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Fumonisin B₂ induces mitochondrial stress and mitophagy in Hek293 cells

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Discipline of Medical Biochemistry

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Table of Contents

Plagiarism Declaration	i
Acknowledgements	ii
Presentations	iii
List of Figures.....	ix
List of Tables.....	xii
List of Abbreviations	xiii
Abstract.....	1
Chapter 1: Introduction.....	3
1.1 Background	3
1.2 Problem statement	5
1.3 Research questions.....	5
1.5 Aims and Objectives.....	6
1.5.1 Aim of study.....	6
1.5.2 Objectives	6
1.6 Hypothesis	6
Chapter 2: Literature Review.....	7
2.1 Mycotoxins	7
2.1.1 Mycotoxin history.....	8
2.1.2 Implications to food safety and health	8

2.2.1 Fumonisin general structure and toxicity	10
2.2.2 Fumonisin detoxification	12
2.3 Fumonisin B ₂	13
2.3.1 Fumonisin B ₂ mechanism of action.....	15
2.4 Mitochondria.....	16
2.4.2 Mitochondrial Toxicity	18
2.4.3 Mycotoxins and Mitochondrial toxicity	19
2.5 Mitochondrial Stress Responses	20
2.5.1 Proteins involved in mitochondrial stress responses	21
2.5.1.1 Sirtuins	21
2.5.1.2 Sirtuin 3	22
2.5.1.3 LON Protease.....	24
2.5.1.4 Heat shock proteins.....	26
2.6 Mitophagy	28
2.6.1 Regulation of mitophagy.....	30
2.6.1.1 Nuclear factor (erythroid-derived 2)-like 2	30
2.6.2 Micro-RNAs	31
2.6.2.2 MicroRNA-27b.....	32
2.7 Kidney structure and function	33
2.7.1 Basic kidney structure	33
2.7.2 Kidney function.....	34

2.7.3 Use of Hek293 cell line.....	34
2.7.4 Nephrotoxicity.....	34
2.7.5 Kidney and mitochondrial toxicity	35
Chapter 3: Materials and Methods	36
3.1 Materials.....	37
3.2 Methods	37
3.2.1 Cell culture and exposure protocol	37
3.2.2 MTT assay.....	37
3.2.2.1 Introduction.....	37
3.2.2.2 Protocol	38
3.2.3 ATP assay	39
3.2.3.1 Introduction.....	39
3.2.3.2 Protocol	41
3.2.4 H₂DCF-DA assay.....	41
3.2.4.1 Introduction.....	41
3.2.4.2 Protocol	42
3.2.5 JC-1 Mitoscreen.....	43
3.2.5.1 Introduction.....	43
3.2.5.2 Protocol	45
3.2.6 Western blots.....	46
3.2.6.1 Introduction.....	46

3.2.6.2 Protein isolation and sample preparation	47
3.2.6.3 SDS-PAGE.....	48
3.2.6.4 Transfer of proteins	49
3.2.6.5 Immuno-blotting.....	49
3.2.6.6 Detection	49
3.2.6.2 Protocol	50
3.2.7 Quantitative PCR	52
3.2.7.1 Introduction.....	52
3.2.7.2. Protocol	53
RNA isolation	53
RNA quantification.....	54
cDNA synthesis.....	54
i. cDNA synthesis for mRNA	54
ii. cDNA synthesis for miRNA.....	55
Gene expression	56
i. mRNA gene expression.....	56
ii. MiRNA gene expression	58
3.2.8 Statistical analysis	59
Chapter 4: Results.....	60
4.1 Cell Viability.....	60
4.2 FB ₂ induced mitochondrial stress	61

4.3 FB ₂ increased ROS production and mitochondrial membrane depolarisation	62
4.4 FB ₂ induced mitochondrial stress responses	63
4.5 FB ₂ promoted mitophagy via Nrf2 activation	64
4.6 FB ₂ induced mitophagy.....	65
Chapter 5: Discussion	67
Chapter 6: Limitations and Recommendations	75
Chapter 7: Conclusion	76
References	77
Appendix A.....	95
Appendix B.....	96

List of Figures

Chapter 2

Figure 2.1: General structure of the fumonisin mycotoxin (Gelderblom et al., 1993)	
.....	10
Figure 2.2: Comparison of fumonisin structures, sphinganine and sphingosine (prepared by author)	
.....	11
Figure 2.3: Structure of FB ₂ (Carrasco-Sánchez et al., 2017)	14
Figure 2.4: Mechanism of action of FB ₂ (Voss et al., 2007)	15
Figure 2.5: Structure and selective functions of mitochondria (prepared by author)	
.....	17
Figure 2.6: Summary of SIRT3 activity during stress conditions (prepared by author)	
.....	23
Figure 2.7: LONP1 proteolysis mechanism for damaged proteins (prepared by author)	
.....	25
Figure 2.8: Simplified diagram of the mitophagic process (Youle and Narendra, 2011)	
.....	29
Figure 2.9: Function of miRNA (prepared by author)	32

Chapter 3

Figure 3: Experimental approach used to determine the mitochondrial toxicity of FB ₂ on Hek293 cells (Prepared by author).	36
Figure 3.1: Conversion of MTT salt to formazan via mitochondrial reductase in viable cells (prepared by author).	38

Figure 3.2: Principle of the CellTiter-Glo® assay used to measure concentration of intracellular ATP (Deshpande, 2001).	40
Figure 3.3: Principle of DCF assay for ROS detection	42
Figure 3.4: Diagram showing different results produced in healthy and unhealthy mitochondria following the JC-1 mitoscreen (Sivandzade et al., 2019).	44
Figure 3.5: Summary of western blot procedure (Bass et al., 2017)	46
Figure 3.6: Principle of qPCR (Garibyan and Avashia, 2013).	53

Chapter 4

Figure 4.1: FB ₂ is cytotoxic to Hek293 cells. A dose dependent decline in viability of the cell occurred after treatment with varying concentrations (0 -500µM) of FB ₂	60
Figure 4.2: FB ₂ induced mitochondrial stress. HSP60 protein expression increased in a dose-dependent manner (A). ATP concentrations exhibited a biphasic response following treatment with FB ₂ (B). <i>*p<0.05; **p<0.001; ***p<0.0001 relative to control</i>	61
Figure 4.3: FB ₂ increased ROS production and mitochondrial membrane depolarisation. ROS production was suppressed at lower concentrations however, significant increases were noted at higher treatments (A). Mitochondrial membrane depolarisation also significantly increased at higher concentrations (B).	62
Figure 4.4: FB ₂ induced a biphasic mitochondrial stress responses. SIRT3 protein expression was up-regulated at 100µM and 500µM, but down-regulated at IC ₅₀ when compared to the control (A). <i>SIRT3</i> gene expression is suppressed at the IC ₅₀ (C). LONP1 expression increases at lower concentrations but decreases at higher concentrations (B). <i>*p<0.05; **p<0.001; ***p<0.0001 relative to control.</i>	63

Figure 4.5: FB₂ promoted mitophagy. Significant upregulations in phosphorylated Nrf2 (Ser40) expression following FB₂ treatment. ***p<0.001; ***p<0.0001 relative to the control.* **64**

Figure 4.6: FB₂ increased mitophagy markers in Hek293 cells. PINK1 protein (A) and gene expression (B) was significantly up-regulated at the higher concentrations of FB₂. Significant up-regulation in p62 protein as compared to the control was noted at high treatment concentrations (C). *MiR-27b* expression exhibited downregulation in comparison to the control (D) **p<0.05; **p<0.001; ***p<0.0001 relative to control* . **66**

List of Tables

Chapter 2

Table 2.1: Summary of location and function of various HSPs (prepared by author)	
.....	27

Chapter 3

Table 3.1: cDNA synthesis (iScript cDNA synthesis kit, Bio-Rad).	55
Table 3.2: cDNA synthesis (miScript® II RT kit, Qiagen).....	56
Table 3.3: Reaction mix for qPCR (iQ™ SYBR® Green PCR kit)	57
Table 3.4: Primers sequences with respective annealing temperatures for genes assessed	58
Table 3.5: Reaction mix for qPCR (MiRNA SYBR® Green PCR Kit).....	59

List of Abbreviations

ADP	Adenosine diphosphate
AKI	Acute kidney toxicity
ARE	Anti-oxidant response element
BCA	Bicinchoninic acid
CCM	Complete culture medium
cDNA	Complementary DNA
CKD	Chronic kidney disease
Cu ²⁺	Cupric ion
Cu ⁺	Cuprous ion
DCF	Dichlorofluorescein
DMEM	Dulbecco's minimum essential medium
DMSO	Dimethyl sulphoxide
dNTP	Deoxynucleotide triphosphates
dsDNA	Double stranded DNA
ELEM	Equine leukoencephalomalacia
ETC	Electron transport chain
FB ₁	Fumonisin B ₁
FB ₂	Fumonisin B ₂
FOXO3a	Forkhead box O3
H ₂ DCF-DA	2, 7-dichlorohydrofluorescein diacetate
Hek293	Human embryonic kidney cells
hr	Hours
HRP	Horseradish peroxidase

HSPs	Heat shock proteins
HSP60	Heat shock protein 60
IARC	International Agency for Research on Cancer
IC ₅₀	Half maximal inhibitory concentration
KEAP1	Kelch like-ECH-associated protein
min	Minutes
mir-27b	microRNA-27b
miRNA	Micro-RNA
mnSOD	Manganese superoxide dismutase
mRNA	Messenger RNA
MTT	Methylthiazol terazolium
NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide
Nrf2	Nuclear factor (erythroid-derived 2)-like 2
OXPHOS	Oxidative phosphorylation
p62	Ubiquitin-binding adaptor p62
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PINK1	PTEN-induced putative kinase 1
PPAR γ	Peroxisome proliferator-activated receptor gamma
qPCR	Quantitative PCR
RBD	Relative band density
RFU	Relative fluorescent units
RLU	Relative light units
RNA	Ribose nucleotide acid

ROS	Reactive oxygen species
RT	Room temperature
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SIRT	Sirtuins
SIRT3	Sirtuin 3
ssDNA	Single stranded DNA
UPR ^{mt}	Mitochondrial unfolded protein response
UTR	Untranslated region
WHO	World Health Organisation
$\Delta\psi_m$	Mitochondrial membrane potential

Abstract

Food insecurity poses a significant socio-economic problem in third world economies, particularly in countries that rely heavily on maize and maize products. Ubiquitous soil fungi parasitize agricultural commodities and produce mycotoxins. Fumonisin B₂ (FB₂), a neglected mycotoxin, is produced by several *Fusarium* species. The aim of this study was to investigate mitochondrial stress responses in human embryonic kidney (Hek293) cells exposed to FB₂ for 24 hours (hr). Cell viability was assessed via the methylthiazol tetrazolium (MTT) assay and the half maximal inhibitory concentration (IC₅₀) value (317.4 μM) was generated. Additional concentrations of 100 μM and 500 μM were selected to achieve a broader toxic profile of FB₂. Reactive oxygen species (ROS) was quantified (fluorescence), mitochondrial membrane depolarisation (fluorescence) was assessed and adenosine triphosphate (ATP) concentration was evaluated (luminometry) to assess mitochondrial integrity. The relative expression of mitochondrial stress response proteins, Sirtuin 3 (SIRT3), Nuclear factor (erythroid-derived 2)-like 2 (Nrf2), LON protease (LONP1), PTEN-induced putative kinase 1 (PINK1), ubiquitin-binding adaptor p62 (p62) and heat shock protein 60 (HSP60) was determined by western blots. Transcript levels of *SIRT3*, *PINK1* and *microRNA-27b* (*miR-27b*) was assessed using quantitative PCR (qPCR). Results indicated that both low and high concentrations of FB₂ that were within the naturally occurring concentration range of the compound were able to induce mitochondrial dysfunction. FB₂ (IC₅₀) downregulated mitochondrial stress proteins and upregulated mitophagy markers. Despite upregulation of mitochondrial stress maintenance proteins at the highest concentration (500 μM) of FB₂, mitophagic markers increased with subsequent cell death; whilst at a lower concentration (100 μM) of FB₂, mitochondrial stress protein expressions were upregulated resulting in decreased expression of mitophagic

markers and cell proliferation. In conclusion, FB₂ was cytotoxic to the kidney derived Hek293 cells via induction of mitochondrial stress and mitophagy.

Keywords: Fumonisin B₂, mitophagy, mitochondrial stress, PINK1, Nrf2, SIRT3, human kidney cells, microRNA

Chapter 1: Introduction

1.1 Background

Extreme weather conditions cause ubiquitous soil fungi to parasitize agricultural commodities and produce mycotoxins (Hussein and Brasel, 2001). Mycotoxins are beneficial to fungi as they are toxic to other organisms, and are considered as a fungal survival mechanism (Hussein and Brasel, 2001).

