for 5 min. The mRNA expression of *IL-1β*, *IL-6*, *IL-10*, *NFkb (p65),* and *COX-2* was assessed using the PowerUp™ SYBR™ Green Master Mix (Thermo-Fisher Scientific, A25777). The housekeeping gene, GAPDH, was used to normalize mRNA expressions. qPCR was conducted in the CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA), with the following thermocycler conditions: initial denaturation (8 min, 95°C) followed by 40 cycles of denaturation (15 s, 95°C), annealing (40 s, see Table 4.1 for temperature) and extension (30 s, 72°C). Primer sequences for each gene are recorded in Table 4.1. qPCR data analysis was conducted on the CFX manager software version 3.1 with the comparative threshold (Ct) method (Livak and Schmittgen, 2001) and represented as a mean fold-change relative to the control.

Table 4.1: Primer sequences and annealing temperatures used to assess gene expression via qPCR.

Gene	**Forward Sequence**	**Reverse Sequence**	**Annealing Temperature**
IL-6	5'-AAA TTC GGT ACA TCC TCG ACG G-3'	5'-GGA AGG TTC AGG TTG TTT TCT GC-3'	58ºC
IL-10	5'-GAC TTT AAG GGT TAC CTG GGT TG-3'	5'-TCA CAT GCG CCT TGA TGT CTG-3'	58.6ºC
NF–κB (p65)	5'-TGA ACC GAA ACT CTG GCA GCT G-3'	5'-CAT CAG CTT GCG AAA AGG AGC C-3'	56ºC
GAPDH	5'-TCC ACC ACC CTG TTG CTG TA-3'	5'-ACC ACA GTC CAT GCC ATC AC-3'	---

Protein Isolation and Western Blot

The western blot assay was performed to determine the protein expressions of IL-1β, TNF-α, BDNF, and COX-2. Crude protein was isolated from control and FB_1-treated U87MG cells by incubating the samples on ice (30 mins) with 200 µl Cytobuster reagent supplemented with phosphatase and protease inhibitors. Thereafter, cells were mechanically lysed, transferred to fresh 1.5 ml microcentrifuge tubes, and centrifuged (4ºC, 12 000 x*g*, 10 min). These protein samples were then quantified by the bicinchoninic acid (BCA) assay and standardized to 1 mg/ml. Standardized samples were boiled (100ºC, 5 min) after being diluted in 1x Laemmli buffer [dH_2O, 0.5M Tris-HCl (pH 6.8), glycerol, 10% SDS, 5% β-mercaptoethanol and 1% bromophenol blue]. Thereafter, proteins were separated in a sodium dodecyl sulfate polyacrylamide gel (10% resolving gel, 4% stacking gel) by electrophoresis (150 V, 1 h) and transferred onto nitrocellulose membranes using the Bio-Rad Trans-Blot® Turbo Transfer System (20 V, 30 min). These membranes were blocked in 5% BSA (RT, 1 h), made up in Tris-buffer saline (TTBS) [0.05% Tween 20, dH_2O, 3mM KCl, 25mM Tris, 150mM NaCl, pH 7.5], followed by an overnight incubation (4ºC) with primary antibody (1:1000 in 5% BSA). Upon washing with TTBS (5 times, 10 min), membranes were probed with horse-radish peroxidase (HRP)-conjugated secondary antibody (RT, 1 h) and washed again with TTBS (5 times, 10 min), before protein bands were visualized using the Clarity Western ECL Substrate detection reagent (Bio-Rad). Images were captured on the Invitrogen iBright™ CL1500 instrument. Subsequently, membranes were quenched in 5% hydrogen peroxide (37ºC, 30 min) and incubated in blocking solution after one wash with TTBS. Membranes were then probed with HRP-conjugated anti-β-actin (Sigma-Aldrich), which served as the housekeeping protein used to normalise protein expression. The iBright™ analysis software (version 5.2.1) was used to analyse the results. Results were expressed as relative band density. The antibodies and dilutions used are presented in Table 4.2.

Table 4.2: Primary and secondary antibody dilutions used for Western blot.

Antibody	**Dilution**	**Company**	**Catalogue Number**
IL-1β (D3U3E) Rabbit mAb	1:1000	Cell Signalling	12703S
TNF-α (D5G9) Rabbit mAb	1:1000	Cell Signalling	6945S
BDNF Antibody Rabbit mAb	1:1000	Cell Signalling	47808S
Cox2 (D5H5) XP ® Rabbit mAb	1:1000	Cell Signalling	12282T
Anti-Rabbit IgG HRP	1:5000	Cell Signalling	7074S
Anti-β-Actin	1:5000	Sigma-Aldrich	A3854

Statistical Analysis

Statistical analysis was performed using GraphPad Prism version 5.0 (GraphPad Prism Software Inc.). All data were analysed using an unpaired t-test, with $p<0.05$ considered as significant. All data were represented as mean ± standard deviation (SD) of 3 independent experiments (n=3).

Results

FB_1 suppressed pro-inflammatory cytokines and NF-κB responses in U87MG cells at a high IC_{50} concentration

Inflammation occurs when the body detects injury. We assessed the impact of FB_1 in U87MG cells through key inflammatory markers. The mRNA expressions of *IL-6* and *NF-κB* were determined by qPCR while protein expressions of IL-1β and TNF-α were determined by western blot. FB_1 significantly decreased the expressions of inflammatory cytokines IL-1β (p=0.0019; Figure 4.1A), *IL-6* (p=0.0004; Figure 4.1B), and TNF-α (p=0.0120; Figure 4.1C), while the expression of the transcription factor *NF-κB* (p=0.0210; Figure 4.1D) was also decreased by FB_1 in comparison to the control.

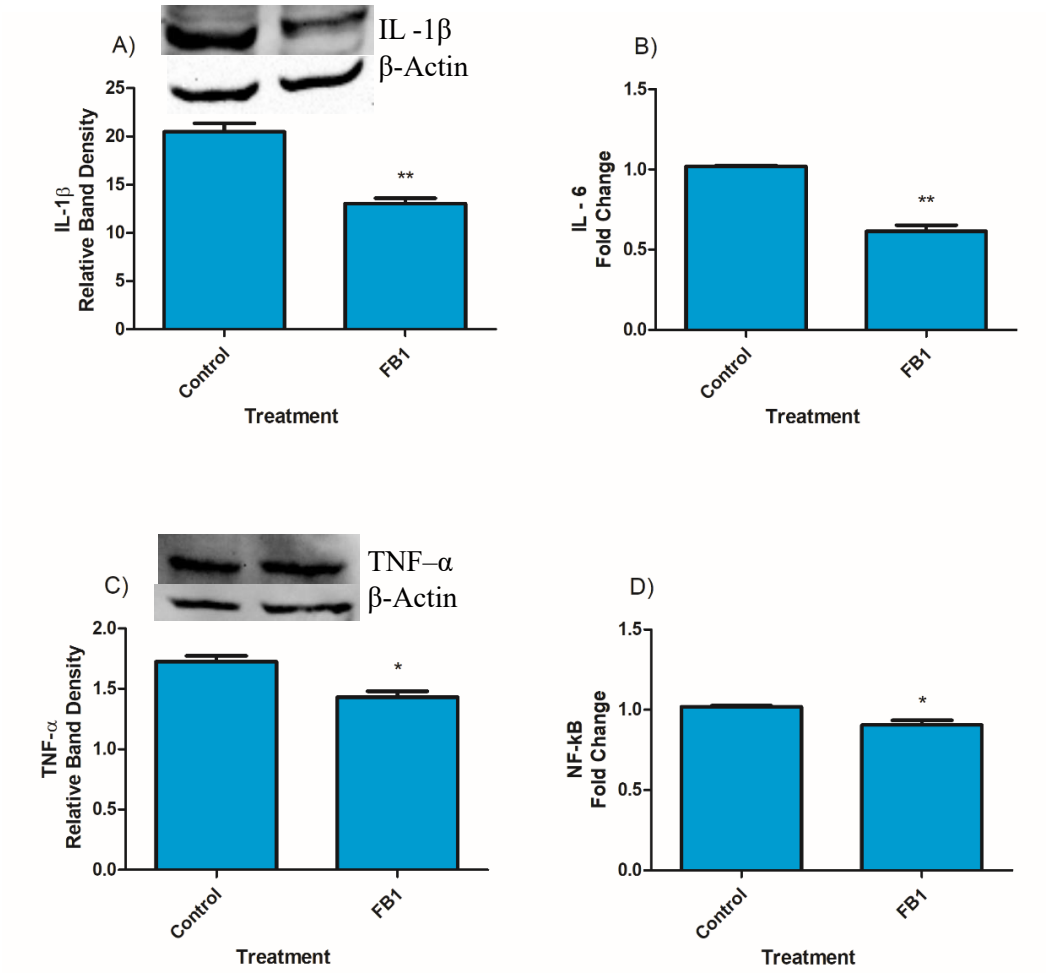

Figure 4.1: Exposure to FB_1 significantly reduced the expressions of pro-inflammatory markers IL-1β (A), *IL-6* (B), TNF-α (C), and *NF-κB* (D) in U87MG cells. Protein expression of IL-1β (A) and TNF-α (C) were determined by western blot and are expressed as band density relative to the control ± SD (n=3). mRNA expression for *IL-6* (B) and *NF-κB* (D) were determined by qPCR and are expressed as mean fold change ± SD (n=3). Statistical significance for control vs FB_1 was determined by unpaired t test (*p<0.05, **p<0.01). IL: Interleukin; TNF-α: Tumor Necrosis Factor-α; NF-kβ: Nuclear factor-kappa B.

FB_1 enhanced *IL-10* expression in U87MG cells following a 24 h treatment

IL-10 is an anti-inflammatory cytokine that protects against inflammation and autoimmune pathologies. Thus, following the decrease in pro-inflammatory markers observed, we assessed the expression of the anti-inflammatory cytokine, IL-10, in FB_1-treated U87MG cells. qPCR analysis revealed that *IL-10* expression (p<0.0001; Figure 4.2) significantly increased in U87MG cells after FB_1 exposure compared to the control. This data is concomitant with reduced pro-inflammatory cytokine expressions.

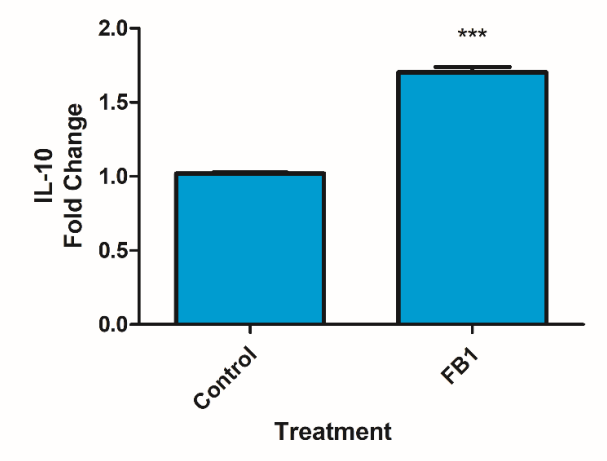

Figure 4.2: Anti-inflammatory cytokine *IL-10* expression increased in U87MG cells post FB_1 treatment. *IL-10* mRNA expression was determined by qPCR and is expressed as mean fold change ± SD (n=3). Statistical significance for control vs FB_1 was determined by unpaired t test (***p<0.0001). IL: Interleukin.

FB_1 suppressed COX-2 protein expression in U87MG cells

COX-2 is expressed in response to inflammation and produces prostaglandins which support the inflammatory process. To further understand the pro-inflammatory response, we assessed the effect of FB_1 on COX-2 expression in U87MG cells by western blot. Protein expression of COX-2 was significantly decreased (p=0.0367; Figure 4.3) by FB_1 compared to the control. COX-2 downregulation further suggests an anti-inflammatory role of FB_1 in U87MG cells.

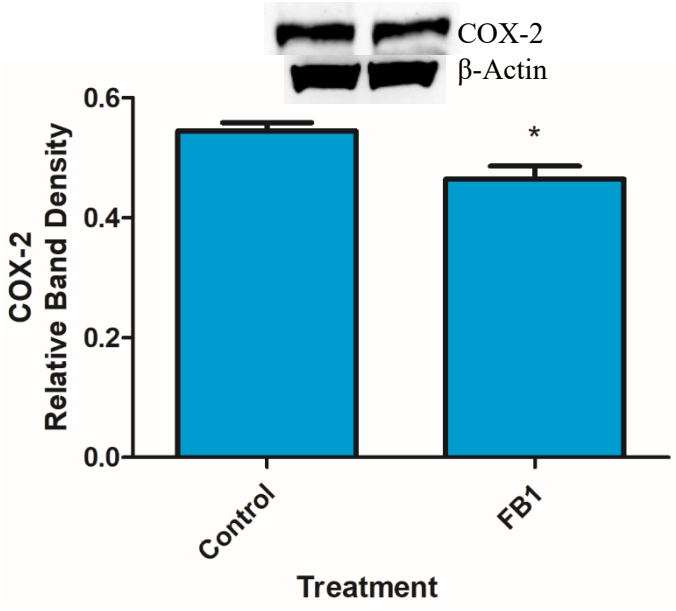

Figure 4.3: FB_1 reduces the protein expression of COX-2 following treatment in U87MG cells. COX-2 protein expression was determined by western blot and is expressed as band density relative to the control ± SD (n=3). Statistical significance for control vs FB_1 was determined by unpaired t test (*p<0.05). COX-2: Cyclooxygenase-2

FB_1 enhanced BDNF protein expression in U87MG cells

BDNF is important in neuronal health regulation and is known to convey neuroprotection in neurons and microglia. The anti-inflammatory cytokine response observed led to the assessment of BDNF expression in FB_1-treated U87MG cells by western blot. Concomitantly, BDNF protein expression was significantly enhanced (p=0.0162; Figure 4.4) by FB_1 compared to the control, suggesting a protective role of FB_1 in U87MG cells.