FB₂ is a highly prevalent mycotoxin produced mainly by *Fusarium verticillioides*, a common contaminant of maize (Stockmann-Juvala and Savolainen, 2008). The World Health Organisation (WHO) has reported that industrial countries have low exposure to the toxin however, exposure often exceeds the provisional maximum tolerable intake of 2 µg/kg in developing countries such as South Africa, Nigeria, Malawi and China with daily exposure exceeding 15 µg/kg body weight (Shephard et al., 2007).

Despite the high prevalence of FB₂, there is a dearth of toxicity studies on FB₂ in comparison to its structural analogue Fumonisin B₁ (FB₁) which has been extensively studied. FB₁ has been implicated in diseases such as equine leukoencephalomalacia (ELEM), pulmonary oedema, neural tube defects and oesophageal cancer in humans. The toxin exerts its noxious effects in humans via numerous pathways including induction of apoptosis, oxidative stress and epigenetic changes (Stockmann-Juvala et al., 2004, Chuturgoon et al., 2014). The mechanisms of FB₂ toxicity however, remains elusive despite FB₂ showing greater cytotoxic potential than FB₁ (Riley et al., 1997).

FB₂ has a higher polarity than FB₁ due to the presence of an additional hydroxyl group. This allows for FB₂ to be rapidly excreted via kidney as it is highly water soluble, causing the kidney to be susceptible to FB₂–induced toxicity. The amino group of FB₂

allows it to utilise endogenous amino acid transporters to cross cell membranes and therefore interact with cellular structures resulting in cellular dysfunction and cell death (Stockmann-Juvala and Savolainen, 2008). Previous studies, using equine cells, have demonstrated the ability of FB₂ to inhibit *de novo* sphingolipid biosynthesis however, no data to date exists on its physiological and biochemical effects in humans (Riley et al., 1997).

Studies have shown the ability of some mycotoxins to upregulate mitochondrial stress in cells however, limited evidence is available to demonstrate the effects of fumonisins on mitochondria (Domijan and Abramov, 2011, Arumugam et al., 2019). Mitochondrial function is imperative to overall cell health and loss of function results in many adverse effects including decreased ATP concentrations, increased oxidative stress and apoptosis or necrosis (Picard et al., 2011b). Upon exposure to toxins the mitochondria activate antioxidant and mitochondrial stress responses (Youle and Narendra, 2011). Proteins such as SIRT3 and LONP1 are activated in an attempt to maintain mitochondrial function. Activation of such proteins can directly ameliorate mitochondrial stress or result in the upregulation in the expression of other proteins that can reduce stress (Ngo and Davies, 2007, Bause and Haigis, 2013).

Failure to do so results in mitophagy, i.e., degradation of the mitochondria mediated by the proteins PINK1 and p62 (Youle and Narendra, 2011). This process is further regulated by a group of small non-coding RNA's namely micro-RNAs (miRNA). Specifically, the upregulation of *miR-27b* expression leads to a suppression of *PINK1* expression at a translation level directly through binding to the 3'-untranslated region (3'-UTR) of its messenger RNA (mRNA), thus inhibiting the occurrence of mitophagy within cells. Conversely, the inhibition of *miR-27b* expression promotes mitophagy in cells (Kim et al., 2016). Additionally, transcription factors normally involved in stress

responses associated with the mitochondria such as Nrf2, allows for the transcriptional activation of *PINK1* in an attempt to promote mitophagy. The subsequent removal of damaged mitochondria has been shown to encourage cell survival by reduction of overall stress on cellular processes (Murata et al., 2015).

The mechanisms of FB₂-induced mitochondrial stress responses in kidney cells remains unknown. Thus the purpose of this study was to determine the effects of FB₂ on the mitochondrial stress responses in Hek293 cells by determining mitochondrial output and dysregulation of mitochondrial maintenance following exposure.

1.2 Problem statement

FB₂ has been found to commonly contaminate maize which is the staple diet in South African and many other third world countries. Contamination of crop poses a serious threat to consumers' health and increases food insecurity. Little is known about the effect of FB₂ on humans as opposed to FB₁ despite the toxin potentially being more cytotoxic and co-produced. Although the toxin has been implicated in sphingolipid metabolism inhibition, the biochemical pathways surrounding its human toxicity are yet to be elucidated. Furthermore, no data exists to show the toxicity of FB₂ in kidney cells. This is essential as FB₂ has a high polarity causing it to be excreted rapidly by kidneys permitting toxic interactions. Additionally, FB₂ is excreted unmetabolised via the kidney increasing the kidneys susceptibility to toxicity.

1.3 Research questions

The study focussed on looking at the mitochondrial toxicity induced by FB₂ in a kidney cell line. The kidney is particularly susceptible to the toxic effects of FB₂ due to the high polarity of the toxin thus increasing retention time of the unmetabolised compound. Additionally, the kidney is densely populated with mitochondria which increases the

risk of mitochondrial toxicity occurring. The following research questions were proposed:

- Does FB₂ interfere with kidney cell mitochondrial output?
- Does FB₂ induce mitochondrial stress in kidney cells?
- Does FB₂ induce mitophagy in kidney cell?

1.5 Aims and Objectives

1.5.1 Aim of study

The aim of this study was to determine the mechanism of mitochondrial toxicity of FB₂ in Hek293 by investigating its role on mitochondrial stress response proteins and mitophagy bio-markers.

1.5.2 Objectives

- To determine the mitochondrial output following FB₂ exposure
- To determine the biochemical mechanism of mitochondrial stress induction and mitophagy by FB₂

1.6 Hypothesis

FB₂ induces human kidney toxicity via upregulation of mitochondrial stress and induction of mitophagy within kidney cells.

Chapter 2: Literature Review

2.1 *Mycotoxins*

Mycotoxins are secondary metabolites that are primarily produced by the mycelial structure of filamentous fungi bearing no major biochemical importance to the growth of the fungi. The term originates from Greek words meaning fungus (mykes) and poison (toxicum) (Goldblatt, 1972). These metabolites are produced when moulds release enzymes that degrade macromolecules for growth and metabolism. In doing so they gain the ability to absorb low molecular weight nutrients and produce secondary metabolites (mycotoxins). These have no effect on primary metabolism however, they may possess biological activity and be toxic to other organisms (Moss, 1991)

Tropical climates with high temperatures, increased humidity and rainfall promote fungal growth and mycotoxin formation (Bhat and Vasanthi, 2003). Thus, the production of mycotoxins is promoted by both favourable ecological conditions and storage environments (Hussein and Brasel, 2001). Mycotoxins account for detrimental loss in global wealth due to contamination of agricultural commodities. Therefore, they hold great significance in the public health and agro-economic sector due to adverse effects elicited upon exposure. The Food and Agriculture Organisation has estimated that roughly 25% of condemned agricultural products are contaminated with mycotoxins (Boutrif and Canet, 1998). The most studied groups of contaminants include with aflatoxins, ochratoxins and fumonisins (Heidtmann-Bemvenuti et al., 2011).

2.1.1 Mycotoxin history

Mycotoxicoses refers to acute diseases induced by mycotoxins in animals and humans (Hoerr, 2020). This phenomenon was noted for several years however, in England in 1960, the outbreak of Turkey X disease led to the discovery of aflatoxins. Furthermore, it was discovered that minute doses of secondary metabolites in consumables can induce disease (Ostry et al., 2017). This led to an increase in studies revolving around mycotoxins and its toxic effects. Several mycotoxins existing today were initially proposed as potential antibiotics but were later disregarded due to their high toxicity (Alshannaq and Yu, 2017).

2.1.2 Implications to food safety and health

Mycotoxins can cause both direct and indirect contamination. Direct contamination refers to growth of moulds on food itself. This may occur during production of consumables during storage or transportation. The contaminated food is consumed resulting in direct exposure. Although this seems unlikely, in areas with food shortages consumers are forced to ingest contaminated foods to prevent starvation. In other areas mould consumption is common due to it always being present such as in areas with tropical climates whereas many countries use fungi in production of food. Commercial use of mould has been proved to be non-mycotic and safe however, home fermentation is unreliable and potentially hazardous (Alshannaq and Yu, 2017).

Indirect consumption occurs when a contaminated food ingredient is used for production and is less frequent than direct exposure. This is more prevalent in food manufacturing or processing. Additionally, it may arise when animal products containing mycotoxin residues are consumed and is more common in developed

countries such as America, European states and Canada (Hesseltine et al., 1966, Alshannaq and Yu, 2017)

The most notorious food borne mycotoxins include aflatoxins, ochratoxins and fumonisins. Aflatoxin has been classified as a class I carcinogen by the International Agency for Research on Cancer (IARC) (IARC, 2002). They have the ability to induce stunted growth if exposure prior to birth or during early childhood development occurs. The toxin suppresses cell-mediated immune responses posing huge threats to human health (Pitt, 2013).

Ochratoxin A induces chronic nephrotoxicity compromising kidney function in both humans and animals. The carcinogenic mechanism remains elusive with its outcomes being less potent than its kidney toxicity with both genomic and non-genomic modes of action (Pitt, 2013).

Previously mycotoxin contamination in Africa was often overlooked due to ignorance regarding its existence and implications in health safety. In more recent times, the *Fusarium* and *Aspergillus* genera have received special attention as they are considered to be the most hazardous to health in Africa (WHO, 2006). Fumonisin demonstrate several of adverse effects. Most commonly they induces ELEM in horses and pulmonary oedema in pigs. Studies in humans link exposure to oesophageal cancer especially in South Africa where maize consumption is high (Pitt, 2013).

2.2 Fumonisin

Fumonisin are mycotoxins produced by the *Fusarium* species amongst others. The mycotoxin was first discovered in South Africa in 1988 (Marasas et al., 1988, Marasas, 2001). They are frequently found as contaminants in maize and maize-based products (Shephard et al., 1996). Previously, data had only showed the occurrence of

fumonisin in maize however, most recently, it was found in garlic bulbs (Seefelder et al., 2002), onion powder (Boonzaaijer et al., 2008), peanuts (Liu et al., 2008) and soybeans (Aoyama et al., 2010). In South Africa detection of the toxin in dietary and medicinal wild plants (Sewram et al., 2006) has been documented. Fumonisin B₁ and B₂ are the most prevalent and contribute to 70% of fumonisin abundance (Seo et al., 2001).

2.2.1 Fumonisin general structure and toxicity

In comparison to other mycotoxins, fumonisins have long hydroxylate hydrocarbon chains without cyclic structures. FB₁ is the diester of propan-1,2,3 tricarboxylic acid and 2-amino-12,16-dimethyl 3,5,10,14,15-pentahydroxyeicosane in which C₁₄ and C₁₅ hydroxyl groups are esterified with the terminal carboxyl group or tricarboxylic acid. R-groups refer to different side chains present on different types of fumonisins (Figure. 2.1). FB₂ is the C₁₀ deoxy analogue of FB₁ in which the corresponding stereogenic units of the eicosane backbone have the same configurations (Desjardins and Proctor, 2007).

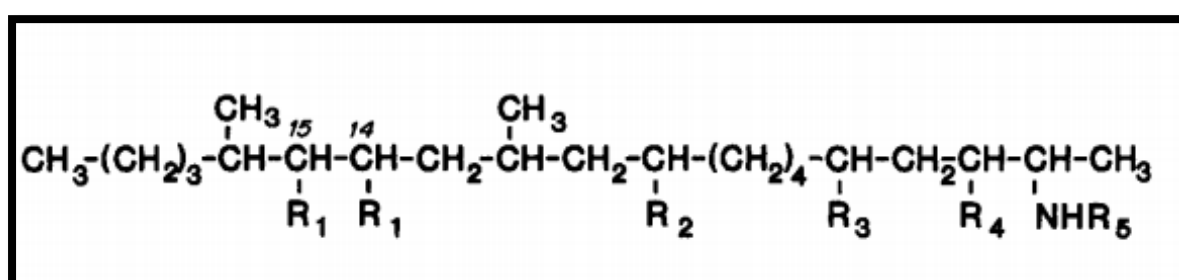


Figure 2.1: General structure of the fumonisin mycotoxin (Gelderblom et al., 1993)

Fumonisin B₁ has a structure very close to that of the free sphingoid base sphinganine and sphingosine (Figure 2.2).