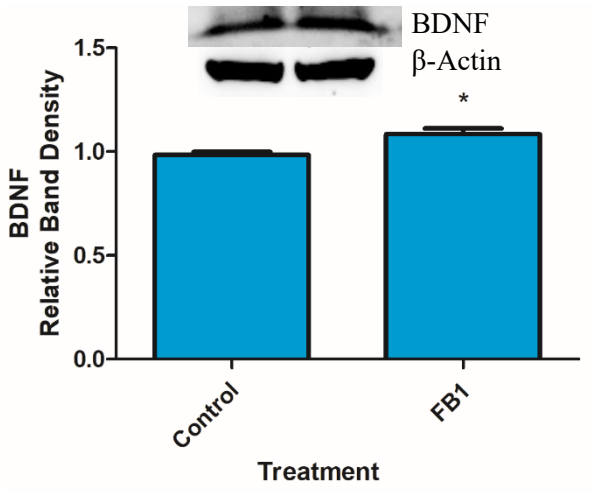

Figure 4.4: BDNF protein expression was increased in U87MG cells following FB_1 treatment. BDNF protein expression was determined by western blot and is expressed as band density relative to the control ± SD (n=3). Statistical significance for control vs FB_1 was determined by unpaired t test (*p<0.05). BDNF: Brain-derived neurotrophic factor

Discussion

Inflammation acts as the body's defense mechanism, in which the immune system identifies and eliminates harmful stimuli and initiates the healing process. Inflammatory responses may influence the central nervous system via various mechanisms. The tight junctions of the brain create the blood-brain barrier (BBB) to limit pathogen diffusion from the blood into the brain (Porter and O'Connor 2022). Peripheral inflammation may obstruct this barrier causing an increase in BBB permeability, thus allowing infiltration of cytokines and monocytes to the brain (Porter and O'Connor 2022). Peripheral cytokines can influence brain homeostasis via vagus nerve signalling (Zanos et al. 2018) or through

signalling of pattern recognition receptors on endothelial cells of the BBB (Engblom et al. 2002; Capuron and Miller 2011). These inflammatory signalling responses initiate neuroinflammatory reactions in the brain. Accumulating data indicates that a positive and negative cytokine feedback system, along with a balanced pro-inflammatory and cytokine inhibitory system, is vital for optimal brain cellular response modulation (Plata-Salaman 2002). Several researchers have drawn their attention to cytokine function and expression in the brain, with the literature rapidly expanding. Likewise, our interest in neuroinflammation led us to investigate the inflammatory response in the human glioblastoma U87MG cell line after exposure to the common food-borne mycotoxin FB_1. Current knowledge suggests that FB_1 has marked immunological effects. Therefore, we first evaluated the impact of FB_1 on U87MG cells through the expressions of transcription factor NF-κB and pro-inflammatory cytokines IL-1β, IL-6, and TNF-α.

IL-1β is a potent member of the IL-1 family, which is associated with various autoimmune and inflammatory diseases (Tsai 2017). IL-1β is synthesized and released by astrocytes and microglia, thus it is highly expressed in the brain, with key regulatory roles in mood and memory (Tsai 2017). The IL-1 family contributes to neurodegeneration and induces IL-6 production (Rauf et al. 2022). IL-6 is produced in response to tissue injury and infections thus providing defense to the host. IL-6 expression is regulated by transcriptional and post-transcriptional mechanisms; however, its continued dysregulated synthesis contributes to the pathological effects of chronic inflammation and autoimmunity (Tanaka, Narazaki, and Kishimoto 2014b). TNF-α is also a vital inflammatory marker that regulates acute phase inflammation and is responsible for various physiological functions (Parameswaran and Patial 2010). Upon injury, TNF-α protects against infection, neuronal toxicity, and neurodegeneration; however, its dysregulated expression may result in chronic inflammation (Rauf et al. 2022). NF-κB is a key inflammatory response mediator as it controls various aspects of innate and adaptive immunity (Liu, Zhang, et al. 2017). In response to inflammation, NF-κB induces pro-inflammatory cytokines to maintain optimal immune responses and cell survival. Consequently, altered NF-κB activation is linked to pathogenic processes of inflammatory diseases (Liu, Zhang, et al. 2017).

In U87MG cells, FB_1 significantly decreased the expressions of pro-inflammatory cytokines IL-1β (Figure 4.1A), *IL-6* (Figure 4.1B), and TNF-α (Figure 4.1C), along with *NF-κB* (Figure 4.1D) compared to the control, suggesting that U87MG cells induced an appropriate response to counteract the toxicity of FB_1, during the exposure period of 24 hrs. In the healthy brain, IL-1 is expressed at low levels and is only rapidly expressed in response to trauma. In ischemic mice, IL-1β deficiency reduced neuronal loss and infarct volume (Boutin et al. 2001a). In rodents, increased IL-1β gene and protein expressions have been observed in the brain following cerebral ischemia, excitotoxin infusion, or traumatic brain injury

(Patel, Boutin, and Allan 2003). In contrast, administration of IL-1β neutralizing antibody ameliorates cerebral ischemic damage in rats (Relton and Rothwell 1992; Mulcahy et al. 2003). Animals lacking the interleukin-1 converting enzyme, which is essential for IL-1β processing and activation, display reduced IL-1β expressions and infarct volumes (Hara et al. 1997; Liu et al. 1999). Also, mice lacking IL-1β show reduced brain injury after cerebral artery occlusion (Ohtaki et al. 2003; Boutin et al. 2001b). Immunotoxicity assessment in swine alveolar macrophages revealed that FB_1 downregulated IL-1β, suggesting that FB_1 partially exerts its toxicity in macrophages through cytokine production (Liu et al. 2002). Given these findings, it is evident that IL-1β is increased in detrimental states, while its decrease proves favourable to the host. Thus, the decreased IL-1β protein expression observed in this study implies that FB_1 is not fully toxic in U87MG cells over 24 hrs. In human dendritic cells, a 6 h and 24 h lipopolysaccharide treatment significantly increased *IL-1β* and *IL-6* expressions; however, upon FB_1 administration, the lipopolysaccharide-induced expressions of *IL-1β* and *IL-6* were significantly reduced (Stockmann-Juvala, Alenius, and Savolainen 2008). The study also showed that FB_1 alone or in combination with lipopolysaccharide had no effect on *TNF-α* expression (Stockmann-Juvala, Alenius, and Savolainen 2008).

In the central nervous system, IL-6 is normally expressed at low levels and increases in pathological circumstances (Wei, Alberts, and Li 2013). Considering this notion, the FB_1-induced decrease of *IL-6* in U87MG cells (Figure 1B) would indicate a physiological state, in which an inflammatory response was not stimulated. While enhanced IL-6 expression has been widely associated with autism development (Wei, Alberts, and Li 2013), Smith et al. (2007) demonstrated that an anti-IL-6 antibody improved autism-like behaviours and normalized the autism-induced gene expression changes by inactivating IL-6 in mice brain tissues. In a preclinical model, genetic deletion of IL-6 and neutralizing IL-6 antibody reduced disease signatures of multiple sclerosis (Gijbels et al. 1995; Okuda et al. 1998; Kummer et al. 2021). In mice with traumatic brain injury, IL-6 upregulation promoted peripheral inflammation; however, inflammation, brain injury, and motor impairment were alleviated by treatment with an anti-IL-6 antibody (Yang et al. 2013; Rowe et al. 2016). In murine bone marrow-derived dendritic cells the lipopolysaccharide-induced secretion of IL-6 was suppressed by FB_1 in a dose-dependent manner (Li et al. 2017).

Like most cytokines in a healthy adult brain, TNF-α is expressed at low levels and is only highly expressed in neurodegenerative brains (Cheng et al. 2010). TNF-α also facilitates the release of the pro-inflammatory cytokines IL-1β and IL-6 through macrophage stimulation (Abbas, Lichtman, and Pober 1997). In U87MG cells, TNF-α protein expression was significantly suppressed by FB_1 exposure. This decrease in TNF-α expression aligns with the observed decrease in IL-1β and *IL-6* expressions seen in

FB_1-treated U87MG cells. TNF-α is a key mediator of ischemic brain injury. Direct administration of TNF-α into the cerebroventricular system elicits focal brain ischemia, while in TNF-α-treated rats, ischemic injury led to increased infarct size and neurological damage (Feuerstein, Wang, and Barone 1998). However, a specific neutralizing TNF-α antibody completely eradicated the effects of TNF-α in ischemic injury (Feuerstein, Wang, and Barone 1998). In mice subjected to radiotherapy, increased astrogliosis, vessel permeability, and leukocyte adhesion were significantly ameliorated by anti-TNF-α treatments (Wilson et al. 2009). In human dendritic cells, TNF-α gene and protein expressions were not affected by FB_1 exposure (Stockmann-Juvala, Alenius, and Savolainen 2008); however, in pig lungs exposed to 0.5mg FB_1/kg body weight/day for 7 days (Halloy et al. 2005) and mouse peritoneal macrophages injected with 0 to 6.75 mg/kg/day FB_1 for 5 days (Dugyala et al. 1998) TNF-α expression was enhanced. On the contrary, FB_1 exposure (2 and 10 μg/ml for 24 h) in swine alveolar macrophages reduced TNF-α expression (Liu et al. 2002), suggesting that FB_1 might affect TNF-α expression in a dose-, time-, and cell type-dependent manner. Accumulating data highlights the importance of low-level TNF-α expression in a healthy host, thus suggesting that the observed FB_1-induced decrease in TNF-α protein expression was favourable to U87MG cells.

NF-κB is a transcription factor that also plays a role in brain function following injury and neurodegeneration. Several studies have shown an increase in NF-κB due to brain injury. In an ischaemia-reperfusion injury model, NF-κB was increased in the ischaemia cortex (Salminen, Liu, and Hsu 1995) whereas in a traumatic brain cortical injury model, a persistent NF-κB increase was observed (Yang, Mu, and Hayes 1995). These models suggest that upon injury, increased IL-1 and TNF production activates NF-κB and a range of pro-inflammatory genes. In U87MG cells, *NF-κB* expression (Figure 4.1D) was decreased by FB_1, which is parallel with the decrease observed in IL-1β, *IL-6,* and TNF-α expressions (Figure 4.1A – 4.1C). In rats, Yuan et al. (2020) suggested a neuroprotective effect against ischaemia reperfusion injury via the downregulation of NF-κB along with reduced IL-1β, IL-6, and TNF-α levels. In astroglia, NF-κB inhibition was found to reduce inflammation and improve recovery of spinal cord injury (Brambilla et al. 2005). In an optic neuritis study, transgenic mice with selectively inactive NF-κB in astrocytes were significantly protected, averting axonal demyelination, and attenuating retinal ganglion cell death, while also displaying a decrease in pro-inflammatory cytokine expression (Brambilla et al. 2012). Similarly, in a mouse model, NF-κB inactivation resulted in decreased pro-inflammatory cytokine expression, suggesting that NF-κB-dependent expression is vital for inflammation and tissue injury in autoimmune demyelinating disease in the central nervous system (van Loo et al. 2006).

IL-10 is the most studied anti-inflammatory cytokine of the immune system. IL-10 limits inflammation and autoimmune diseases by suppressing the immune response to pathogens (O'Garra and Vieira 2007). IL-10 expression is regulated by cell-specific mechanisms including epigenetics, the activation of transcription factors, signalling pathways, and post-transcriptional modifications (Saraiva and O'Garra 2010a). IL-10 production has been documented in the brain; however, its cellular and regulatory mechanisms are less understood compared to those in the periphery. This lack of knowledge on the IL-10 regulatory immune response in the brain hampers the development of immune modulatory strategies. The decreased pro-inflammatory response in FB_1-treated U87MG cells prompted us to determine the expression of the anti-inflammatory cytokine IL-10. FB_1 significantly increased *IL-10* expression (Figure 4.2), corresponding with the prevention of inflammation in U87MG cells. This increase in anti-inflammatory response suggests a mechanism for the reduced inflammatory state observed in FB_1-treated U87MG cells. Several studies have implicated IL-10 deficits in patients and animals with neurological diseases such as Alzheimer's, Parkinsons, multiple sclerosis, and neuropathic pain. Therefore, IL-10 production is considered a mechanism to hinder exaggerated neuroimmune responses. In neurons, IL-10 receptor signalling has been linked to the enhancement of cellular survival (Zhou et al. 2009) and adult neurogenesis regulation (Perez-Asensio et al. 2013). Prior to relapse, a decrease in IL-10 secretion by peripheral blood mononuclear cells has been documented in multiple sclerosis patients; however, during remission IL-10 is increased, signifying the importance of IL-10 for recovery (Waubant et al. 2001; Rieckmann et al. 1994). IL-10-based therapies have been tested in animal models which displayed decreases in inflammation, dopaminergic cell damage, and its related microglial activation (Kwilasz et al. 2015). Pérez-de Puig et al. (2013) described the role of IL-10 in ameliorating inflammatory responses in permanent ischemia, where IL-10 deficiency worsened the outcomes of permanent middle cerebral artery occlusion and inflammation. Also, patients suffering from ischemic stroke display low levels of IL-10, indicating the down-regulation of the anti-inflammatory response in acute stroke patients (Perini et al. 2001). In a neurotoxicant model of Parkinsons disease, adenoviral-mediated expression of human IL-10 reduced the neurodegenerative impact upon toxin administration in the striatum of mice (Joniec-Maciejak et al. 2014). In BALB/c mice fed FB_1 for 2 weeks, IL-10 expression was increased with an associated decrease in TNF-α expression (Abbès et al. 2016). Similarly, in murine bone marrow-derived dendritic cells, FB_1 suppressed IL-10 secretion but not TNF-α in a dose-dependent manner following lipopolysaccharide exposure (Li et al. 2017).

COX-2 is stimulated by inflammatory cytokines such as TNF-α, IL-1, and IL-2 (Rauf et al. 2022), therefore its expression in the brain has been linked to pro-inflammatory states. Transcriptional factor NF-κB also influences the expression of COX-2 thus COX-2 inhibition can be achieved through factors that alter NF-κB expression, such as IL-10 (Consilvio, Vincent, and Feldman 2004). Considering the parallel expressions of pro-inflammatory cytokines with COX-2, herein, we also examined the effect of

FB_1 on COX-2 expression. In U87MG cells, FB_1 significantly downregulated COX-2 protein expression (Figure 4.3) compared to the control. COX-2 is induced in neurons under pathological conditions. In the hippocampal and cortical neurons, COX-2 is promptly expressed upon a single maximal electroconvulsive seizure (Consilvio, Vincent, and Feldman 2004). In mice neurons, COX-2 overexpression led to increased excitotoxicity while COX-2 knockout mice displayed lower neuronal death when exposed to ischemia (Iadecola et al. 2001). COX-2 upregulation has been implicated in Alzheimer's disease therefore, many researchers have shown interest in synthesizing COX-2 inhibitors to minimize inflammation and halt Alzheimer's disease progression (Moussa and Dayoub 2023). Studies also suggest that NF-κB activation may promote Alzheimer's disease development by increasing COX-2 in microglia and subsequently increasing pro-inflammatory molecules to aggravate neuroinflammation (Amor et al. 2010; Shabab et al. 2017). It is also documented that IL-1β, IL-6, IL-18, and TNF-α overexpression weakens the learning capacity of mice by inducing COX-2 expression (Wang et al. 2017; Ghosh et al. 2013). In our study, COX-2 expression was downregulated, along with the pro-inflammatory cytokines, implying a positive role for FB_1 in U87MG cells compared to other findings.

BDNF is abundantly expressed in the brain, where it plays a role in a range of physiological processes including inflammation. BDNF plays a key role in neuron growth and development, while it is also known to improve neurons in a pathological state (Liang, Deng, and Huang 2019). Since the anti-inflammatory cytokine response was increased in U87MG cells, we further investigated the anti-inflammatory effect of FB_1 via the expression of BDNF in U87MG cells. We found a significant increase in BDNF protein expression in U87MG cells exposed to FB_1 compared to the control (Figure 4.4). Mounting literature has described the anti-inflammatory properties of BDNF. BDNF pretreatment in a pneumococcal meningitis study, reduced pathological and clinical disease severity, while also improving hippocampal apoptosis and the inflammatory response (Xu et al. 2017). In this pneumococcal meningitis study, BDNF treatment also led to decreased gene and protein expressions of TNF-α, IL-1β, and IL-6 while increasing IL-10 gene and protein expression (Xu et al. 2017). NF-κB nuclear translocation was also inhibited thus neutralizing the harmful impact of excessive inflammation (Xu et al. 2017). In ischemic stroke rats, intranasal BDNF administration suppressed TNF-α but increased IL-10 gene and protein expressions, suggesting that BDNF might protect against ischemic insult by regulating cytokine expressions in rat brains (Jiang et al. 2011). Another ischemic stroke rat model showed that exogenous BDNF upregulated IL-10 expression, downregulated TNF-α and NF-κB expressions, and improved vestibulomotor and sensorimotor functions (Jiang et al. 2010). In a neuroinflammation and epileptogenesis rat model, intra-hippocampal administration of BDNF eased recurrent seizures and the inflammatory marker IL-1β (Bovolenta et al. 2010). Our observation of the inflammatory response in FB_1-treated U87MG cells (Figure 4.1 – 4.4) is consistent with these previous

findings, where enhanced BDNF and IL-10 expressions resulted in reduced IL-1β, IL-6, TNF-α, and NF-κB expressions.

Conclusion

FB_1 was found to significantly decrease the inflammatory response in U87MG cells by suppressing the activity of the pro-inflammatory markers IL-1β, IL-6, TNF-α, and NF-κB (Figure 4.5). This reduced inflammatory state was further supported by the increase in anti-inflammatory cytokine IL-10 expression (Figure 4.5). COX-2 is commonly expressed in response to inflammation; however, FB_1 reduced COX-2 expression in U87MG cells, while enhancing the negative neuroinflammatory regulator BDNF (Figure 4.5). These findings suggest an anti-inflammatory role for FB_1 in U87MG cells, albeit over short exposure times.

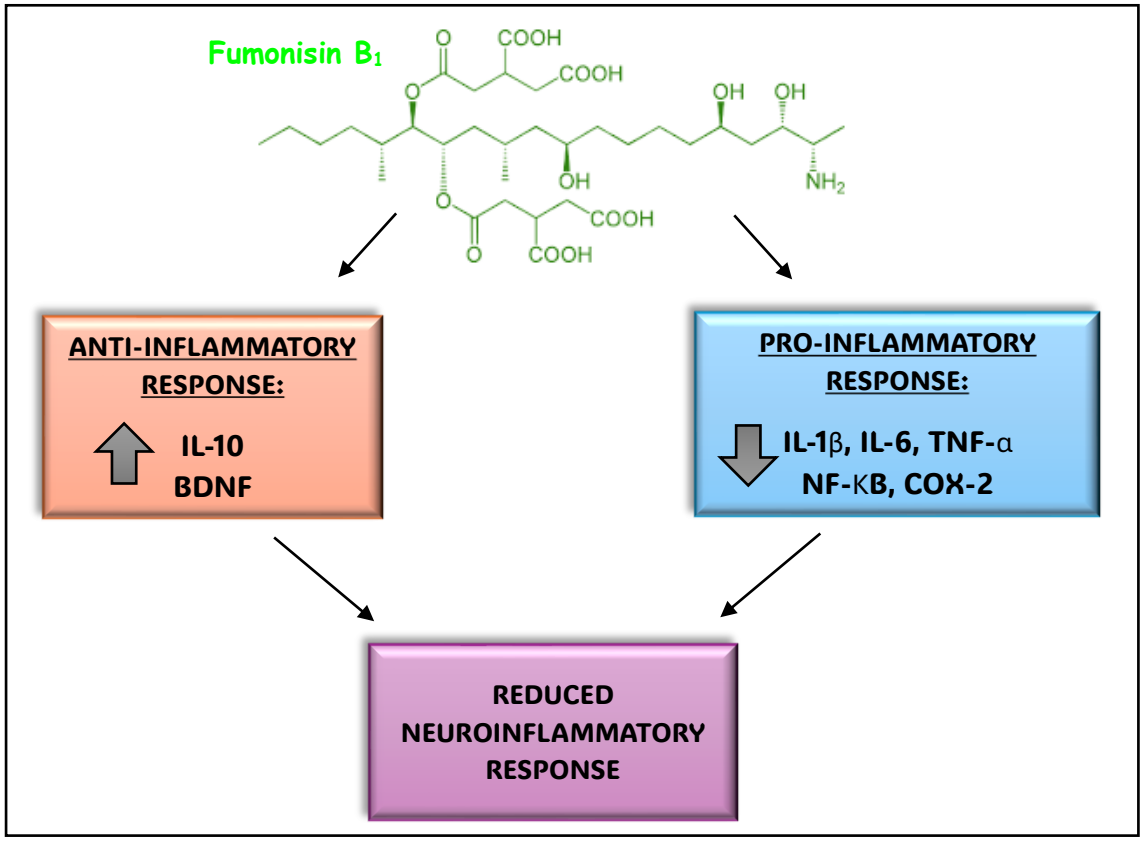

Figure 4.5: FB_1 attenuates the inflammatory response in U87MG cells. FB_1 downregulated the expression of IL-1β, IL-6, and TNF-α which are key pro-inflammatory cytokines that drive inflammatory responses. FB_1 also reduced expression of the transcription factor NF-κB and COX-2, which serve as crucial regulators of inflammation by activating cytokines and promoting the production of inflammatory mediators. In contrast, the expression of the anti-inflammatory cytokine IL-10 and BDNF was elevated, indicating a shift towards a protective, anti-inflammatory cellular environment. FB_1: Fumonisin B_1; IL-1β: Interleukin-1β; IL-6: Interleukin-6; TNF-α: Tumour necrosis factor-alpha;

NF-κB: Nuclear factor-kappa B; COX-2: Cyclooxygenase-2; IL-10: Interleukin-10; BDNF: Brain-derived neurotrophic factor.

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CHAPTER 5

5.1 Synthesis/Discussion

Fusarium verticillioides, the producer of FB_1, is a significant fungal contaminant in maize, particularly in homegrown crops meant for human consumption (Marasas, Jaskiewicz, et al. 1988). The extent of maize infection is largely influenced by environmental factors, such as average temperatures and frequent rainfall. In certain regions of South Africa, maize forms part of the staple diet thus increasing the risk of Fumonisin consumption among these populations. In rural areas of Africa, research has found that certain processing methods deliver added risks of Fumonisin consumption (Shephard et al. 2005). A significant factor contributing to this is the widespread cultivation of maize as a cereal in Sub-Saharan Africa (Dutton 2009). Furthermore, maize is used to make locally brewed alcoholic and non-alcoholic beers (Phoku et al. 2012a). These products are consumed daily, imposing major health hazards among impoverished communities.

In the context of Fumonisin toxicity, oxidative stress and ROS are crucial in inducing damage to lipids, DNA, and proteins. Oxidative damage to cell membrane lipids leads to increased lipid peroxidation, which can be measured by TBARS. MDA is the most prevalent individual aldehyde produced from lipid peroxidation, and its concentration serves as a marker for lipid oxidation (Klarić et al. 2007; Pirinccioglu et al. 2010). In U87MG cells, MDA levels decreased upon FB_1 exposure, which led to concomitant decreases in SOD2, CAT and GPx gene and protein expressions. Although FB_1 is known to be a strong inducer of oxidative stress, studies show that its effect on ROS production and other oxidative stress-related events varies in the brain depending on the cell line (Stockmann-Juvala et al. 2004a). The reason for this is unclear but could be due to differences in how cells recognize FB_1 or variations in sphingolipid metabolism. Similar decreases in antioxidant enzymes due to FB_1 exposure were previously observed in Balb/c mice spleen (Abbès et al. 2016) as well as female rat liver and kidney (Hassan et al. 2015). The decreased activities of antioxidant enzymes during oxidative stress are thought to play a role in FB_1 neurotoxicity (Domijan and Abramov 2011; Stockmann-Juvala et al. 2004a). The brain, being rich in mitochondria, is continuously exposed to oxidative stress and free radicals, which may contribute to adverse health outcomes.