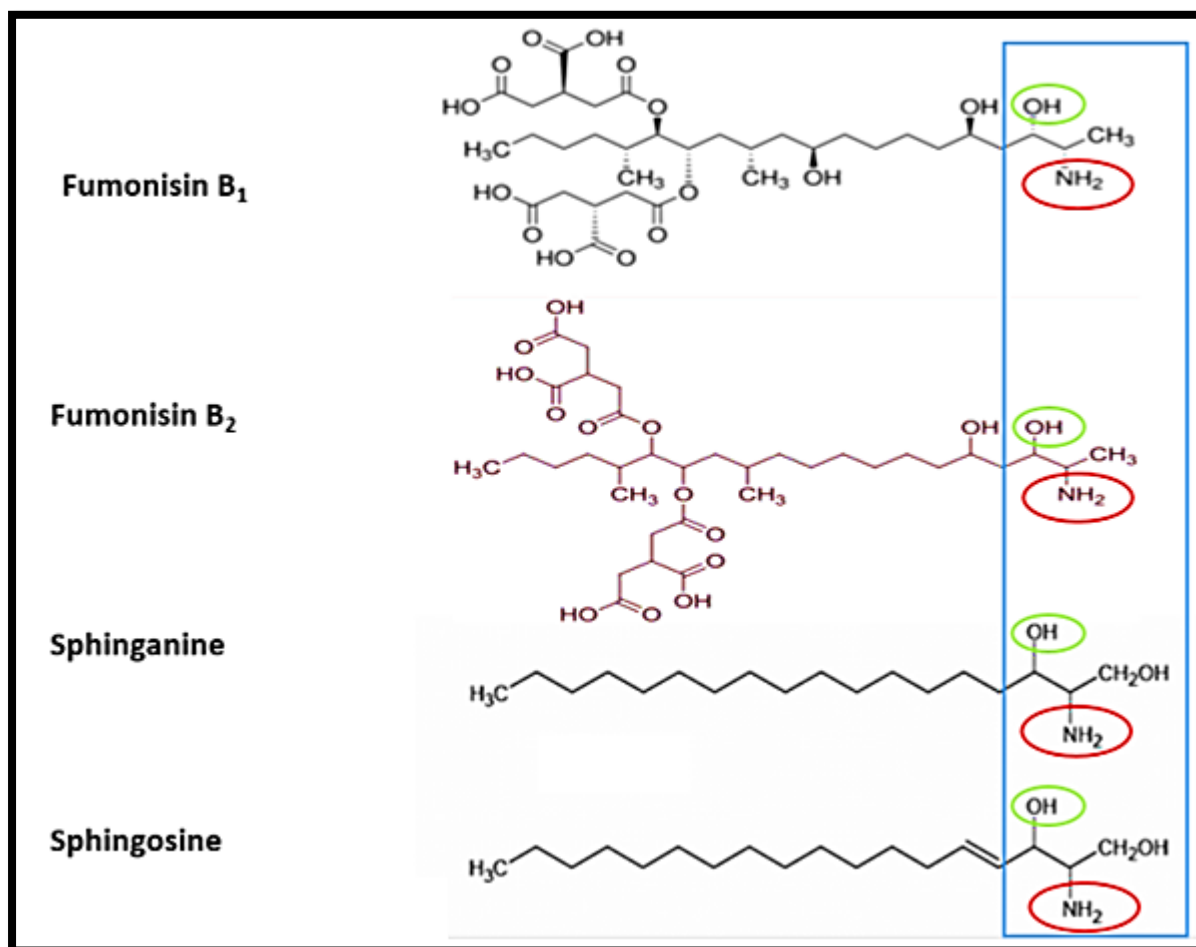


Figure 2.2: Comparison of fumonisin structures, sphinganine and sphingosine (prepared by author)

Therefore, it was concluded that fumonisins exert their toxicity via disrupting sphingolipid metabolism and inhibiting sphingolipid function. Sphingolipids hold essential roles in membrane and lipoprotein structure, cell-cell communications and as secondary messengers (Soriano et al., 2005).

Disruption of sphingolipid metabolism results in a series of events alluding to altered cell growth, in both *in vivo* and *in vitro* models. This causes growth inhibition, and both

apoptotic and oncotic cell death in liver and kidneys. Aside from this mechanism of toxicity the mycotoxin can induce oxidative stress initiating a cascade of adverse effects (Theumer et al., 2010).

The highly studied FB₁ has been implicated as a causative agent in various pathologies affecting farm animals. Furthermore, studies demonstrated the hepatotoxic and carcinogenic effects of the toxin. Research involving humans associate the toxin with oesophageal cancer and neural tube defects more specifically in the Transkei (Marasas, 2001, Missmer et al., 2005). Recent toxicology studies demonstrate the ability of FB₁ to induce various adverse effects in humans however, it was established that hydrolysed FB₁ was less toxic compared to its counterpart (Voss et al., 2009). Additionally, FB₁ has the ability to cross the blood brain barrier inducing neurotoxic effects in young carp (Kovačić et al., 2009). Studies surrounding the toxicity of FB₂ are limited however, it has been implicated in neurotoxicity in horses (Thiel et al., 1991).

2.2.2 Fumonisin detoxification

Cholestyramine, a bile acid sequestrant was shown in both *in vivo* and *in vitro* models to effectively bind both FB₁ and FB₂ (Avantaggiato et al., 2005). Enzymatic detoxification processes use recombinant enzymes (carboxylesterase) from bacteria to hydrolyse fumonisins resulting in the loss of tricarboxylic groups. This is followed by deamination processes in the presence of pyruvate and pyridoxal phosphate (Heinl et al., 2010). Lactic acid has been shown to bind FB₁ and FB₂ via use of peptoglycan binding sites. FB₂ is more bound than FB₁ with a minimum of one tricarboxylic acid moiety involved in binding (Niderkorn et al., 2009).

2.3 Fumonisin B₂

Initially *Fusarium* species were thought to be the only producers of FB₂ however, more recent studies have shown the production of the toxin by *Aspergillus niger* isolated from coffee and grapes. Additionally, the toxin has been detected in wine and beer contamination thus increasing the list of food commodities affected by fumonisin exposure other than maize (Scott, 2012). FB₂ has been classed as a type 2B carcinogen by IARC (IARC, 2002). Previous studies have showed that the toxin has similar cancer initiating properties as FB₁ however, comparative cytotoxic test showed that FB₂ is more toxic than FB₁ in rat hepatocytes (Gelderblom et al., 1993, Shephard et al., 1996). This was confirmed by FB₂ being able to reduce the body weight of rats by a greater percentage than other fumonisin subtypes which was attributed to its ability to disrupt sphingolipid metabolism (Gelderblom et al., 1993, Shephard et al., 1996).

FB₂ is the C₁₀ deoxy analogue of FB₁ in which the corresponding stereogenic units of the eicosane backbone have the same configurations. The amino group present on the hydrocarbon chain has been implicated in the mechanism of toxicity of the compound (Figure 2.3) (Desjardins and Proctor, 2007).

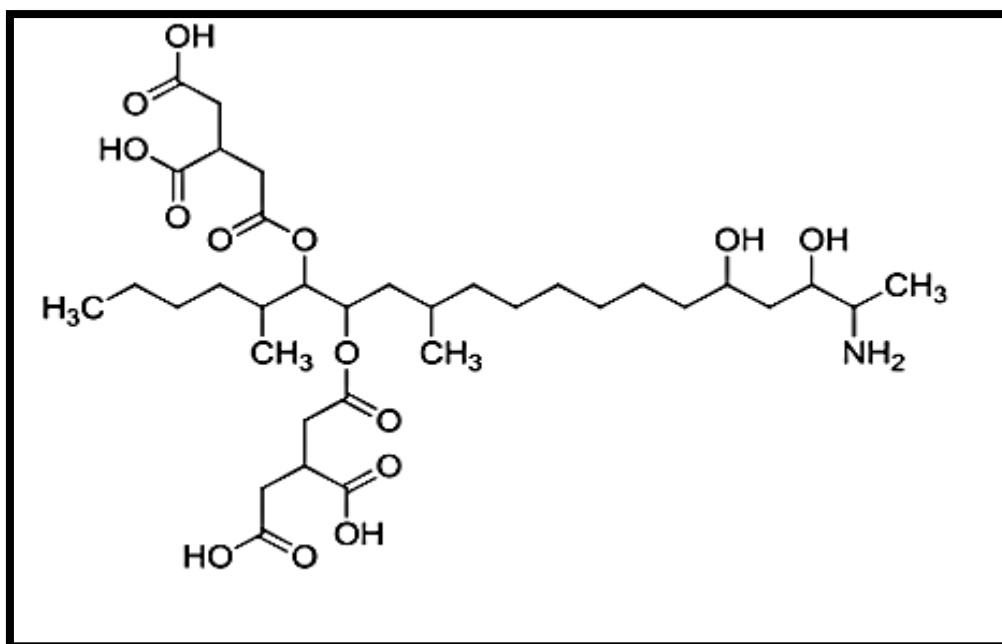


Figure 2.3: Structure of FB₂ (Carrasco-Sánchez et al., 2017)

FB₂ has demonstrated to be excreted unmetabolised through urine, bile and faeces (Shephard et al., 1995). The amino group of FB₂ has been shown to play a major role in the toxicity of the compound as endogenous systems are able to recognise the group and mistaken FB₂ for native structures. This allows FB₂ to bind to enzymes and complexes in cells resulting in competitive inhibition (Gelderblom et al., 1993, Soriano et al., 2005). The structure of FB₂ remains intact during excretion as it is unmetabolised. This enhances its toxicity as the amino group is able to interact with cellular entities. Furthermore, the high polarity of FB₂ increases its water solubility causing it to be excreted at a faster rate than other fumonisin subtypes (Gelderblom et al., 1993). This increases the susceptibility of excretory organs such as the kidney as the compound is not structurally modified and excretion via the kidney permits toxic interactions with cellular structures.

The most common noted side effect of FB₂ exposure is observed in horses largely annotated to its interference with sphingolipid metabolism (Thiel et al., 1991). Aside

from this the toxin has exhibited phytotoxicity when introduced to maize and tomato seedlings through its ability to activate pathogen infection induced hypersensitive responses (Lamprecht et al., 1994, Saucedo-García et al., 2011).

2.3.1 Fumonisin B₂ mechanism of action

FB₂ is responsible for the inhibition of *de novo* sphingolipid biosynthesis (Figure 2.4).

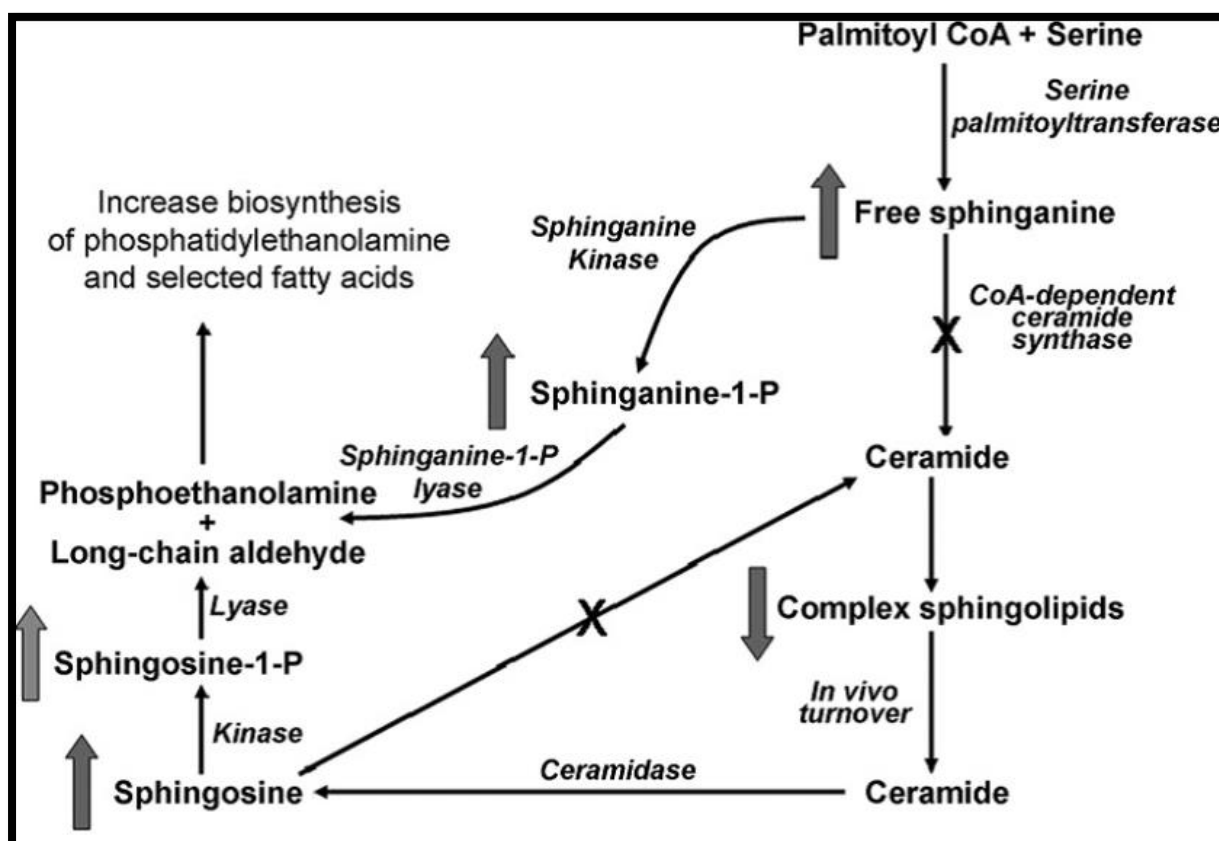


Figure 2.4: Mechanism of action of FB₂ (Voss et al., 2007)

They act as competitive inhibitors for the substrate of ceramide synthase which is essential for the transfer of fatty acids from fatty acyl –COA's to sphinganine and sphingomyelin (Enongene et al., 2000, Soriano et al., 2005). The structural similarity of FB₂ and sphinganine allows ceramide synthase to recognise the toxin as a substrate. More specifically the amino group of FB₂ binds to ceramide synthase

causing inhibition of the enzymes activity (Gelderblom et al., 1993, Enongene et al., 2000).