In this study, NRF-2 protein and gene expressions were decreased, corresponding with the decreases in lipid peroxidation and antioxidant enzyme levels in FB_1-treated U87MG cells. The human *OGG1* gene encodes a DNA glycosylase that facilitates the removal of 8-hydroxyguanine from damaged DNA and suppresses its mutagenic ability (Nash et al. 1996). Downregulated mRNA expression of *OGG1* was

observed in U87MG cells following FB_1 treatment. This reduction in *OGG1* may indicate the absence of base lesions by 8-hydroxyguanine, thus resulting in the deactivation of the repair mechanism for oxidatively damaged DNA. Since 8-hydroxyguanine serves as a biomarker for DNA damage caused by oxidative stress, assessing its levels with a DNA Damage ELISA Kit would be valuable for future research. Our laboratory previously demonstrated that FB_1 treatment to HepG2 cells induces a significant increase in oxidative DNA damage, as indicated by elevated levels of 8-hydroxy-2′-deoxyguanosine (Arumugam, Ghazi, and Chuturgoon 2020)

Considering the reduced oxidative state of FB_1-treated U87MG cells, as observed by reduced levels of lipid peroxidation and antioxidant enzymes including NRF-2 expressions, we explored whether mitochondrial stress levels were also constrained in this study. Neurons have significant energy requirements and are particularly susceptible to oxidative stress because of their high oxygen consumption, elevated levels of membrane polyunsaturated fatty acids, and moderate antioxidant defense (Cenini, Lloret, and Cascella 2019). Sirtuins are a group of nicotinamide adenine dinucleotide (NAD^+)-dependent deacetylases that are evolutionarily conserved from bacteria to humans. SIRT3 interacts with its essential cofactor NAD^+ to deacetylate mitochondrial protein substrates, producing by-products in the process. This activity allows SIRT3 to regulate metabolic adaptations by altering the overall protein acetylation landscape in mitochondria (Hebert et al. 2013). By deacetylating substrates involved in both ROS production and detoxification, SIRT3 is emerging as a key defender against stressors, as its deficiency can lead to decreased efficiency of electron transfer, resulting in increased ROS generation and reduced ATP production (Wu et al. 2019). Contrastingly, elevated SIRT3 helps maintain low steady-state levels of ROS, thereby coordinating metabolic and genetic processes by preventing excessively high ROS levels (Wu et al. 2023). Here, we observed significant increases in gene and protein expressions of SIRT3 following FB_1 treatment, suggesting that SIRT3 maintained mitochondrial integrity in U87MG cells. While FB_1 appears to preserve mitochondrial integrity in U87MG cells, it may be worth considering an alternative explanation, particularly given that SIRT3 is highly expressed in the brain (Sidorova-Darmos et al. 2014). The FB_1-induced upregulation of SIRT3 expression could lead to NAD^+ depletion, potentially impairing metabolic processes by reducing available NAD^+ for mitochondrial function, rather than promoting mitochondrial maintenance. Further investigation is needed to confirm this hypothesis and elucidate its underlying mechanisms. Mitochondria possess a family of proteins that help address mitochondrial protein misfolding during oxidative stress. Another important regulator of mitochondrial activity is LonP1, which was upregulated in FB_1-treated U87MG cells. LonP1 is a nuclear-encoded, mitochondrial ATP-dependent serine peptidase that facilitates the breakdown of oxidatively modified proteins in the mitochondrial matrix (Ngo and Davies 2007b), helping to maintain mitochondrial homeostasis. In U87MG cells, the expression of molecular stress protein HSP60 was also enhanced following FB_1 exposure. HSPs are a

group of highly conserved protective proteins that are expressed in all cells. HSP60 is responsible for the folding and assembly of newly imported proteins in the mitochondrial matrix (Cheng, Hartl, and Norwich 1990). Recent studies have revealed that HSP60 knockout reduces mitochondrial activity while increasing cell proliferation (Teng et al. 2019). Knocking out HSP60 has also proven lethal to mice (Christensen et al. 2010) and zebrafish (Pei et al. 2016). Additionally, patients with disease-related variations in HSP60 are susceptible to neurodevelopmental disorders, with impaired myelination being a common characteristic (Yamamoto et al. 2018; Magen et al. 2008). mtDNA is also vulnerable to oxidative stress, as it lacks histone proteins and has inefficient DNA repair mechanisms (Santos et al. 2013). Physiologically, mtDNA is more prone to oxidative damage than nuclear DNA, as the mitochondrial respiratory chain is a significant source of ROS and mtDNA is situated close to this ROS generator (Kang and Hamasaki 2002). TFAM is a DNA-binding protein that plays a vital role in maintaining the normal function of mtDNA (Aasumets et al. 2021). It stabilizes mtDNA and initiates its replication, making it essential for mtDNA metabolism (Song et al. 2024). TFAM is known for its role of protecting mtDNA from ROS, while mtDNA helps shield TFAM from degradation by Lon protease (Lu et al. 2013b). Following exposure to FB_1, TFAM expression was significantly downregulated in U87MG cells suggesting altered mtDNA integrity due to impaired protection. The expression of human TFAM suppresses TFAM promoter activity and enhances mitochondrial activity or increases mtDNA copy number, which can be viewed as a negative feedback mechanism for TFAM expression (Lee et al. 2014). Mitochondrial dysfunction frequently results in declined mtDNA copy number or mutations, with research indicating that a reduction in mtDNA copy number is linked to neurodegenerative diseases (Song et al. 2024). Thus, the FB_1-induced downregulation in TFAM expression could also lead to reduced mtDNA copy number in U87MG cells, proving detrimental to neuronal health. A reduction in mtDNA copy number can also cause defects in the mitochondrial respiratory chain, ultimately leading to mitochondrial dysfunction (Song et al. 2024). In a skeletal muscle atrophy study on mitochondrial dysfunction, Theilen, Kunkel, and Tyagi (2017) found that TFAM binds to and encircles mtDNA, providing protection against ROS-induced degradation while also promoting mitochondrial function. These findings highlight the importance of TFAM regulating optimal mitochondrial function.

Previous studies have revealed that oxidative stress and ROS generation are closely linked to the immunotoxic potential of mycotoxins (Theumer et al. 2010; Mary et al. 2012). During oxidative stress, cytokine production is often elevated (Jena et al. 2023), triggering a stronger inflammatory response. Contrary, cytokines can also modulate the oxidative stress environment, such that certain cytokines can promote ROS production by activating cellular pathways that involve enzymes like NADPH oxidase and contribute to mitochondrial dysfunction (Jena et al. 2023). This bidirectional relationship indicates that cytokines and oxidative stress are interconnected in a feedback loop, where each factor can amplify

the effects of the other. If not properly regulated, this cycle can contribute to chronic inflammation and tissue damage. Inflammation is a crucial biological response to injury, infection, and trauma affecting cells or tissues. An effective inflammatory response removes invading pathogens and initiates wound healing and angiogenesis (Carson, Thrash, and Walter 2006). However, in the brain, inflammation can negatively influence both acute and chronic brain disorders (Harry and Kraft 2008). Therefore, to counteract these adverse effects, neurons engage in neuroprotective activities by clearing cellular debris and regulating the secretion of neurotrophic factors, cytokines, and proteases (Shabab et al. 2017). Neuroinflammation is a response that engages all cell types in the CNS, including neurons, macroglia, and microglia. Microglia are the brain's resident macrophages and play a vital role in the organism's defense and tissue repair. In microglia, mitochondrial dysfunction results in increased production of ROS, contributing to redox imbalance and triggering the transcription of pro-inflammatory genes, as well as the release of cytokines like IL-1β, IL-6, and TNF-α, which in turn induces neuroinflammation (Shabab et al. 2017). Additionally, microglia interact with various damage-associated molecular patterns (DAMPs) with recent data suggesting that TFAM may function as a specific DAMP in peripheral tissues (Little et al. 2014). Impaired mitochondrial function is also associated with both acute and chronic inflammatory diseases (Naik and Dixit 2011). Mitochondria play a central role in regulating the pro-inflammatory response, primarily by influencing innate immunity through redox-sensitive inflammatory pathways or by directly activating the inflammasome. The inflammasome is a protein complex whose activation triggers the immediate activation of caspase-1, which in turn cleaves and activates the inactive precursors IL-1β and IL-18 (Strowig et al. 2012). Moreover, these pathways can work in concert to stimulate the release of inflammatory cytokines, potentially leading to an excessive and prolonged inflammatory response (Escames et al. 2012).

We investigated the inflammatory response of FB_1 in U87MG cells and found that the pro-inflammatory markers IL-1β, IL-6, TNF-α, NF-κB and COX-2 were significantly downregulated, which led to the upregulation of the anti-inflammatory markers IL-10 and BDNF, indicating a low inflammatory response in this study. IL-1β plays a crucial role in triggering and amplifying the immune response to infections. It contributes to immune cell recruitment and activation, stimulates the production of pro-inflammatory cytokines, and influences adaptive immunity (Duque and Descoteaux 2014; Pyrillou, Burzynski, and Clarke 2020). IL-6 is produced in response to infections and tissue damage, playing a key role in host defense by triggering acute phase responses, thus supporting haematopoiesis, and regulating immune reactions (Tanaka, Narazaki, and Kishimoto 2014a). While its expression is tightly controlled at both transcriptional and posttranscriptional levels, persistent dysregulated IL-6 production contributes to chronic inflammation and autoimmune diseases (Tanaka, Narazaki, and Kishimoto 2014a). TNF-α is an inflammatory cytokine produced by macrophages and monocytes during acute inflammation (Idriss and Naismith 2000). TNF-α plays a crucial role in cellular signalling, promoting

necrosis or apoptosis and is also essential for immune defense, contributing to resistance against infections and cancer (Idriss and Naismith 2000). NF-κB plays a role in regulating both innate and adaptive immune functions and acts as a central mediator of inflammatory responses. It drives the expression of various pro-inflammatory genes, including cytokines and chemokines, and is also involved in inflammasome regulation (Liu, Zhang, et al. 2017). Additionally, NF-κB is essential for controlling the survival, activation, and differentiation of innate immune cells and inflammatory T cells (Liu, Zhang, et al. 2017). COX-2 is a major enzyme of prostaglandins synthesis from arachidonic acid, which plays a central role in inflammation (Abdel-Latif et al. 2009). Its increased expression has also been observed in various other human cancers (Gandhi et al. 2017). IL-10 is a powerful anti-inflammatory cytokine, vital for immune response regulation during infectious diseases. IL-10 helps minimize immunopathology by reducing excessive inflammation, preventing tissue damage, and limiting immune-related pathology to maintain homeostasis (Saraiva and O'garra 2010b). The role of IL-10 is especially critical in chronic infections, where prolonged inflammation can negatively impact the host.

Upon activation due to injury, microglia present antigens to T-cells and trigger the release of various antioxidants, genes and proteins including cytokines IL-1β, TNF-α and COX-2, ROS and other neurotoxins which may induce neuronal dysfunction and cell death (Park et al. 2011). Cytokines and ROS are intricately linked and can impact each other in a variety of physiological and pathological processes. Certain cytokines, such as TNF-α, IL-1β, and IL-6, can promote ROS production across different cell types. In U87MG cells, ROS production was limited along with the reduction in expression of pro-inflammatory cytokines, reflecting the link between ROS and cytokine expression. In neonatal cardiac myocytes, exposure to TNF-α resulted in increased oxidative stress (Suematsu et al. 2003b). Additionally, TNF-α-exposed adult cardiac myocytes displayed elevated lipid peroxidation and ROS, via 2′,7′-Dichlorofluorescin Diacetate (Kaur et al. 2006). In THP-1 cells, recombinant human TFAM triggered the expression of the pro-inflammatory cytokines IL-1β, IL-6, and IL-8. This effect was further amplified when IFN-γ was present. The increased secretion of IL-6 in response to recombinant human TFAM and IFN-γ was also confirmed in primary human microglia (Little et al. 2014). Here, we observed decreased TFAM expression which could be linked to the decreased expression of the pro-inflammatory cytokines.

IL-1β and TNF-α are key players in pathological inflammation and disease progression (Lyman et al. 2014a). They contribute to BBB breakdown, increased adhesion molecule expression, and the diffusion of harmful substances like nitric oxide (Blamire et al. 2000). In the CNS, IL-1β is increased upon trauma to peripheral nerves, astrocytes, and microglia (Yan et al. 1992), while its role is critical in chronic

neurodegenerative diseases (e.g., Alzheimer's, Parkinson's) and acute conditions like stroke and brain injury (Swaroop et al. 2016). IL-6 also disrupts BBB integrity, facilitating leukocyte infiltration and influencing neuronal survival or death post-injury (Gadient and Otten 1997). High IL-6 levels have been linked to cognitive decline, Alzheimer's, bipolar disorder, and depression (Bradburn, Sarginson, and Murgatroyd 2018; Howren, Lamkin, and Suls 2009; Del Giudice and Gangestad 2018). TNF-α, a multifunctional cytokine, triggers apoptosis through death domain receptors (Shabab et al. 2017) and is rapidly elevated in response to injury and neurodegenerative diseases (Figiel 2008). TNF-α also regulates various cellular processes, including apoptosis and necrosis, and plays a role in immune defense (Idriss and Naismith 2000).

NF-κB has multiple roles within the mammalian cell. Interestingly, ROS can also regulate the activation of transcription factors such as NF-κB which is critical in mediating cytokine production and inflammatory responses (Bhol et al. 2024). In Jurkat T cells, H_2O_2 has been demonstrated to activate NF-κB, highlighting the direct relationship between these inflammatory and oxidative stress markers (Schreck, Rieber, and Baeuerle 1991). In U87MG cells, a similar pattern was noted, with reduced levels of NF-κB accompanying lower oxidative stress levels. Additionally, studies have indicated an increased sensitivity to NF-κB activation in the context of mitochondrial dysfunction, suggesting that NF-κB binding sites play a critical role in the expression of inflammatory genes (Ungvari et al. 2007; Vaamonde-García et al. 2012). NF-κB is stimulated by inflammatory cytokines such as ILs and TNF-α (Nennig and Schank 2017), hence the decrease in pro-inflammatory cytokines in FB1-treated U87MG cells, would stimulate a decrease in NF-κB expression. Impairment of NF-κB signalling contributes to chronic inflammatory diseases, including neurodegenerative disorders and cancer (Lawrence 2009). Increased NF-κB expression was observed in the ischemic cortex in a rat ischemia-reperfusion model (Salminen, Liu, and Hsu 1995), whereas persistent NF-κB activation was noted in a rat model of traumatic brain injury (Yang, Mu, and Hayes 1995). When activated, NF-κB triggers the production of pro-inflammatory cytokines, chemokines, inducible nitric oxide synthase and COX-2, which all contribute to neuroinflammation (Glass et al. 2010). COX-2 expression is primarily found in neurons, where it is linked to synaptic function and memory formation. In U87MG cells, COX-2 expression was decreased upon exposure to FB_1. Research has shown that COX-2 inhibition or deletion improves ischemia-related neurological deficits and reduces infarct volume in the early stages after ischemia (Iadecola et al. 2001; Candelario-Jalil et al. 2003; Sasaki et al. 2004). Inhibition of mitochondrial respiratory chain activity can upregulate COX-2 expression, leading to ROS production and NF-κB activation (Biniecka et al. 2010; Cillero-Pastor et al. 2008). Limiting the pro-inflammatory response is thus imperative for optimal brain function.

In U87MG cells, IL-10 expression was upregulated by FB_1. IL-10 plays a role in inhibiting inflammation and autoimmune pathologies by downregulating pro-inflammatory cytokines to counteract their production and function (Zhang and An 2007). Released by immune cells and glia as part of a negative feedback mechanism during inflammation, IL-10 helps mitigate neuroimmune disorders, including neurodegeneration, nerve injury, peripheral inflammation, and autoimmune diseases, all of which often feature IL-10 deficiency and chronic inflammation (Ledeboer et al. 2002; Moore et al. 2001; Kettenmann et al. 2011; Kwilasz et al. 2015), while advanced techniques to boost IL-10 have shown sustained therapeutic effects with a single injection (Kwilasz et al. 2015). In animal and patient models, IL-10 has been reported as an immune regulator of the brain as its deficit has been implicated in diseases such as Parkinson's (Arimoto et al. 2007), Alzheimer's (Kiyota et al. 2012), multiple sclerosis (Hesse et al. 2011) and neuropathic pain (George et al. 2004). Apart from its role in inflammation, IL-10 has been demonstrated to reduce the production of ROS in isolated macrophages (Dokka et al. 2001) and is thought to play a role in modulating TNF-α-mediated, oxidative stress-induced acute lung injury (Mulligan et al. 1993; Shanley et al. 1995). In non-challenged cardiac myocytes, IL-10 did not affect intracellular ROS levels or lipid peroxidation at any concentration. However, IL-10 was effective in alleviating TNF-α-induced oxidative stress and cell injury (Kaur et al. 2006). Gunnett et al. (2000) revealed that IL-10 can mitigate the increase in ROS triggered by inflammatory stimuli. In models of renal ischemia–reperfusion, IL-10 treatment decreased lipid peroxidation, improved the redox ratio, and enhanced CAT and SOD activities (Köken et al. 2004). These findings suggest that IL-10 may exert an antioxidant-like effect in biological systems experiencing elevated oxidative stress. BDNF expression was also elevated in FB_1-treated U87MG cells. BDNF levels are closely linked to brain function, with lower levels associated with reduced synaptic plasticity and neuronal atrophy (Kuipers et al. 2003), whereas higher levels promote neuronal survival and differentiation (Ventimiglia et al. 1995). Studies within the brain have displayed the effect of inflammation on BDNF expression. Raetz and Whitfield (2002) reported that administration of lipopolysaccharide or pro-inflammatory cytokines significantly lowered BDNF levels whereas in rat models, IL-1β and lipopolysaccharide injections reduce BDNF in the hippocampus and cortical regions (Lapchak, Araujo, and Hefti 1993; Guan and Fang 2006; Schnydrig et al. 2007). Additionally in schizophrenia, increased MDA and decreased BDNF levels were correlated with BDNF showing a negative relationship with SOD (Zhang et al. 2015). Similarly, in U87MG cells, BDNF expression inversely correlates with MDA and SOD levels.

Various cancer models indicate that elevated ROS levels play a role in cancer development and progression by influencing both genetic and epigenetic mechanisms. In terms of epigenetic changes, ROS can trigger hypermethylation of tumour suppressor genes as well as global DNA hypomethylation (Wu and Ni 2015). Notably, DNA methylation occurs primarily on CpG islands. Guanine is particularly

susceptible to oxidation, which results in the formation of 8-oxo-deoxyguanosine, while cytosine can also undergo modifications, producing 5-hydroxymethylcytosine (Menezo et al. 1989). 5-hydroxymethylcytosine is a physiological product of oxidation and plays a key role in DNA demethylation. Oxidative changes in CpG sites can alter the interactions between these sites and transcription factors (Dattilo et al. 2016). Consequently, DNA oxidative damage can lead to heritable epigenetic modifications, affecting chromatin structure (Donkena, Young, and Tindall 2010b; Maltseva et al. 2009), inducing aberrant hypermethylation of specific gene promoters, and contributing to global hypomethylation. CpG islands are hotspots for mutations (Wachsman 1997), and ROS-induced alterations in DNA methylation patterns have been implicated in the malignant transformation and progression of various cancers (O'Hagan et al. 2011; Valinluck et al. 2004a). Research has also demonstrated the role of DNA methylation in regulating inflammatory cytokine expression (Liu, Sun, et al. 2019) while also serving as a key regulator of immune cell proliferation, differentiation, and response (Zhang et al. 2023).

Epigenetic mechanisms are often overlooked as potential pathways of toxicity as much of the focus in toxicology has traditionally been on more immediate and visible processes like inflammation, oxidative stress, and apoptosis. DNA methylation is a crucial epigenetic modification (Baylin and Jones 2016) that plays vital roles in regulating gene expression, genomic imprinting, X chromosome inactivation, and tumorigenesis (Smith and Meissner 2013). In recent years, there has been growing interest in the link between DNA methylation and human diseases. Looking at the reduced states of inflammation and oxidative stress in U87MG cells and considering previous studies reporting FB_1 as a neurotoxin, we investigated an alternative mechanism of toxicity via the effect of FB_1 on global DNA methylation status. FB_1 significantly enhanced the expression of DNA maintenance and establishment methyltransferases, DNMT1, DNMT3A and DNMT3B while it reduced the expression of the demethylase MBD2, thus inducing global DNA hypermethylation in U87MG cells. DNMTs are extensively expressed in the brain, regulating gene transcription through epigenetic mechanisms. Their expression varies by brain region and cell type, and appear to serve both partially redundant (Feng et al. 2010) and distinct functions (Morris and Monteggia 2014; Morris et al. 2016). DNMT3A is crucial for learning (Morris and Monteggia 2014), while DNMT1 is linked to anxiety (Morris et al. 2016), though the mechanisms remain unclear. DNMT1 overexpression is linked to abnormal DNA methylation in solid tumours, metastasis, and poor prognosis (Zhao et al. 2011; Peng et al. 2006; Saito et al. 2003). Likewise, elevated levels of DNMT3A and DNMT3B have been observed in many patient samples. Elevated DNMT3A is associated with hepatocellular carcinoma (Zhao et al. 2010), while high DNMT3B levels contribute to BRCA1 inactivation in breast cancer (Butcher and Rodenhiser 2007) and are essential for colonic micro-adenoma growth (Lin et al. 2006; Linhart et al. 2007). Furthermore, DNMTs are closely linked to the regulation of inflammatory cytokine expression. For instance, elevated

levels of DNMT1 have been shown to enhance the release of pro-inflammatory cytokines, such as TNF-α and IL-6, in response to lipopolysaccharide stimulation in macrophages (Cheng et al. 2014). Overexpression of DNMT3A can lead to increased DNA methylation at the IL-6 promoter in synovial fibroblasts (Yang et al. 2017). Additionally, abnormal DNA methylation of the promoters of pro-inflammatory cytokines like IL-6, TNF-α, IL-1α, and IL-11 can disrupt the normal expression of these genes, thereby exacerbating the inflammatory response (Arroyo-Jousse et al. 2016; Notley et al. 2017). MBD2 regulates gene expression by binding to methylated CpGs in DNA (Berger and Bird 2005a). MBD proteins generally repress gene expression by interacting with chromatin-modifying complexes (Nan, Cross, and Bird 2007). BD2 is implicated in cancer, immunity, and potentially neurodevelopment, though its exact role in brain function remains unclear. Loss of MBD2 is associated with behavioral deficits, including impaired maternal care, poor nest building, and mild memory issues (Wood et al. 2016; Hendrich et al. 2001b) as well as cognitive, social, and emotional challenges (Lax et al. 2023a). An ever-growing number of studies are exploring the role of DNA methylation in the brain to gain a deeper understanding of their involvement in disease-related pathophysiology, which is essential for determining their potential as therapeutic targets.

Gliomas, pose major challenges in neuro-oncology while its treatment outcomes and prognosis are heavily impacted by genetic and epigenetic factors (Chen et al. 2024). Gliomas frequently display aberrant DNA methylation, which can silence tumour suppressor and other key regulatory genes. The interaction between genetic mutations and epigenetic changes plays a vital role in glioma pathogenesis. Glioma CpG island methylator phenotype is a distinctive hypermethylation pattern that is linked to better prognosis and survival (Christensen et al. 2011). This phenotype arises from IDH mutations, resulting in global DNA hypermethylation, which impacts gene expression and tumour behaviour (LeBlanc and Marra 2016). Studies have identified specific DNA methylation patterns in gliomas, particularly in promoter regions of genes regulating the cell cycle, apoptosis, and DNA repair (Weng and Salazar 2021). For example, aberrant O6-Methylguanine-DNA methyltransferase (MGMT) promoter methylation is a well-known marker associated with better responses to alkylating agents and increased survival (Aoki and Natsume 2019). Moreover, DNA methylation profiles are beneficial for classifying gliomas into prognostic subgroups, with certain methylation markers indicating poorer survival outcomes (Chen et al. 2019). Moreover, DNA methylation functions not only as an epigenetic modification that influences chromatin structure and regulates gene transcription but also increases the mutation rate of methylated DNA sequences by prompting the transition of cytosine to thymine (J. Dabrowski and Wojtas 2019). As a result, multiple cellular processes can be impacted. Additionally, a significant overexpression of DNMT1 and DNMT3B was observed in glioma tumours and the glioblastoma cell lines U87MG and LN18, resulting in aberrant genomic stability and impaired cell cycle progression (Rajendran et al. 2011). These authors suggested that DNMT1 and DNMT3B

overexpression in gliomas cause hypermethylation of several tumour suppressor genes, contributing to poor prognosis, and serve as a cancer cell marker and potential target for cancer therapies. Sun et al. (2017) evaluated the expression of DNMT1 to assess its role in the regulation of tumour suppressor gene neurofibromatosis type 2 (NF2) in gliomas. They observed increased *DNMT1* mRNA expression in GBM cell lines (U251, U87, T98-G and A172) compared to normal glial cells, suggesting that NF2 may play a role in GBM development, potentially via DNMT1 regulation (Sun et al. 2017)."

Given the persistent neurotoxic effects of FB_1, understanding its long-term effects on neuronal health and function can be significant. Establishing a causal link between chronic exposure to these mycotoxins and adverse effects in humans has been challenging. This difficulty arises due to the absence of well-defined biomarkers for exposure and effects, the presence of multiple mycotoxins in the same food sources, and the uncertainty associated with estimating dietary intake levels (van den Brand et al. 2022). Experimental animal models are employed to study general and developmental neurotoxicity as well as the underlying mechanisms, since they closely replicate human biochemical and physiological conditions (Obafemi et al. 2025). *In vitro* models are commonly used to complement animal studies, as they help address ethical concerns associated with animal research. Additionally, they provide consistent and reproducible results since they originate from a single ancestor cell, leading to homogeneous cell populations (Obafemi et al. 2025). *In vitro* studies have demonstrated that FB_1 exposure (10–100 μM) increases neuronal and network excitability in cultured neurons and hippocampal slices from 18-day-old rats. Hippocampal cells exposed to 20–100 μM FB_1 exhibited c-fos activation, altered neuronal excitability, reduced neuronal labelling in the hippocampus, and increased cell death (Bódi et al. 2020). Further research using embryonic rat telencephalon-derived brain cells exposed to 3–40 μM FB_1 for 10 days showed neurotoxicity, disrupted myelin formation, and impaired oligodendrocyte maturation, though glial cells remained unaffected (Monnet-Tschudi et al. 1999). Studies on murine neuroblastoma (N2a) and microglial (BV-2) cells with both long-term (0.625–50 μM for 4 days) and short-term (25 μM for 24 h) FB_1 exposure revealed necrotic cell death, disrupted sphingolipid metabolism (increased sphinganine and decreased sphingosine), and differential cytokine expression, with TNF-α and IL-1β downregulated in BV-2 cells but not in N2a cells (Osuchowski and Sharma 2005b). Furthermore, FB_1 contributes to neuroinflammation by increasing pro-inflammatory cytokines such as IL-1β, IL-6 and TNF-α, resulting in chronic activation of microglia and astrocytes which promote neurodegeneration and synaptic dysfunction (Wang et al. 2015).