The inhibition of ceramide synthase prevents the incorporation of serine into the backbone of dihydroceramides and complex sphingolipids. Furthermore, FB₂ inhibits the conversion of sphinganine to sphingosine via acylation which adds a double bond to the substrate. In healthy functioning cells, reacylation of sphingosine occurs, which is released during hydrolysis of complex sphingolipids however, this is prevented when FB₂ is introduced. Ultimately, this initiates the accumulation of sphinganine in cells and promotes formation of sphinganine 1-phosphate and sphingosine 1-phosphate with simultaneous cleavage of the sphingoid backbone to produce fatty aldehydes and ethanolamine 1-phosphate (Merrill Jr et al., 2001).

Increased concentrations of sphingoid bases is known to inhibit the activity of protein kinase C and increase protease activity. Inhibition of protein kinase C is associated with elevated susceptibility to apoptosis (Merrill et al., 1993, Spiegel and Merrill Jr, 1996). Sphingosine accumulations affect insulin receptor tyrosine kinase (Arnold and Newton, 1996), calmodulin-dependent kinase activity (Jefferson and Schulman, 1988) and tyrosine kinases of epidermal growth factor receptors (Davis et al., 1988). Furthermore, sphingosine causes dephosphorylation of retinoblastoma protein thus inducing growth inhibition. Concentration of sphingosine 1-phosphate in cells has been shown to induce the release of calcium, thus causing perturbations in calcium homeostasis (Olivera et al., 1994).

2.4 Mitochondria

The mitochondria are commonly highlighted as energy sources of cells leading to classification of the organelle as an essential but independent functional subunit in

cells (Wallace and Starkov, 2000). Early research described mitochondrion as the location for the production of ATP via breakdown of fatty acids and sugars, oxidative phosphorylation (OXPHOS) and the citric acid cycle (Figure 2.5). Additional functions include lipid and steroid metabolism, DNA replication/transcription and protein translation. Furthermore, mitochondrial function plays a vital role in cell cycle regulation and differentiation. However, studies have focused on dynamic mitochondrial pathways including fission, fusion and motility events which are closely regulated by mitochondrial proteins (McBride et al., 2006).

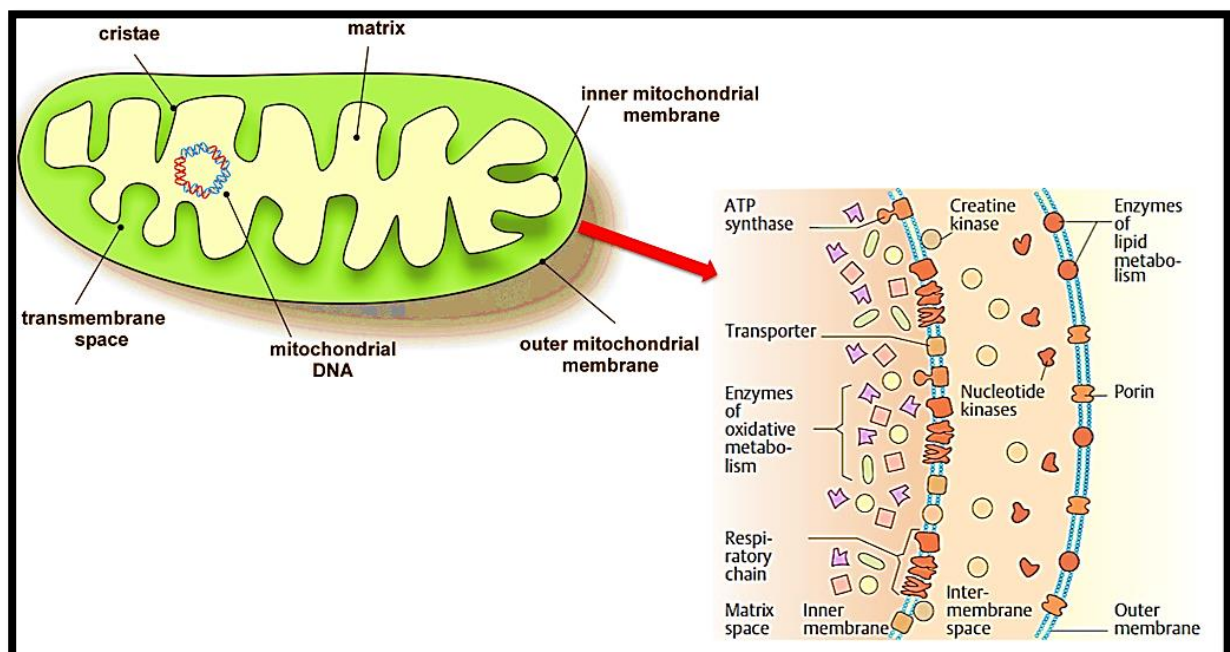


Figure 2.5: Structure and selective functions of mitochondria (prepared by author)

The unusual double membrane of mitochondria separates the organelle into four areas namely the outer membrane, intermembrane space, inner membrane and matrix. The citric acid cycle occurs within the matrix. The inner membrane is strategically folded into cristae to increase the surface area of the organelle (Figure. 2.5). This area

contains complexes that are required for the electron transport chain (ETC) and thus being a controller of the rate of cell metabolism (Figure. 2.5) (McBride et al., 2006).

Aside from the common production of ATP, the mitochondria are responsible for the endogenous production of ROS. This is essential for cell survival as ROS acts as redox messengers thus mediating intracellular signalling pathways. The majority of ROS production within the mitochondria occurs during the ETC and disruptions in the process leads to unwarranted ROS production leading to states of oxidative stress (Kowaltowski and Vercesi, 1999). Additionally, the mitochondria is the site for the intrinsic apoptotic pathway which is programmed cell death induced by caspases (proteins) within the organelle (Mayer and Oberbauer, 2003). Due to mitochondria being the prime location for several crucial processes, any irregularities pose a major threat to overall cell function and health.

2.4.2 Mitochondrial Toxicity

Essential mitochondrial pathways play an important role in maintaining overall cell homeostasis. Several xenobiotics have been shown to selectively target the mitochondria causing dysregulation in processes and inducing mitochondrial toxicity as a mechanism of action (Liu and Wang, 2016, Tsai et al., 2016). Due to the mitochondria being a target for toxicity, mitochondrial dysfunction has been implicated in several pathologies including Alzheimer's disease, Parkinson's disease, cardiomyopathy, cancer and diabetes (King et al., 2006, Duncan, 2011, Exner et al., 2012, Wang et al., 2014).

The most prominent consequence of mitochondrial dysfunction is reduced energy production. However, notably increases in mitochondrial catalysed side reactions occur during dysfunction. These include free radical production and exothermic

combustion of oxygen, which result in cellular damage (Wallace and Starkov, 2000). Primary toxicity may arise when inhibition of the respiratory chain ATP synthase and uncoupling of OXPHOS occurs. Secondary mitochondrial toxicity refers to interference with mitochondrial biogenesis, protein synthesis, gene expression and inhibition of membrane transporters (Zoll et al., 2003, Picard et al., 2011a).

The most common mechanism of mitochondrial toxicity is interference with the ETC resulting in exacerbated ROS production. The imbalance of ROS and antioxidants within cells leads to oxidative stress. ROS has the ability to interact with macromolecules such as proteins and lipids causing conformational changes, which further induce stress (Kowaltowski and Vercesi, 1999). The inability to ameliorate oxidative stress induces cell death. In other cases, manipulation in mitochondrial apoptotic pathways induces uncontrolled cell death which has been commonly observed in several cancers (Reed, 1999).

2.4.3 Mycotoxins and Mitochondrial toxicity

A multitude of studies have been carried out testing the effects of mycotoxins on cellular function with specificity to mitochondrial function. Studies have revealed the ability of certain mycotoxins to induce potent toxicity via inhibition of state 3 of mitochondrial respiration as well as inducing uncoupling effects (Kawai et al., 1983). Furthermore, a study using a *Fusarium* mycotoxin (Fusaric acid) illustrated abilities to induce lipid peroxidation and upregulation in mitochondrial stress mechanisms. In the same study aberrant mitochondrial biogenesis and upregulations in apoptosis was noted (Abdul et al., 2016). Other mycotoxins such as trichothecenes induce mitophagy as an adaptive response to toxicity thus reducing mycotoxin induced oxidative stress with inhibition in protein synthesis noted (Umer and Anwar, 2014). An unusual

mechanism of mitotoxicity is that of FB₁ which deregulates calcium homeostasis and inhibits mitochondrial respiration via inhibition of complex I of the respiratory chain. This leads to increased mitochondrial membrane depolarisation, increased ROS production and interruption in calcium signalling hampering overall cell functionality (Domijan and Abramov, 2011).

2.5 Mitochondrial Stress Responses

In order to maintain mitochondrial integrity, the mitochondria has several mechanisms to combat deviations that may arise due to toxicity or endogenous irregularities. This is imperative to the overall functioning of cells and aids in reducing stress within the mitochondria (Wallace and Starkov, 2000).

The mitochondria have been shown to release peptides to mediate stress responses. A key example is the mitochondrial unfolded protein response (UPR^{mt}) which is initiated by a collection of unfolded proteins in the mitochondria (Benedetti et al., 2006, Hill et al., 2018). During this response misfolded proteins induce an increase in ATP production to aid in the cleavage of misfolded proteins as certain mediators are ATP-dependent. The organelle has dedicated a multitude of molecular chaperones and enzymes to ensure correct folding of proteins with a set of quality control enzymes to degrade misfolded proteins (Pellegrino et al., 2013). The tightly regulated process is disturbed by the ETC generation of ROS and mitochondrial DNA vulnerabilities to damage. The UPR^{mt} is a mitochondrial to nuclear signalling pathway that allows for the transcription of mitochondrial stress genes and chaperones to maintain homeostasis (Benedetti et al., 2006).

Furthermore, the mitochondria is able to mediate redox signalling through the endogenous production of ROS. ROS are required for the adequate functioning of

cells such as the production of ATP in the mitochondria and for oxygen-dependent killing however, excess amounts of ROS (oxidative stress) may result in damage to DNA, proteins, lipids and disruption of cellular processes. Due to the mitochondria producing most of the cellular ROS, oxidative insult within the organelle occurs at a higher rate. Therefore, the mitochondria have mechanisms to reduce oxidative stress within the cell via the antioxidant defence system (Wallace and Starkov, 2000).

Programmed cell death within the mitochondria is initiated by mitochondrial caspases and occurs in order to prevent the spread of stress and dysfunction to other cells. The process results in the release of pro-apoptotic proteins such as cytochrome c which aid in cell death. The most common trigger of the intrinsic apoptotic pathway is a loss in mitochondrial membrane potential that generally occurs during mitochondrial dysfunction. This is a tightly regulated process and is vital to ensure the health of the organism (Mayer and Oberbauer, 2003).

2.5.1 Proteins involved in mitochondrial stress responses

2.5.1.1 Sirtuins

Sirtuins (SIRT) are nicotinamide adenine dinucleotide (NAD⁺) dependent proteins that belong to a family of deacetylase enzymes with SIRT1-7 being present in humans. Primarily, they function to remove an acetyl group from the amino group of a lysine residue thus activating or inhibiting the protein (David Adams and Klaidman, 2007). More specifically, sirtuins deacetylate proteins and transcription factors involved in the regulation of stress, metabolism, survival and aging. Human SIRT (SIRT1-7) share a conserved Sir2 catalytic core however, they have variable amino and carboxy terminal extensions, which provide uniqueness in location and catalytic function (Huang et al., 2010).

2.5.1.2 Sirtuin 3

Sirtuin 3 (SIRT3) is located in the mitochondrial matrix and is responsible for the activation or inhibition of several proteins. SIRT3 is able to induce post translational modifications to proteins thus regulating their activity. The post translation modifications may increase the activity of the protein or cause suppression of activity. In doing so, SIRT3 has been described as a modulator of several pathways through its deacetylating effects on essential enzymes and proteins (Figure 2.6) (Bause and Haigis, 2013).

SIRT3 has been shown to target enzymes in the citric acid cycle namely isocitrate dehydrogenase 2 and aconitase and therefore indirectly regulates respiration. Furthermore, activation of SIRT3 has proven to increase OXPHOS with coinciding increases in deacetylation of enzymes in complex I and II of the ETC further verifying its regulation of respiration (Ahn et al., 2008, Cimen et al., 2009, Bao et al., 2010).