Overall, FB_1 is a significant health concern due to its widespread contamination in staple food for both humans and animals. While existing studies have extensively highlighted the toxic effects of FB_1, there remains limited information on effective strategies to mitigate its toxicity in humans compared to

animals (Obafemi et al. 2025). Here, we briefly highlight some strategies in mitigating the toxic effects of FB_1 in humans and animals. Extrusion cooking, a high-temperature and high-pressure process, effectively reduces FB_1 levels in maize, lowering its concentration by 64% in grits and up to 99% when combined with glucose (Voss et al. 2011). This method also prevents sphingolipid metabolism disruption and kidney lesions in rats consuming FB_1-contaminated maize (Voss et al. 2011). Nixtamalization, an alkaline cooking process using calcium hydroxide, is another approach to reducing FB_1 contamination. While it alters FB_1 by removing tricarboxylic groups, the exact impact on toxin levels remains unclear (Voss et al. 2017). Interestingly, organic and inorganic compounds can bind or adsorb FB_1 in the gastrointestinal tract, preventing its absorption. Calcium montmorillonite (NovaSil), a smectite clay, has been shown to reduce urinary FB_1 levels in rats by 20% within 24 h and 50% after 48 h (Robinson et al. 2012). In a human trial, 3 g/day of NovaSil eliminated 90% of FB_1. The binding occurs due to the protonation of FB_1's amino group in acidic conditions, allowing interaction with the clay's negatively charged surface. Nanosilicate platelets from montmorillonite, with high surface area and binding capacity, reduced FB_1 levels, restored sphingolipid balance, and prevented neural tube defects in mice exposed to FB_1-contaminated diets (Liao et al. 2014). Nanocellulose compounds have also shown promise. Jebali et al. (2015) developed polylysine-modified nanocellulose (NMPL), which binds to FB_1's carboxyl groups, while Zadeh and Shahdadi (2015) coated nanocellulose with free fatty acids to target its hydrophobic tail. Both effectively adsorbed FB_1 and reduced liver toxicity in mouse cells, but NMPL's stability in varying pH needs further evaluation. Additionally, Adidetox™, a mycotoxin inactivator (2–5 g/kg), moderately reduced FB_1 toxicity in rats, though it led to sphingolipid accumulation (Denli et al. 2015). In silver juvenile catfish, diphenyl diselenide $(PhSe)_2$ was found to ameliorate FB_1-induced neurotoxicity by attenuating brain lesions caused by FB_1 and inhibiting oxidative damage as indicated by increased CAT, GPx, glutathione S-transferase (GST) and glutathione reductase (GR) activities (Baldissera et al. 2020). Pre-treatment of a phenolic antioxidant, apocynin protected Balb/c mice from FB_1-induced neurotoxicity by restoring serotonin levels and reducing oxidative stress markers including ROS, MDA, protein carbonylation, and caspase-3 and -8 activation (Krishnaswamy et al. 2024). In another study on Balb/c mice, apocynin pre-treatment averted FB_1-induced oxidative brain damage via increased ROS and lipid peroxidation but decreased GSH along with *CAT*, *GPx*, *caspase-3* and *caspase-8* mRNA levels (Krupashree and Rachitha 2022). Similarly, astrocyte-like C6 cells treated with a plant-derived lignan called magnolol displayed low oxidative stress by ameliorating SOD, CAT, GSH and MDA levels (Wang et al. 2024). Microorganisms are also being explored as potential agents for FB_1 detoxification, as their inclusion in the diet may reduce FB_1 absorption in the gastrointestinal tract. Twelve *Lactobacillus* strains and six *Saccharomyces cerevisiae* strains were found to lower FB_1 levels by 62–77% and 67–74%, respectively, through weak noncovalent binding to their cell walls (Chlebicz and Śliżewska 2020). These microorganisms can also absorb FB_1 and aflatoxin simultaneously without losing effectiveness (Pizzolitto, Salvano, and Dalcero 2012). Probiotic strains such as *Lactobacillus delbrueckii* and *Pediococcus acidilactici* have been shown to

mitigate FB_1-induced hepatorenal toxicity and genotoxicity in rats by restoring kidney function, maintaining redox balance, and reducing DNA fragmentation (Khalil et al. 2015; Abdellatef and Khalil 2016). Additionally, *Lactobacillus paracasei* exhibited antioxidant properties by enhancing free radical scavenging, inhibiting lipid peroxidation, and protecting against FB_1-induced immunotoxicity (Abbès et al. 2016). Furthermore, research has identified several enzymes that function as biological detoxifiers of FB_1 by breaking it down into its less toxic hydrolyzed form (HFB_1), offering a strategy for decontaminating agricultural products and animal feed. Among these, FumD, a carboxylesterase derived from *Sphingopyxis* sp. MTA144, is the most extensively studied and is commercially available as FUMzyme®. Another carboxylesterase, FumDSB, is obtained from *Sphingomonas* bacteria. Both enzymes convert FB_1 into HFB_1, reducing its toxicity. A study using piglets demonstrated that FUMzyme® lowered the sphinganine/sphingosine ratio by 48.8%, while FumDSB achieved an 8.2% reduction (Wang et al. 2023; Li, Wang, et al. 2021). The ability of these enzymes to degrade FB_1 highlights their potential in preventing neurotoxicity associated with FB_1-contaminated food and feed (Obafemi et al. 2025). When evaluating the therapeutic potential of neuroprotective compounds for mitigating FB_1-induced neurotoxicity, it is crucial to assess their potential side effects and toxicity, as the boundary between safe and toxic doses is often unclear (Cooper 2004).

5.2 Limitations of the study

This study provides insights into the effects of FB_1 on DNA methylation, oxidative stress and inflammation, using an *in vitro* model in which U87MG cells were exposed to a 24 h treatment. However, the following limitations should be considered when interpreting the results:

1. Short exposure time

   The 24 h treatment period may only capture the immediate or acute cellular responses of FB_1 exposure. Some biological process may only occur after prolonged periods, which could be important for understanding chronic or cumulative cellular changes. Cells may also not fully metabolize the toxin within 24 h, leading to an incomplete understanding of its biological impact. The use of a single time point also reduces the generalizability of the findings. Longer treatment durations (48 – 96 h) are recommended.

2. *In vivo* validation

   The use of a cell culture system under a controlled *in vitro* environment, may not fully replicate the complexity of *in vivo* environments. *In vitro* models lack the complex tissue architecture and cell-cell interactions that occur *in vivo*. The absence of extracellular matrices, diverse cell types, and the structural organization of tissues can lead to a simplified representation of

biological processes. Many compounds also undergo metabolic activation or detoxification in vivo (e.g., by the liver), which cannot be fully replicated in cell cultures. Under *in vitro* environments, cells are maintained in controlled conditions with optimal nutrients, CO_2 and oxygen levels, which do not fully mimic the *in vivo* micro-environment. Therefore, the results may not fully reflect how FB_1 affects global DNA methylation, oxidative stress and inflammation in a living organism. Additionally, *in vitro* models do not fully replicate the body's ability to metabolize and process compounds. FB_1 may be metabolized into active forms *in vivo*, but this is not typically accounted for in cell culture systems, potentially underestimating or misrepresenting the compound's biological effects.

3. Cell line-specific results

   The glioblastoma-derived U87MG cell line may not fully represent the behaviour of normal glial cells or other cell types, which could limit the broader applicability of the findings to other tissues or disease models. As immortalized cells, U87MG cells may exhibit different responses compared to primary cells or those within a natural tissue environment. Additionally, these cell lines can accumulate mutations or genetic drifts over time, potentially altering their reactions to treatments and further reducing the reproducibility of the results to other cell types or organisms.

4. FB_1 concentration

   The concentration of FB_1 used may not precisely mirror the exposure levels typically encountered by humans or other organisms. *In vitro* studies often use higher concentrations of a compound than what would be found *in vivo* to elicit measurable effects, thus high concentrations of FB_1 may induce effects that do not necessarily correspond to environmental or dietary exposure levels.

5. Global DNA methylation assay:

   Global DNA methylation assesses the overall methylation levels across the genome but does not provide insights into the methylation status of specific gene loci or regulatory regions. Consequently, it may miss critical changes in the methylation of particular genes or regions that are biologically important. Methylation changes at promoter regions or enhancers, which influence gene expression, may be overlooked. Global methylation assays often cannot differentiate between 5-methylcytosine and 5-hydroxymethylcytosine, which have distinct biological roles. Additionally, some assays measure only CpG methylation, missing potential methylation at non-CpG sites, which can be relevant in certain tissues Future work should incorporate gene-specific methylation analysis and sequencing techniques to gain deeper

insights into the methylation patterns in these cells, as well as to better understand the role of methylation in FB_1-induced alterations in oxidative stress and inflammatory genes.

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APPENDIX

6.1 UKZN examination rules

The UKZN College of Health Science (CHS) Handbook outlines the requirements for a PhD thesis, specifying that it should be presented in the standard format along with one published paper or an unpublished manuscript that has been submitted to an accredited journal, based on the doctoral research. According to CHS16 (Thesis by Publications), the thesis may consist of at least three published papers or papers in press in accredited journals, with the student listed as the primary author. CHS16 also allows for a thesis by manuscript, where the thesis may include at least three manuscripts, with the student as the primary author, that have not yet been published but are in preparation. At least two of these manuscripts must represent original research. In both cases (thesis by publications or by manuscript), the thesis must include introductory and concluding sections that integrate the work.

6.2 DNA methylation assay

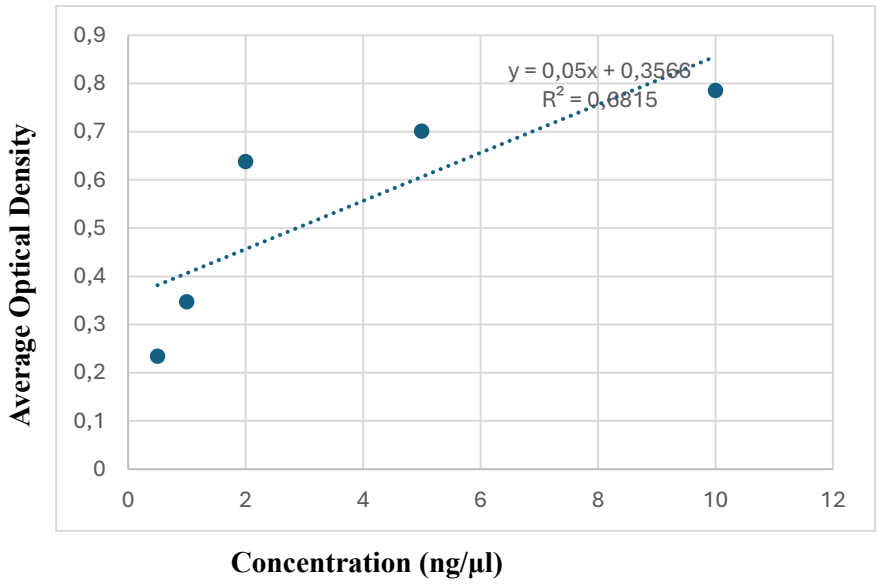


Figure 6.1: DNA methylation standard curve used to determine the 5-methylcytosine content in FB_1-treated U87MG cells.

6.3 BCA assay

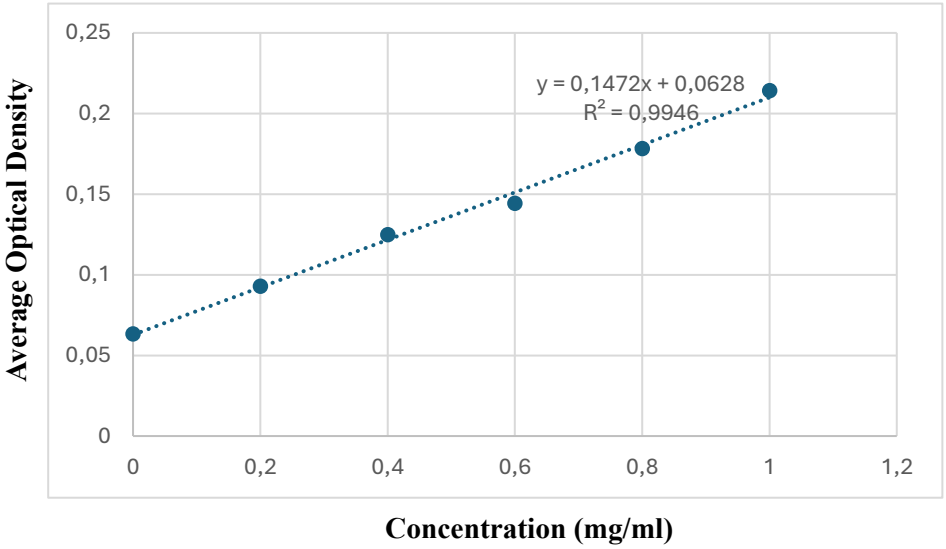


Figure 6.2: The BCA assay standard curve with known bovine serum albumin (BSA) concentrations (0, 0.2, 0.4, 0.6, 0.8, 1 mg/ml) was used to determine the protein concentration of each sample (n=3).

6.4 Ethics statement

This study was carried out using a commercially available cell line in an *in vitro* laboratory setting, with strict adherence to ethical guidelines. As the study was conducted using *in vitro* cell cultures, it was exempt from ethical considerations.

6.5 TBARS assay equation

$$\text{MDA-TBA} = \frac{Sample\ Optical\ Density}{156\ mM^{-1}} \times 1000$$

Note: 156 mM^{-1} is the absorption co-efficient for measuring malondialdehyde (MDA).

6.6 RNA concentrations

Table 6.1: RNA concentrations and absorbances values obtained for control and FB_1 samples

RNA Sample ID	Nucleic Acid Conc	A260 Absorbance	A280 Absorbance	A260/280 ration
Control	539.8 ng/µl	13,496	6,633	2,03
FB_1 880	686 ng/µl	17,151	8,726	1,97

6.7 Example of qPCR melt curves obtained for this study

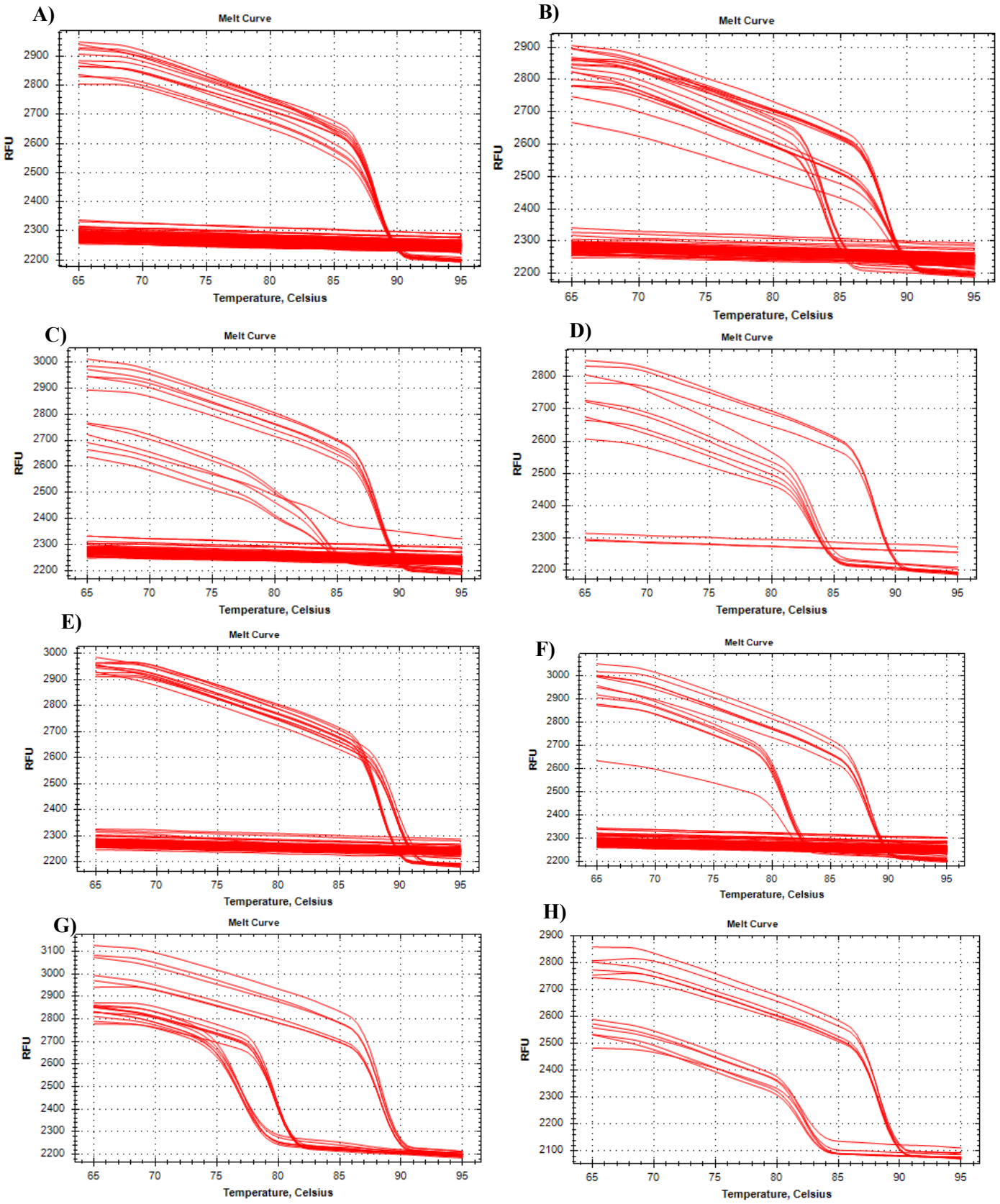
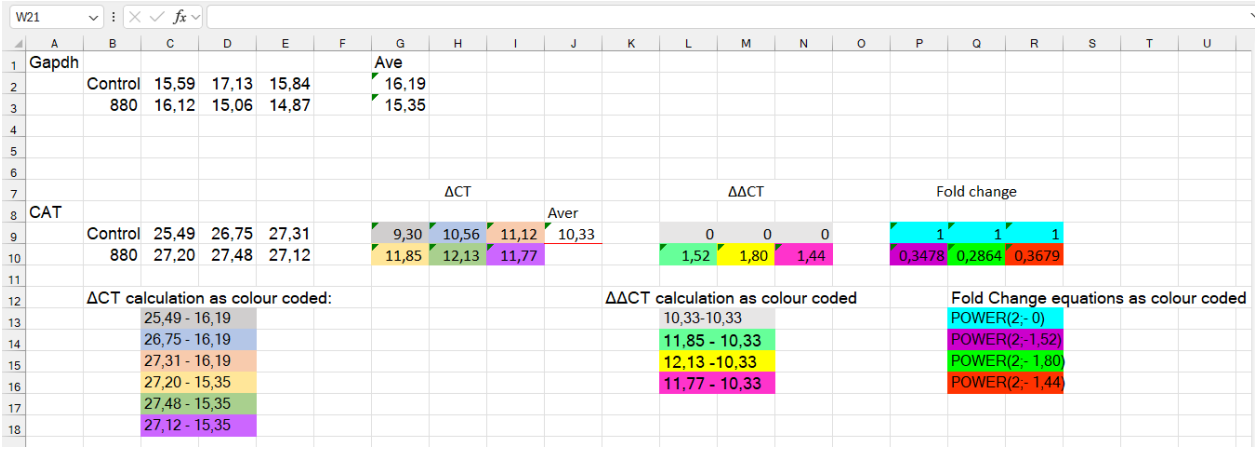


Figure 6.3: Melt curves of *DNMT1*(A), *DNMT3A* (B), *DNMT3B* (B), *MBD2* (C), *CAT* (D), *GPx* (E), *NRF-2* (F), *COX-2* (G), *NF-kβ* (G) and *IL-10* (H) genes from the qPCR analysis of this study. DNMT: DNA methyltransferase; MBD2: Methyl-CpG binding domain 2; CAT: Catalase; GPx: Glutathione

peroxidase; NRF-2: Nuclear factor erythroid 2-related factor 2; COX-2: Cyclooxygenase-2; NF-kβ: Nuclear factor kappa B; IL-10: interleukin-10

6.8 Ct Method explanation



1	Gapdh					Ave														
2		Control	15,59	17,13	15,84	16,19														
3		880	16,12	15,06	14,87	15,35														
7							ΔCT					ΔΔCT			Fold change					
8	CAT									Aver										
9		Control	25,49	26,75	27,31	9,30	10,56	11,12	10,33		0	0	0		1	1	1			
10		880	27,20	27,48	27,12	11,85	12,13	11,77			1,52	1,80	1,44		0,3478	0,2864	0,3679			
12		ΔCT calculation as colour coded:									ΔΔCT calculation as colour coded				Fold Change equations as colour coded					
13			25,49 - 16,19								10,33-10,33				POWER(2;- 0)					
14			26,75 - 16,19								11,85 - 10,33				POWER(2;-1,52)					
15			27,31 - 16,19								12,13 -10,33				POWER(2;- 1,80)					
16			27,20 - 15,35								11,77 - 10,33				POWER(2;- 1,44)					
17			27,48 - 15,35																	
18			27,12 - 15,35																	

Figure 6.4: Example of fold change calculation using the Ct method for Catalase gene expression.

6.9 Cytobuster recipe

Cytobuster was obtained from Novagen (catalogue no. 71009) while the protease and phosphatase inhibitors were obtained from Roche (catalogue nos. 05892791001 and 04906837001, respectively).

6.10 Full blot images for all proteins and their corresponding β-actin expressions assessed in this study

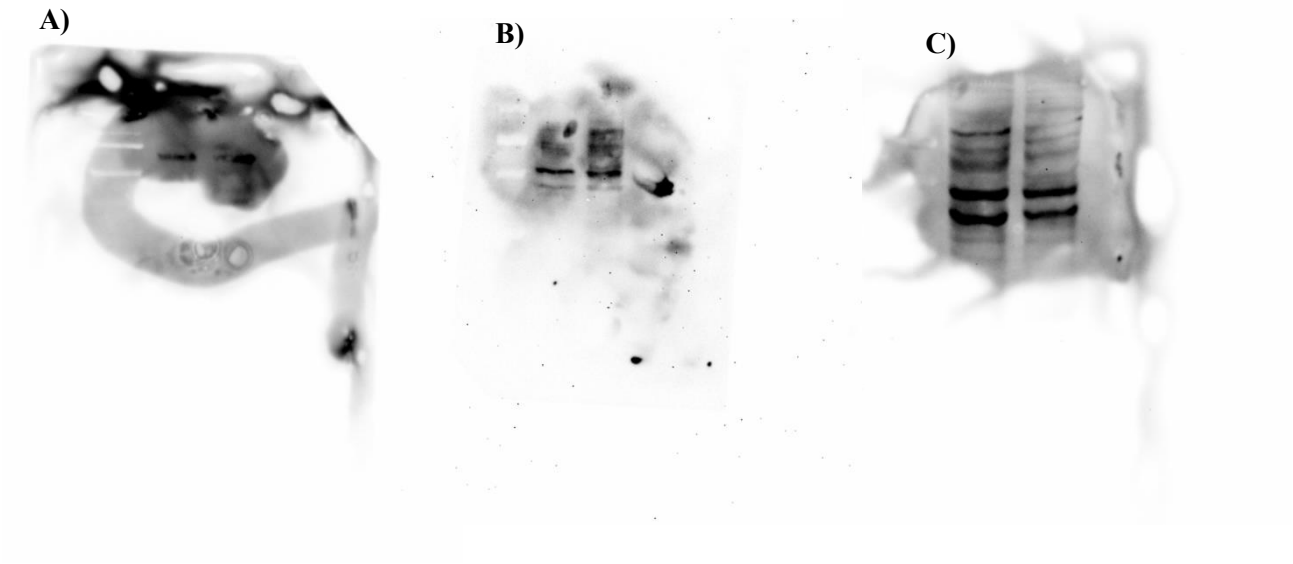


Figure 6.5: DNMT1 (A), DNMT3A (B) and MBD2 (C) full blot images. The MBD2 membrane (C) has 2 bands as the antibody used probes for both MBD2 and MBD3. DNMT: DNA methyltransferase; MBD2: Methyl-CpG binding domain 2.

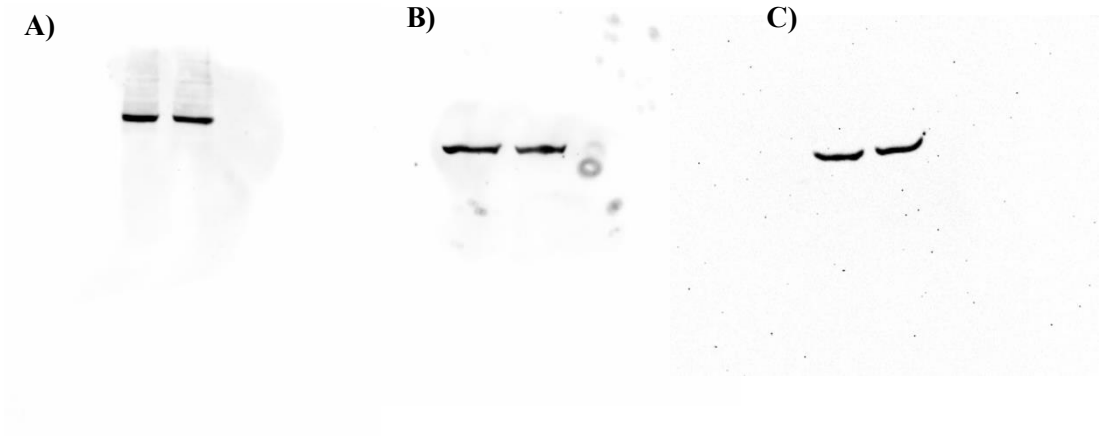


Figure 6.6: Full β-actin blot images used to normalize the protein expressions of DNMT1 (A), DNMT 3A (B) and MBD2 (C). DNMT: DNA methyltransferase; MBD2: Methyl-CpG binding domain 2.