Aside from metabolic regulation, SIRT3 is important for the regulation of mitochondrial ROS production. At substrate level manganese superoxide dismutase (mnSOD), which is responsible for ROS scavenging is deacetylated and activated by SIRT3, thus reducing oxidative stress. Similarly SIRT3, deacetylates forkhead box O3 (FOXO3a) (involved in antioxidant responses), aiding in its translocation to the nucleus (Kim et al., 2010). SIRT3 has a high affinity for FOXO transcription factors during oxidative stress in an attempt to upregulate antioxidant systems. Indirect regulation includes the activation of isocitrate dehydrogenase, which increases nicotinamide adenine dinucleotide (NADH) concentration, thus facilitating the production of reduced glutathione (antioxidant) (Someya et al., 2010).

Studies have shown that SIRT3 protein and gene expression is significantly elevated in states of mitochondrial stress. Inhibition of SIRT3 has prevented amelioration of mitochondrial stress and led to cell death. The expression of the enzyme is dependent on the degree of stress present where increases in stress coincides with upregulation in SIRT3 expression (Weir et al., 2013). In similar studies it was observed that SIRT3 deacetylates and inhibits mitochondrial Ribosomal protein L10 which causes suppression of mitochondrial protein synthesis as a possible energy conservation method during stress (Yang et al., 2010).

Notably, SIRT3 aids in detoxification of ammonia during amino acid metabolism via activation of ornithine transcarbamoylase (Hallows et al., 2011). Additionally, the enzyme functions to reduce the mitochondrial permeability transition by inhibiting the activity of mitochondrial matrix isomerase cyclophilin D. Consequently cyclophilin D is involved in calcium mitochondrial efflux thus giving SIRT3 an indirect role in calcium homeostasis regulation.

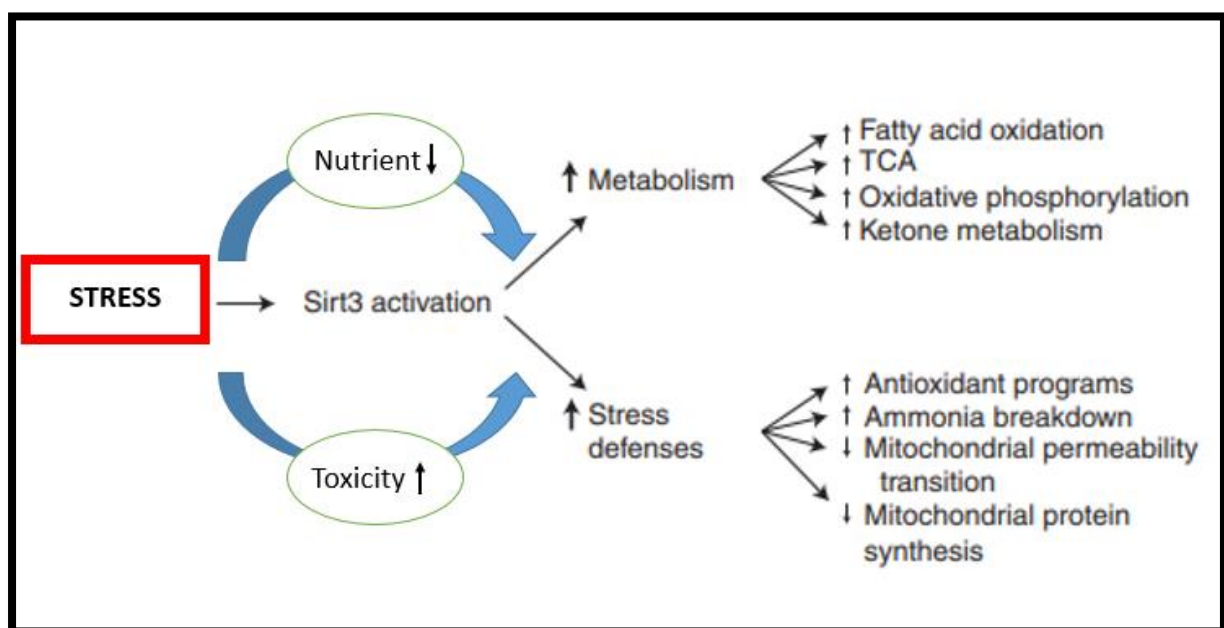


Figure 2.6: Summary of SIRT3 activity during stress conditions (prepared by author)

2.5.1.3 LON Protease

Lon protease (LONP1) is a serine protease involved in the degradation of oxidatively damaged proteins. LONP1 contains a highly conserved ATPase domain with an AAA⁺ module and a proteolytic domain with a N-terminal domain (Ngo and Davies, 2007). The AAA⁺ module partakes in target selection with nucleotide binding and ATP hydrolysis due to a Walker motif (Neuwald et al., 1999). The confirmation of the LON complex is organ specific with 4-8 LON polypeptides binding together to form a homo-oligometric complex.

The site of action occurs at the N-terminus where the hydrophobic amino acid of protein substrates bind. Upon binding, ATP hydrolysis occurs allowing the protein to be passed through the Lon complex and proteolytic chamber. This is followed by protein cleavage (Figure 2.7) (Ondrovičová et al., 2005).

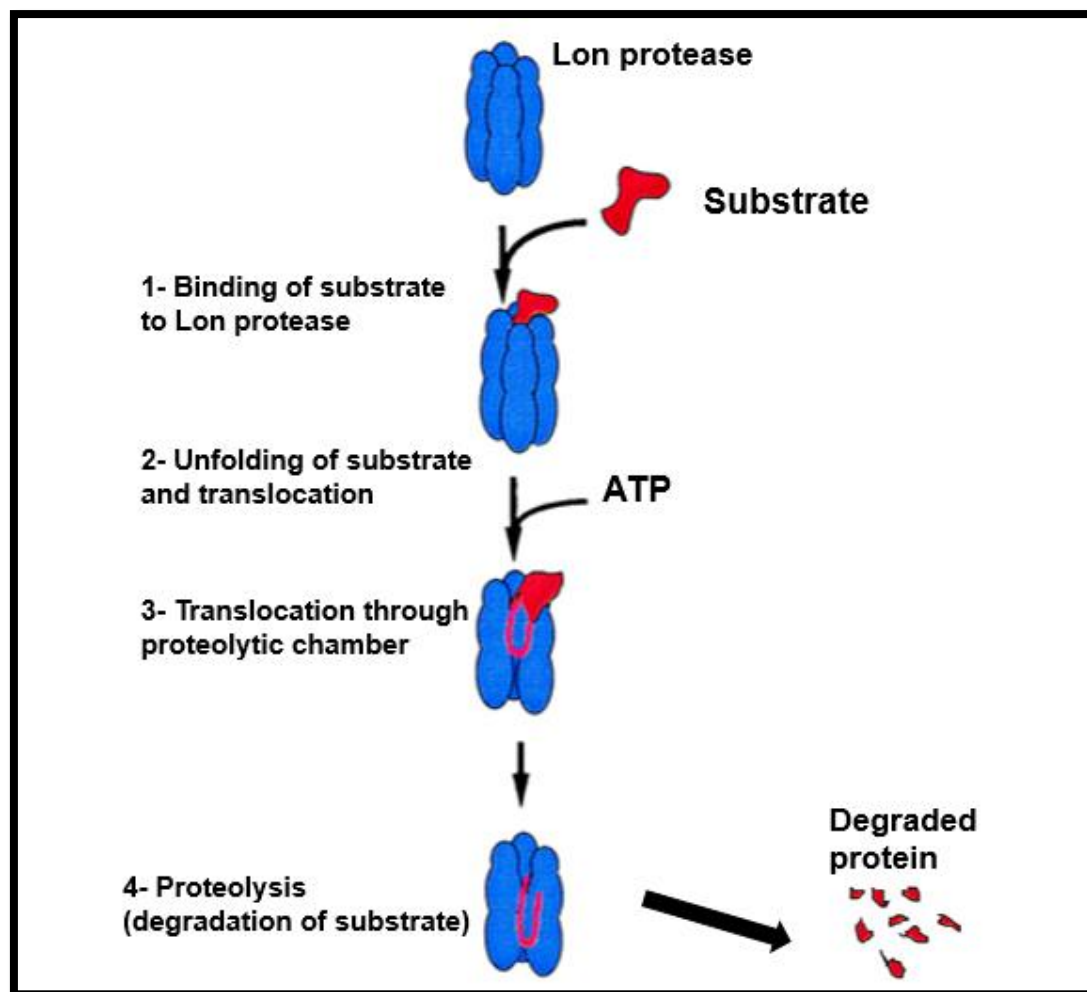


Figure 2.7: LONP1 proteolysis mechanism for damaged proteins (prepared by author)

LONP1 has a high affinity for oxidatively damaged proteins, misfolded proteins and protein substrates exposed to stress conditions which all signal upregulation in LONP1 expression (Ngo and Davies, 2007). A study carried out suggests that LONP1 has the ability to bind to mitochondrial DNA. Such binding is inhibited when LONP1 is sequestered by ATP. Hydrolysis of ATP during stress conditions allows for LONP1 now bound to adenosine diphosphate (ADP) to rebind nucleic acids after regulation during stress. The function of the process remains elusive however, it has been suggested that this keeps LONP1 away from the mitochondrial matrix to prevent

degradation of functional proteins while keeping the complex in close proximity in case of stress induction (Liu et al., 2004).

LONP1 is post translationally regulated by SIRT3. SIRT3 deacetylates LONP1 causing decreases in protein expression. Silencing of SIRT3 causes increases in LONP1 protein levels however, it has no effect on LONP1 mRNA thus allowing for LONP1 activation or suppression in spite of SIRT3 presence (Bota et al., 2005, Gibellini et al., 2014).

2.5.1.4 Heat shock proteins

During stress conditions genes and proteins involved in survival, detoxification, inflammation and death are activated or suppressed accordingly. A special group of stress genes allow for the transcription of proteins namely heat shock proteins (HSPs). HSPs are molecular chaperones involved in correcting the folding of misfolded proteins that have occurred due to stress related incidences. Furthermore, HSPs prevent the aggregation of misfolded proteins. Due to their distinct activity they are used as bio-markers for cellular stress and hazards. HSPs are classified based on their molecular weight, amino acid sequences and function. The proteins are separated into five main families including HSP- 100, -90, -70, -60 and -40 (Table 2.1) (Gupta et al., 2010).

Table 2.1: Summary of location and function of various HSPs (prepared by author)

HSPs family	Cellular Location	Function
HSP40	Cytoplasm and nucleus	Regulates protein folding
HSP60	Mitochondria	Protein assembly, refolding of misfolded proteins and protein stability
HSP70	Cytoplasm, ribosome and nucleus	Assembly and transport of new proteins and removal of denatured proteins
HSP90	Cytoplasm and nucleus	Binds proteins and receptors in specific manner
HSP100	Cytoplasm and nucleus	Prevents aggregation and assist in refolding

Heat shock protein 60 (HSP60) is mainly involved in polypeptide folding and translocation of proteins. Proteins of this family have been identified in the mitochondria of both bacteria and eukaryotes (Cappello et al., 2008).

HSP60 is predominantly located in the human mitochondrial matrix where it aids in the folding of small monomeric soluble proteins and the refolding of misfolded proteins (Pellegrino et al., 2013). Furthermore, the chaperone transports certain proteins to the mitochondria when needed. Studies have shown a direct correlation in the upregulation of HSP60 expression in response to mitochondrial dysfunction, thus

making it a suitable marker for mitochondrial stress (Pellegrino et al., 2013). Additionally, HSP60 is known to play a role in ensuring protein stability through its ability to ensure correct folding of protein structures (Richter-Landberg and Goldbaum, 2003).

2.6 Mitophagy

In the mitochondria, respiration results in the production of ROS. Alterations in mitochondrial respiration chain may cause exacerbated production of mitochondrial derived ROS, thus inducing cytotoxicity. Endogenous production of ROS causes the mitochondria to be susceptible to oxidative damage. This may induce ATP depletion from uncoupled OXPHOS and releases of cytochrome promoting caspase activation and apoptosis (Kim et al., 2007). Furthermore, mitochondria DNA lack histones, thus increasing their vulnerability to damage as opposed to nuclear DNA. Additionally, disease and exposure to toxins may exacerbate ROS production inducing stress and causing occurrence of dysfunctional mitochondria.

Dysfunctional and damaged mitochondria trigger the activation of several proteins to ensure that compromised mitochondria are removed from the cell. This is carried out by autophagic sequestration followed by lysosomal hydrolytic degraded in a collective process named mitophagy (Figure 2.8). Mitophagy is promoted during states of mitochondrial stress and mitochondrial toxicity (Kim et al., 2007, Youle and Narendra, 2011).

Mitophagy was first observed in mammalian cells with increased mitochondrial sequestration by lysosomes following treatment with glucagon. Before mitophagy occurs in mammal cells, mitochondrial fission occurs to split elongated mitochondria in smaller fragments for easy encapsulation and as a quality control technique to

identify damaged mitochondria to be removed by mitophagy (Twig et al., 2008, Westermann, 2010). This may occur in diseased states or to control the required number of mitochondria needed in normal metabolic processes (Tal et al., 2007).