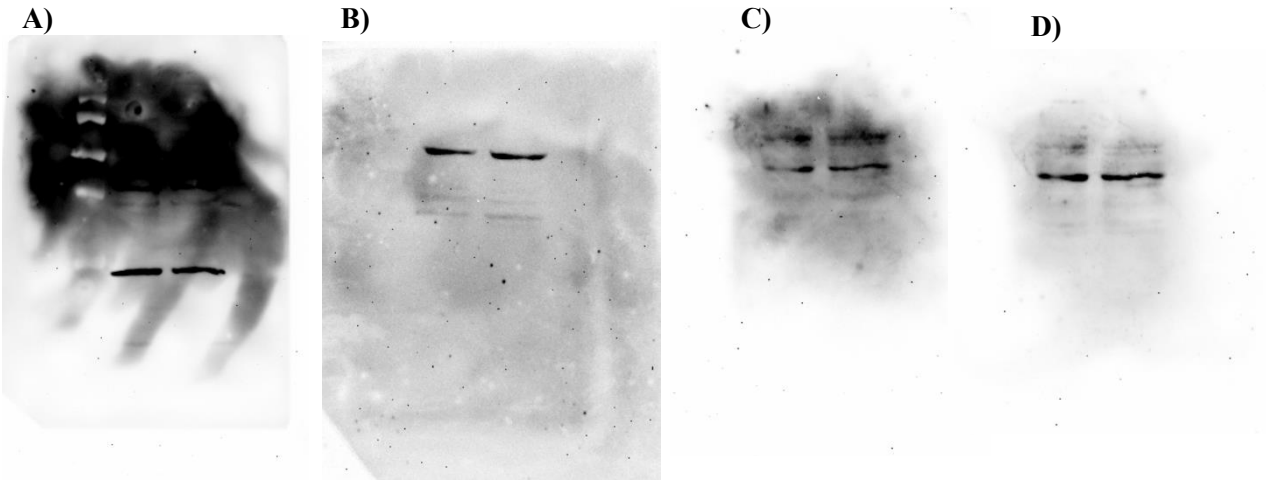


Figure 6.7: SOD2 (A), CAT (B), tNRF-2 (C) and pNRF-2 full blot images used to determine protein expressions. SOD2: Superoxide dismutase 2; CAT: Catalase; NRF-2: Nuclear factor erythroid 2-related factor 2.

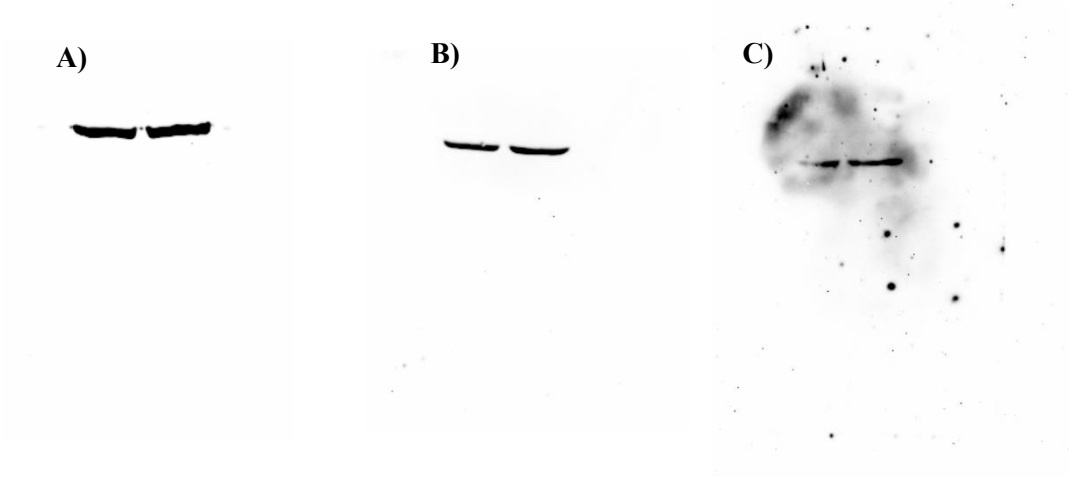


Figure 6.8: Full β-actin blot images used to normalize the protein expressions of SOD2 (A), CAT (B), tNRF-2 (C) and pNRF-2 (C). SOD2: Superoxide dismutase 2; CAT: Catalase; NRF-2: Nuclear factor erythroid 2-related factor 2.

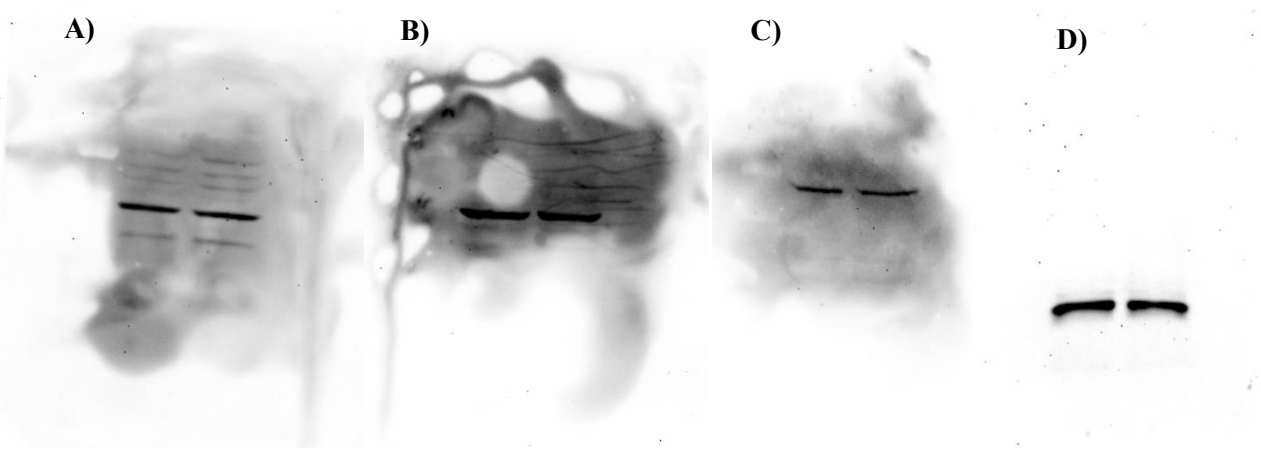


Figure 6.9: SIRT3 (A), LONP1 (B), HSP 60 (C) and TFAM full blot images used to determine protein expressions. SIRT: Sirtuin; LONP1: Lon protease 1; HSP: Heat shock protein; TFAM: Mitochondrial transcription factor A.

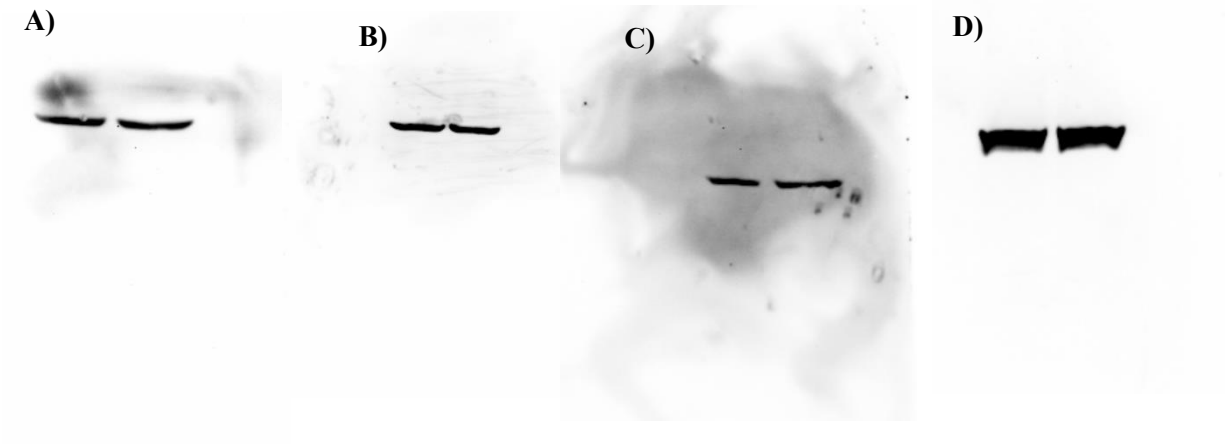


Figure 6.10: Full β-actin blot images used to normalize the protein expressions of SIRT3 (A), LONP1 (B), HSP60 (C) and TFAM (D). SIRT: Sirtuin; LONP1: Lon protease 1; HSP: Heat shock protein; TFAM: Mitochondrial transcription factor A.

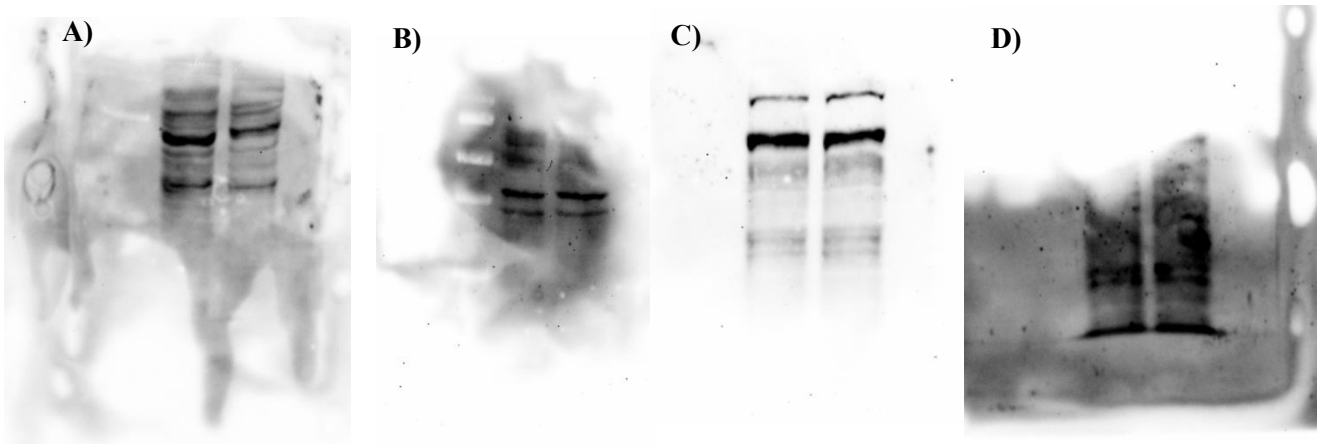


Figure 6.11: IL-1β (A), TNF-α (B), COX-2 (C) and BDNF (D) full blot images used to determine protein expressions. IL-1β: Interleukin 1 beta; TNF-a: Tumour necrosis factor-alpha; COX-2: Cyclooxygenase-2; BDNF: Brain-derived neurotrophic factor.

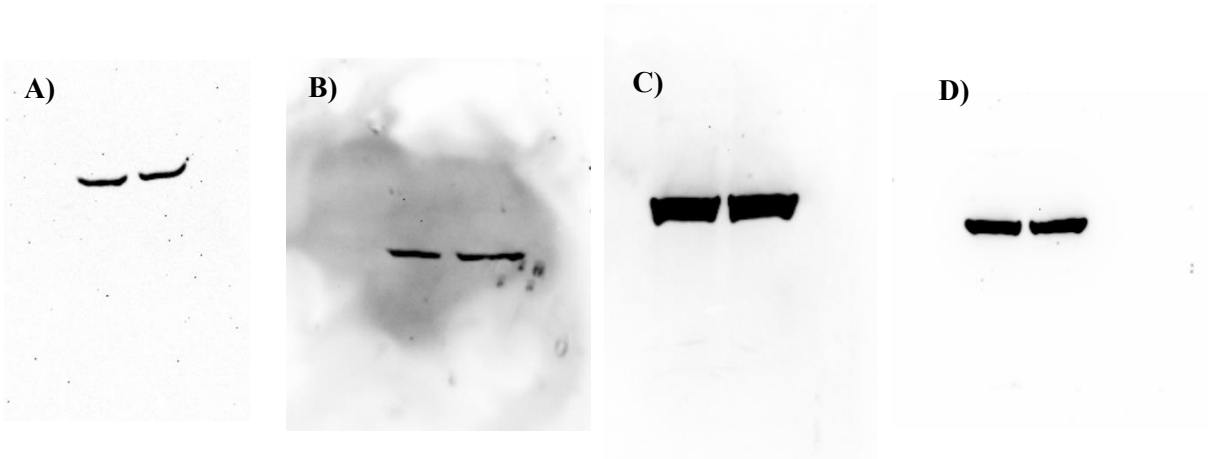


Figure 6.12: Full β-actin blot images used to normalize the protein expressions of IL-1β (A), TNF-α (B), COX-2 (C) and BDNF (D). IL-1β: Interleukin 1 beta; TNF-a: Tumour necrosis factor-alpha; COX-2: Cyclooxygenase-2; BDNF: Brain-derived neurotrophic factor.