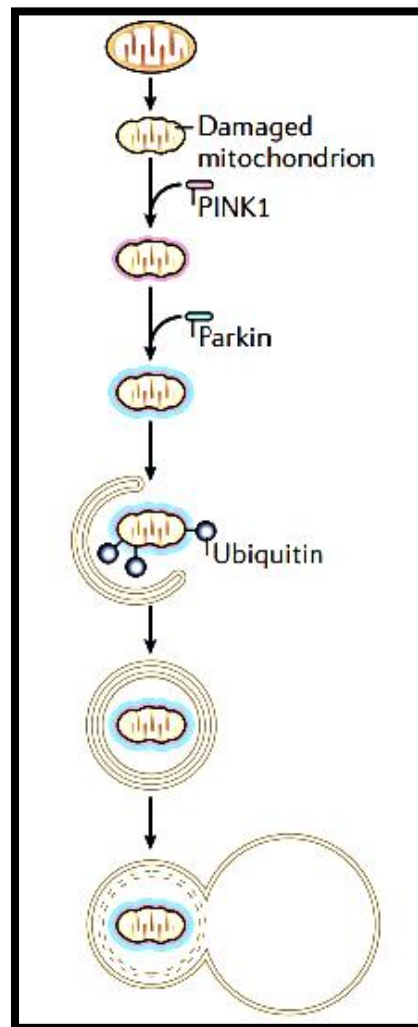


Figure 2.8: Simplified diagram of the mitophagic process (Youle and Narendra, 2011)

Once mitochondria are damaged or begin to lose membrane potential the kinase PTEN induced kinase 1 (PINK1) begins to accumulate around the mitochondria. This initiates the recruitment of the E3 ubiquitin ligase parkin from the cytosol to the injured mitochondria. The ligase causes engulfment of the mitochondria via isolation membranes that are later fused with lysosomes (Youle and Narendra, 2011). PINK1 is expressed in all healthy mitochondria and constantly degraded by proteolysis whilst

maintaining a low level of the protein. When mitochondria are damaged this proteolysis is inhibited to allow for accumulation of PINK1 and progression of mitophagy. Following ubiquitylation via parkin, p62 gathers in the mitochondria. This protein has the ability to aggregate ubiquitylated structures via polymerisation with other p62 molecules whilst recruiting these structures to phagosomes via binding to the protein LC3. In the same way p62 binds to parkin mitochondrial substrates and allows clumping of ubiquitylated mitochondria (Geisler et al., 2010, Narendra et al., 2010a, Narendra et al., 2010b).

2.6.1 Regulation of mitophagy

2.6.1.1 Nuclear factor (erythroid-derived 2)-like 2

Nuclear factor (erythroid-derived 2)-like 2 (Nrf2) is a transcription factor that initiates first line defences to maintain homeostasis when deviations occur in cells. These include irregularities such as oxidative stress, uncontrolled inflammation and xenobiotic exposure (Robledinos-Antón et al., 2019). Several toxicity studies have highlighted Nrf2 as a key marker during stress conditions and the expression of the transcription factor has been a target for manipulation by several toxins (Osburn and Kensler, 2008, Pillay et al., 2015, Loboda et al., 2017).

Nrf2 remains in the cytoplasm when bound to Kelch like-ECH-associated protein (KEAP1), which marks it for ubiquitination and thereafter, proteasomal degradation. During stress conditions, Nrf2 is removed from KEAP1 via the disruption of cysteine residues in KEAP1. The KEAP1 association is disrupted and Nrf2 is no longer degraded but instead translocates to the nucleus. Once in the nucleus, Nrf2 is able to transcribe for various stress relieving genes, thus aiding in the amelioration of stress. The most common phenomenon that induces Nrf2 expression is the increased

production of ROS. Various studies have illustrated the role of Nrf2 in the reduction of oxidative stress via the transcription of antioxidant genes (Scannevin et al., 2012, Robledinos-Antón et al., 2019).

Aside from its common antioxidant transcription function, Nrf2 has been shown to transcriptionally activate the upregulation of *PINK1* and p62 during oxidative stress conditions (Jain et al., 2010, Murata et al., 2015). Therefore, Nrf2 has been described as a promoter of mitophagy. Nrf2 transcriptionally regulates *PINK1* gene expression via activation of the antioxidant response element (ARE) sequence in the *PINK1* promoter region. The Nrf2-PINK1 axis has been shown to promote cell survival (Murata et al., 2015). In similar studies it was found that p62 has an ARE in its promoter region that allows for activation by Nrf2 during oxidative stress. This allows for increase in p62 protein expression and progression of mitophagy (Jain et al., 2010).

2.6.2 Micro-RNAs

MiRNA are small non-coding ribonucleotide acids (RNA) comprising of ~ 22 nucleotides. Research has described the function of these molecules as gene expression modulators via protein translation inhibition (Figure 2.9). This has led to theories suggesting the miRNA can be used as biomarkers in disease as well as targets in therapeutic interventions.

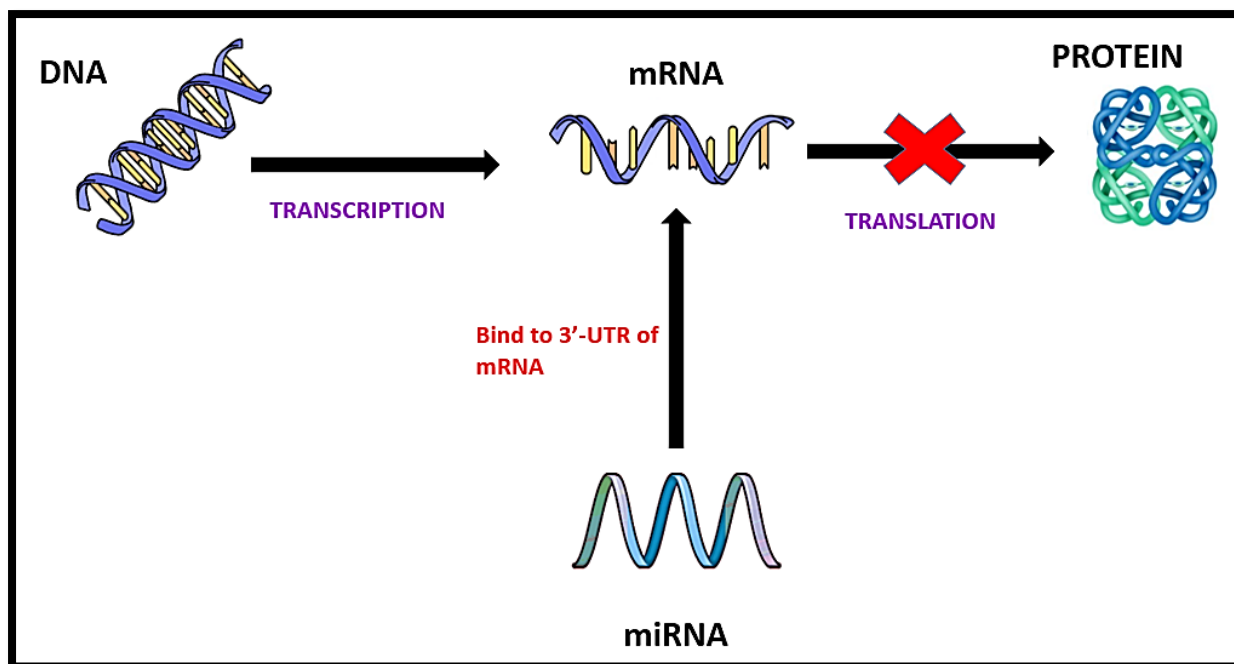


Figure 2.9: Function of miRNA (prepared by author)

MicroRNAs regulate protein expression via binding to the 3'-UTR of mRNA. The interaction between the mRNA and miRNA inhibits protein translation. The function of miRNA allows for regulation of various biological processes including cell proliferation, cell death and metabolism (Maltby et al., 2016, Hammond et al., 2001).

2.6.2.2 *MicroRNA-27b*

Previously miR-27b was known to negatively regulate adipocyte differentiation via repression of peroxisome proliferator-activated receptor gamma (PPAR γ) (Lin et al., 2009). However, more recent studies show a new role of the miRNA in the negative regulation of mitophagy. *MiR-27b* repressed the expression of PINK1 via binding to the 3'-UTR of its mRNA thus preventing translation. This repression prevents the progression of mitophagy and allows for accumulation of damaged mitochondria. The inhibition in PINK1 was coupled with decreases in parkin translocation and

ubiquitylation. Furthermore, *miR-27b* prevents lysosomal degradation of damaged mitochondria (Kim et al., 2016).

2.7 Kidney structure and function

The kidney is an excretory organ consisting of two bean shaped entities. They are located in the posterior section of the abdomen adjacent to both sides of the vertebral column (Radi, 2019). Primarily the kidney functions to regulate blood composition in humans. They control the concentration of water, buffers, molecules and electrolytes in the blood. Additionally, the kidney has the ability to remove organic molecules, metabolic waste, toxins and unnecessary metabolic by-products from the blood (Habuka et al., 2014).

2.7.1 Basic kidney structure

Each kidney comprises of a cortex with renal corpuscles, a medulla with medulla pyramids, renal papilla and renal pelvis. The medulla can be separated into an inner and outer region. The outer section of the medulla houses parts of the proximal tubule, distal tubes and collecting ducts. The proximal tubule is further divided into 3 segments; S1, S2 and S3, all having different nutritional and energy requirements. S1 segments have a high mitochondrial concentration with increased oxidative rates. S2 have fewer mitochondria with more lysosomes. S3 segments houses cytochrome P450 enzymes which are essential for the metabolism of drugs and toxins (Radi, 2019).

The inner medulla may be identified by ascending thick limbs, descending thin limbs and collecting ducts. The cortex and the medulla have urinary producing entities namely nephrons. Nephrons are broken down into glomeruli, renal tubules, interstitium

and juxtaglomerular apparatus. The functional units of the kidney include the nephrons, renal corpuscle, proximal tubule, loop of Henle and distal tube (Radi, 2019).

2.7.2 Kidney function

Primarily the kidney has 6 main functions namely regulation of water and electrolyte in the body, endocrine functions (release of hormones), controls volumes of extracellular fluids, maintaining pH and pressure in blood, excretion of metabolic waste and metabolic function and biotransformation (Radi, 2019).

2.7.3 Use of Hek293 cell line

The Hek293 cell line has been used in numerous toxicology studies including those involving mycotoxins (Pillay et al., 2015, Zhang et al., 2015). The biochemical systems of the cell allow for efficient post-translational folding and processing required for protein and nucleic acid synthesis. Furthermore, the cell line is favoured due to quick reproduction and maintenance. This allows for reputable analysis to profile the action of xenobiotics and their cellular targets (Thomas and Smart, 2005).

Hek293 cells have an abundance of mitochondria and several studies have used the model to test mitochondrial function and dynamics (Mao et al., 2011, Seeland et al., 2015). Furthermore, Hek293 cells have been used in studies determining the effects of mycotoxins on mitochondrial function. This makes the model suitable to test the effects of FB₂ on the mitochondria of kidney cells (Liu et al., 2007, Pillay et al., 2015).

2.7.4 Nephrotoxicity

Blood flows at high volumes to the kidney whilst high oxygen consumption occurs at the site. This enhances organ's susceptibility to exposure and injury caused by xenobiotics and chemicals through circulation (Gwaltney-Brant, 2018).

One of the primary routes of excretion of toxins is the kidney which consists of a vast array of biotransformation enzymes. This increases vulnerabilities to injury as the toxins present in the glomerular filtrate are concentrated, while the cortex receives 80% of blood and as such exposure to higher concentrations of toxins occurs and toxins concentrate in the proximal tubule following reabsorption and secretion (Gwaltney-Brant, 2018).

The resulting exposure and failure in detoxification may result in acute kidney toxicity (AKI) or chronic kidney disease (CKD). Acute renal failure is a rapid decrease in kidney function, which allows for accumulation of waste products. Chronic renal failure is the gradual decrease in kidney function and occurs during long term exposure to toxins. Compromised kidney function induces disturbances in homeostasis and limits excretory capacity (Gwaltney-Brant, 2018)

2.7.5 Kidney and mitochondrial toxicity

The kidney functions at a high rate requiring vast amounts of energy for efficiency. The primary form of energy production occurs via aerobic metabolism for ATP production via OXPHOS. Within the mitochondria of kidney cells, oxygen is reduced during the ETC under normal conditions. Furthermore, the ETC has various sites for ROS production. Dysfunction occurring in the ETC results in the inefficient control of ROS production and ultimately oxidative stress. Consequent decreases in mitochondrial membrane potential is noted and decreases in ATP production (Small et al., 2012). In several case of CKD and AKI impaired mitochondrial function was noted. Furthermore, several xenobiotics target mitochondria of the kidney inducing stress and bringing about injury (Granata et al., 2009).

Chapter 3: Materials and Methods

Hek293 cells were used as a model to study the toxic effects of FB₂ with specificity to mitochondrial toxicity (Figure. 3). The effect of FB at varying concentrations on cell viability over a period of 24 hr was established. The IC₅₀ which is the concentration at which 50% growth inhibition had occurred was selected for treatment in subsequent assay. Furthermore, 100 μ M (128.45% viability) and 500 μ M (67.05% viability) were used in subsequent assay to achieve a broad spectrum toxic profile. The concentrations were selected within close proximity to levels of FB₂ that humans are normally exposed to via consumption of contaminated food. These three concentrations were used in all experiments carried out to achieve the objectives of the study.

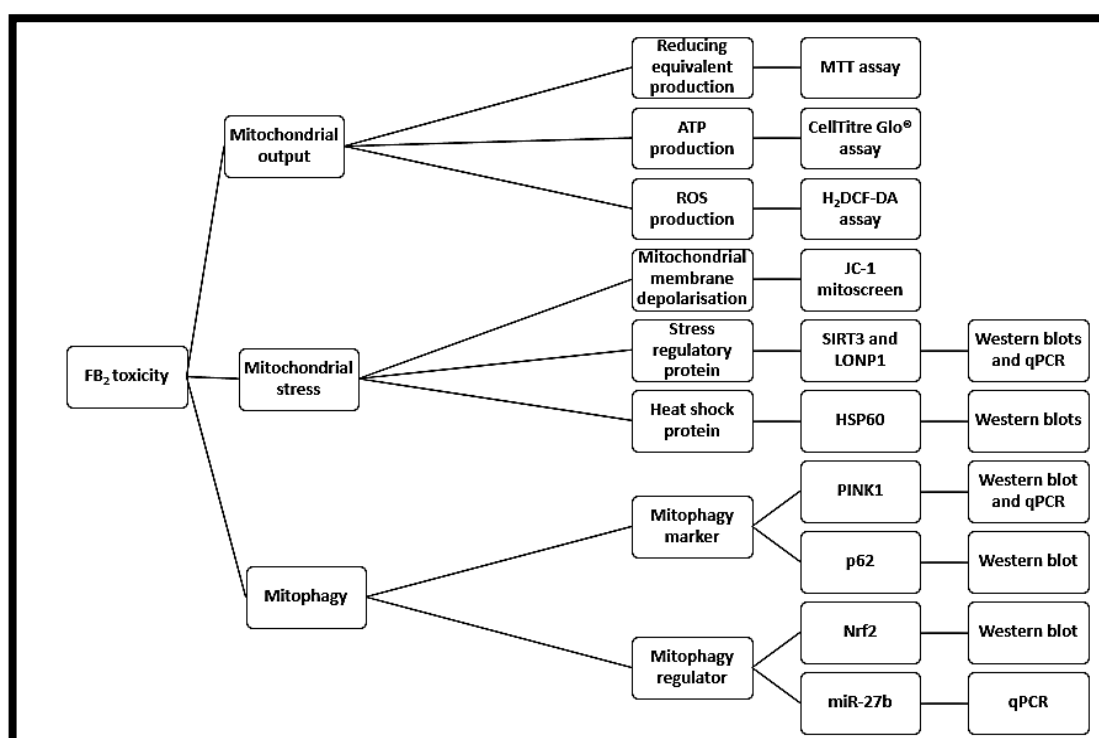


Figure 3: Experimental approach used to determine the mitochondrial toxicity of FB₂ on Hek293 cells (Prepared by author).

3.1 Materials

Hek293 cells were obtained from Highveld Biologicals (Johannesburg, South Africa). Cell culture media and supplements were sourced from Lonza (Basel, Switzerland). Luminometry kits were purchased from Promega (Madison, WI). Western blot reagents were purchased from Bio-Rad (Hercules, CA). All other reagents were purchased from Merck (Darmstadt, Germany) unless otherwise stated.

3.2 Methods

3.2.1 Cell culture and exposure protocol

Hek293 cells were cultured in 25cm³ flasks using Dulbecco's minimum essential medium (DMEM) supplemented with 2.5% HEPES, 10% foetal bovine serum, 1% pen-streptomycin, 1% L-glutamine and maintained in a humidified incubator (5% CO₂) at 37 °C.

FB₂ was purchased from Sigma and a stock solution of 20 mM was made up using 0.1M phosphate buffered saline (PBS). This was diluted using DMEM to produce serial concentrations for the MTT assay (0-500 µM) and thereafter for further experiments.

3.2.2 MTT assay

3.2.2.1 Introduction

The MTT assay is a colorimetric test used to measure cell viability. It is most commonly used to determine cytotoxicity of compounds of varying concentrations. The basic principle of the assay is based on mitochondrial activity where it is postulated that viable cells have constant mitochondrial activity. Therefore, an increase or decrease in cell viability is directly proportional to mitochondrial activity. During the assay

procedure, mitochondrial dehydrogenases convert the MTT salt into formazan yielding a colour change from yellow to purple (Figure 3.1).

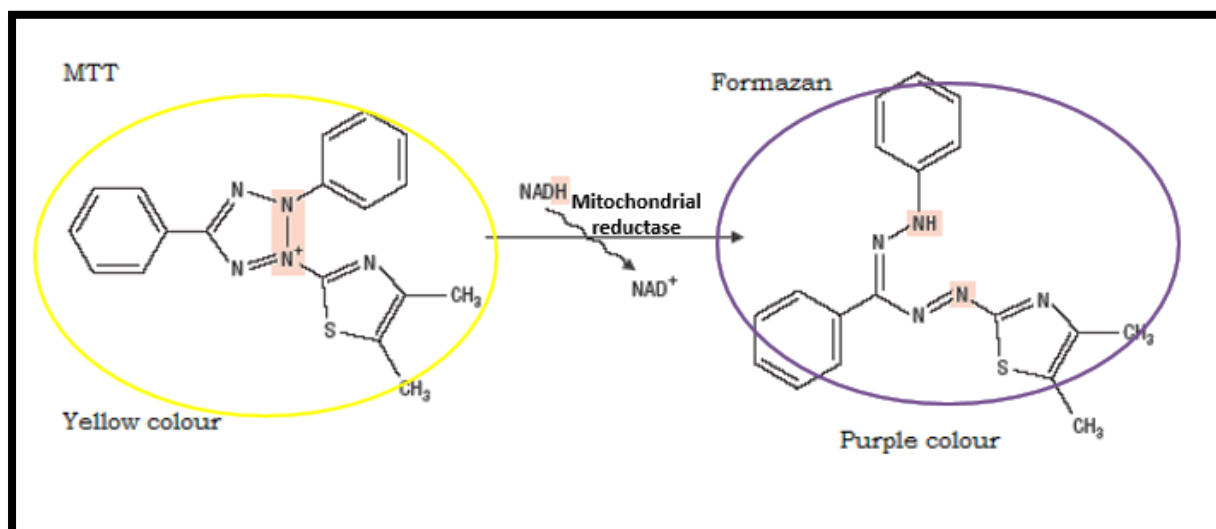


Figure 3.1: Conversion of MTT salt to formazan via mitochondrial reductase in viable cells (prepared by author).

The concentration of solubilised formazan is measured using a spectrophotometer at 570 and 690 nm. In cytotoxicity testing, decreased viability indicates growth inhibition and the toxin sensitivity is expressed at a concentration that induces 50% growth inhibition (IC_{50}) (Van Meerloo et al., 2011).

3.2.2.2 Protocol

The cytotoxicity of FB₂ in Hek293 cells was determined using the MTT assay. Briefly, 20,000 cells/well (triplicate) were seeded and allowed to adhere overnight in a 96-well microtitre plate (37 °C; 5% CO₂). Thereafter, cells were treated for 24 hr with varying concentrations of FB₂ (0-500 μ M). Control wells contained media only. Following the incubation (24hr), treatments were removed, cells were washed using 0.1M PBS and incubated with MTT salt (20 μ l; 5mg/ml in 0.1M PBS) and complete culture medium (CCM) (100 μ l) for 4hr. The MTT salt solution was then removed and 100 μ l of dimethyl

sulphoxide (DMSO) was aliquoted per well, and incubated for 1 hr. Optical density was read using a spectrophotometer (BioTek uQuant) at a wavelength of 570 nm and a reference wavelength of 690 nm. Results were expressed as log concentration versus percentage cell viability.

Following the MTT assay, an IC_{50} (317.4 μ M of FB₂) was determined and was used in all subsequent experiments. In addition, 100 μ M (low dose) and 500 μ M (high dose) of FB₂ were used in experiments. The 100 μ M (128.45% viability) concentration of FB₂ demonstrated cell proliferation, whereas the 500 μ M (67.05% viability) showed the highest inhibition of cell viability.

3.2.3 ATP assay

3.2.3.1 Introduction

ATP is an essential energy source for all cells. The mitochondria produce the highest percentage of ATP via the ETC coupled to OXPHOS and substrate level phosphorylation with cytoplasmic glycolysis accounting for a smaller percentage. To quantify ATP concentration, the CellTiter-Glo® (Promega) kit was used. The assay uses luminometry to measure bioluminescence. Bioluminescence occurs when a chemiluminescent reaction (Figure 3.2) takes place (Deshpande, 2001).

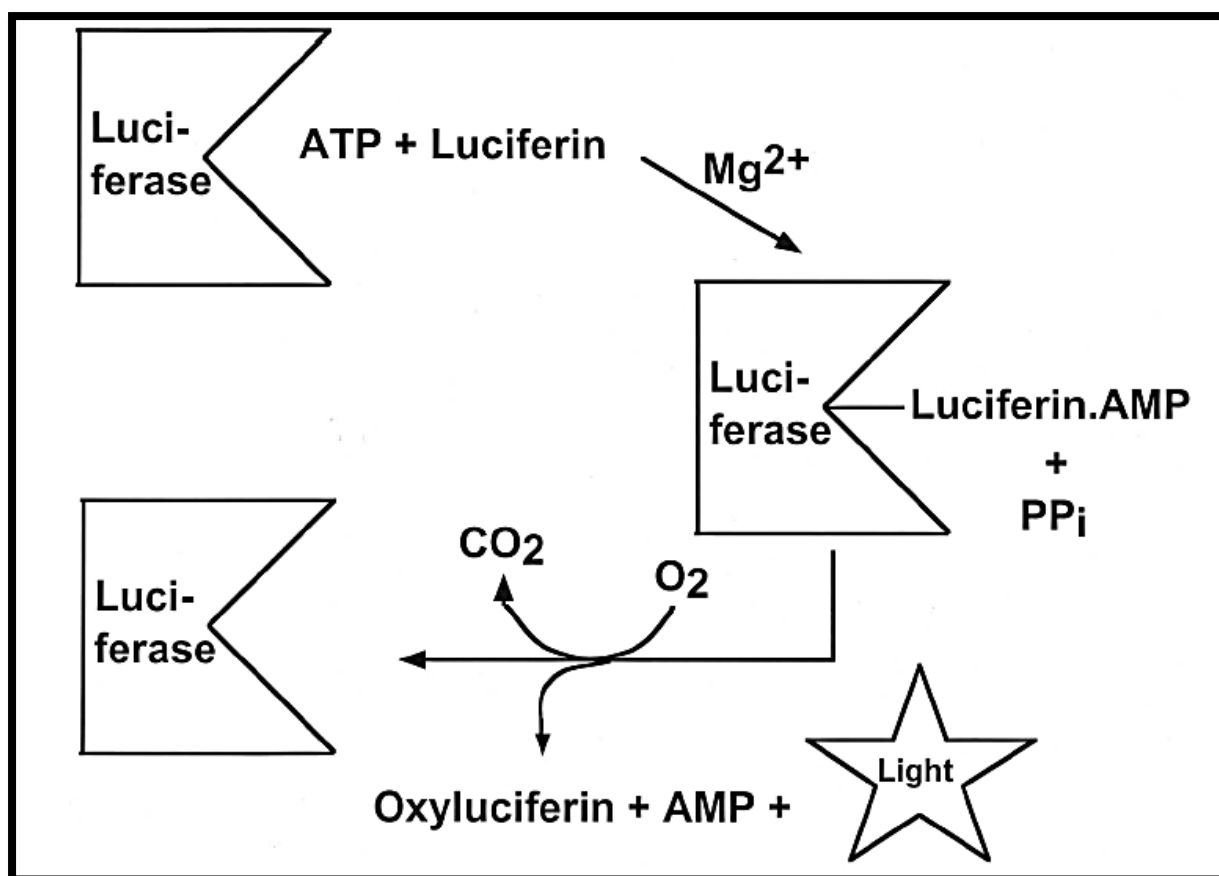


Figure 3.2: Principle of the CellTiter-Glo® assay used to measure concentration of intracellular ATP (Deshpande, 2001).

The reaction results in the conversion of chemical energy to light. The first stage of the reaction is the creation of large amounts of energy using a substrate (D-luciferin), ATP, oxygen and a magnesium co-factor. This reaction is enzymatically regulated by luciferase. Luciferin is converted to oxyluciferin. The chemical energy produced in the first stage excites and increases the energy of the luminescent molecule. This results in decay and photon emission, which then releases light. The light produced is directly proportional to the ATP produced by the cell and light intensity measured using a luminometer (Deshpande, 2001).

3.2.3.2 Protocol

ATP concentration was measured following the CellTiter-Glo® Luminescent Cell Viability Assay (Promega, catalogue no. #G7570). Cells were treated in 6 well plates for 24 hr (37 °C; 5% CO₂). Following treatment, cells were counted and adjusted using the Trypan blue exclusion method and 20,000 cells in 0.1M PBS were seeded per well in triplicates (25 µl/well) using an opaque 96 well microtitre plate. As per manufacturer's instructions, the CellTiter-Glo® Reagent was reconstituted and 25 µl of reagent was added to each well. Plates were incubated in the dark for 20 mins at room temperature (RT), and luminescence was measured using a Modulus™ Microplate Reader. Results were expressed as concentration versus relative light units (RLU).

3.2.4 H₂DCF-DA assay

3.2.4.1 Introduction

Reactive oxygen species (ROS) is endogenously produced by cells however, this production may be stimulated by external factors resulting in an over production of ROS. The imbalance between ROS and antioxidants results in oxidative stress, thus the need to measure ROS level in cells. The most suitable methods for ROS measurement in live cells are fluorescent techniques where the fluorescence indicators are susceptible to fluctuations in ROS concentration. The 2, 7-dichlorohydrofluorescein diacetate (H₂DCF-DA) assay uses a dye that emits low fluorescence in a reduced state but is highly fluorescent in an oxidised state - dichlorofluorescein (DCF). H₂DCF-DA diffuses into cell due to its cell permeable nature and is cleaved by endogenous esterases (Figure 3.3). This process yields the

H_2DCF^- anion which is non-fluorescent. Exposure to ROS results in the anion being reduced to DCF, which is high fluorescent.

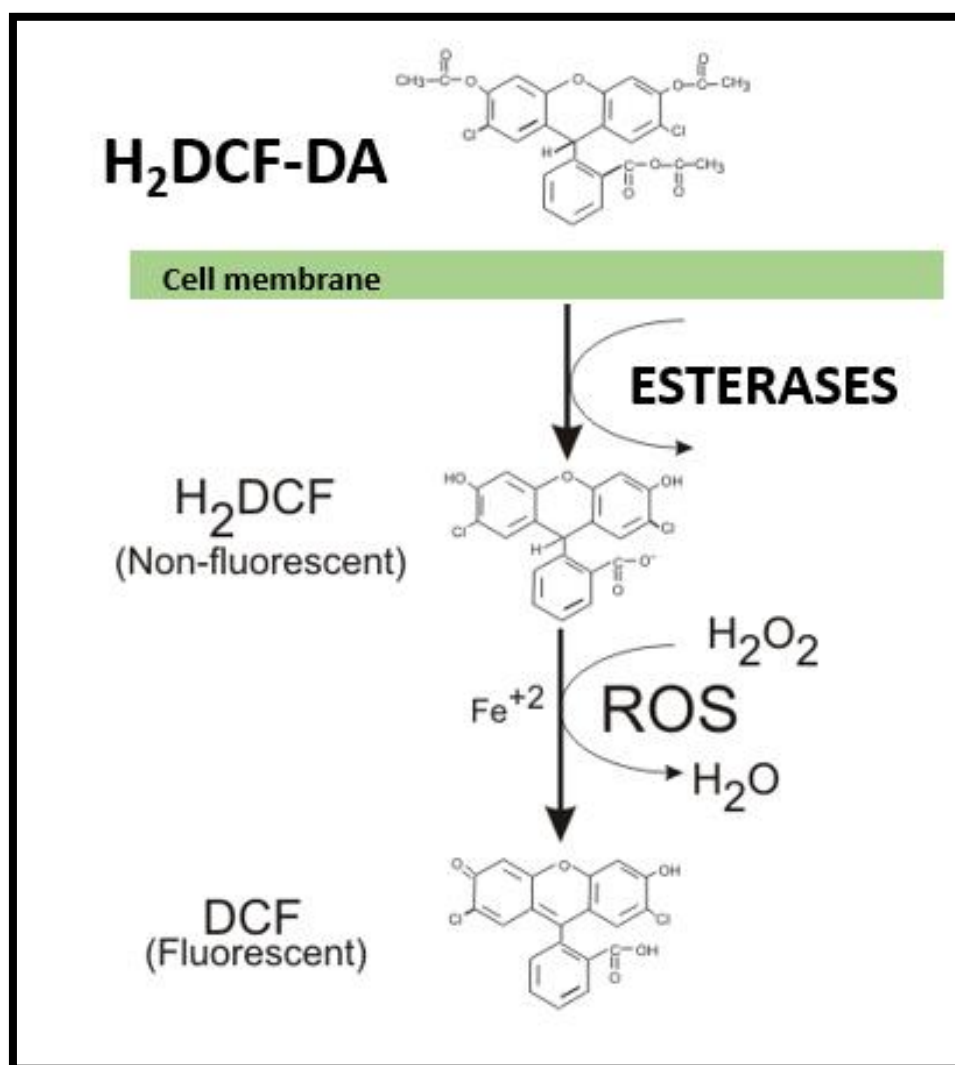


Figure 3.3: Principle of DCF assay for ROS detection (prepared by author)

The fluorescent intensity is directly proportional to the ROS produced by the cell and measured using a fluorometric plate reader (Wang and Roper, 2014).

3.2.4.2 Protocol

ROS concentration was quantified using the DCF assay. Cells were treated in 6 well plates at a confluency of 80% for 24 hr (37 °C; 5% CO₂). Cells were counted and adjusted using the Trypan blue exclusion method and 50,000 cells were seeded in

four separate microcentrifuge tubes. A stock solution of 80 mM H₂DCF-DA was diluted to produce a concentration of 5 μ M in PBS; 100 μ l of the working solution was added to each microcentrifuge tube (37 °C; 30 mins). Cells were washed with PBS and subsequently centrifuged (400 xg; 10 mins). PBS was removed and the process was repeated. Cells were. Fluorescence resuspended in 200 μ l of PBS and transferred to opaque 96-well microtitre plates was measured using a Modulus™ Microplate Reader with a blue filter (excitation wavelength 503nm, emission wavelength 509nm) and results expressed as relative fluorescent units (RFU).

3.2.5 JC-1 Mitoscreen

3.2.5.1 Introduction

The mitochondria are the locations for several major events however, the most significant is the loss of mitochondrial membrane potential ($\Delta\psi_m$) (Green and Reed, 1998). The mitochondria generate an electric gradient across their membrane to produce ATP. The direction of the membrane potential allows for transport of cations in the mitochondria and outward transport of anions. This gradient drives ATP synthesis. However, certain toxins have the ability to decrease $\Delta\psi_m$ leading to dysfunction in mitochondrial process (Sivandzade et al., 2019).

The JC-1 mitoscreen uses the JC-1 dye to detect $\Delta\psi_m$ in cells. The dye is a cation that naturally emits green fluorescence and possesses the ability to enter and accumulate in the mitochondria. Once inside the mitochondria, it begins to form J-aggregates. These complexes exhibit excitation and emission in the red spectrum, as opposed to the JC-1 dye, which is in the green spectrum. Therefore, in healthy mitochondria they enter and form aggregates emitting red fluorescence. Conversely, in dysfunctional mitochondria, they enter less frequently due to the loss in $\Delta\psi_m$ and

the interior of the mitochondria being less negative. This causes the dye to be at insufficient concentrations to form aggregates and it remains in its green fluorescent state (Figure 3.4) (Sivandzade et al., 2019).

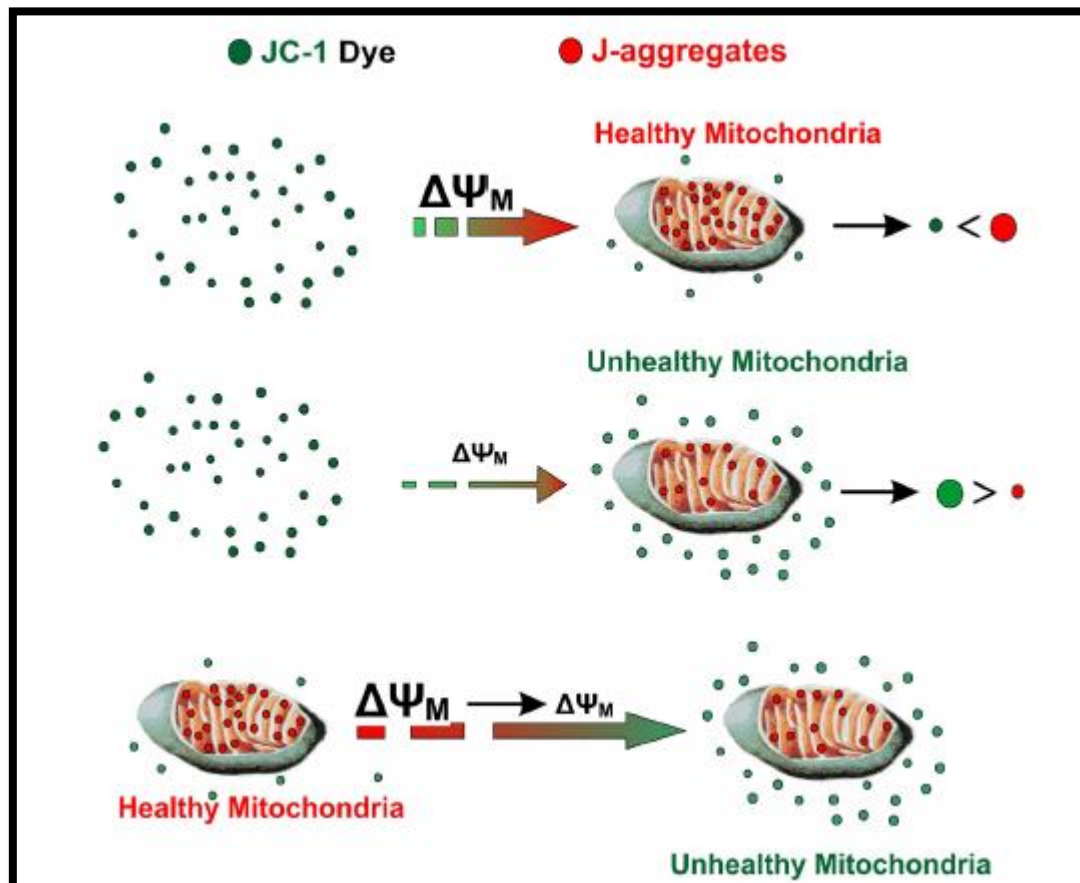


Figure 3.4: Different results produced in healthy and unhealthy mitochondria following the JC-1 mitoscreen (Sivandzade et al., 2019).

The ratio of red-green fluorescence in the mitochondria is proportional to the state of polarisation, the higher the $\Delta\psi_m$, the higher the red emission and the lower the $\Delta\psi_m$, the higher the green emission. Therefore, mitochondrial depolarisation is confirmed by reduced red-green fluorescent ratio (Sivandzade et al., 2019).

3.2.5.2 Protocol

The mitochondrial membrane potential ($\Delta\psi_m$) was measured using the JC-1 stain (Zheng et al., 2013). Control and treated cells (50,000 cells per treatment) were incubated in 200 μ l of 5 μ g/ml JC-1 stain (BD Biosciences, San Jose, NJ, USA) (20 min, 37 °C). The stain was removed via centrifugation (400 x g, 10 min, 24 °C) and the cells were washed twice with JC-1 staining buffer. Cells were re-suspended in 400 μ l of JC-1 staining buffer and seeded in an opaque 96-well plate in triplicate (100 μ l/well). A blank, which consisted of only JC-1 stain buffer was plated in triplicate in wells (100 μ l/well). Fluorescence was quantified on a Modulus™ microplate reader (Turner Biosystems, Sunnyvale, CA). JC-1 monomers are measured with a blue filter (excitation wavelength 488 nm, emission wavelength 529 nm) and JC-1 aggregates are measured with a green filter (excitation wavelength 524 nm, emission wavelength 594 nm). The $\Delta\psi_m$ of the Hek293 cells are expressed as the fluorescence intensity ratio of JC-1 aggregates and JC-1 monomers.

3.2.6 Western blots

3.2.6.1 Introduction

Western blots are used to detect proteins of interest using a multi-step process (Figure 3.5). Proteins are initially separated by size using electric fields and thereafter are transferred to a membrane for detection. Thereafter antibodies may be used to probe for the protein of interest. The technique allows for the determination of protein levels, which are expressed in relation to band density obtained (Manns, 2011, Bass et al., 2017).

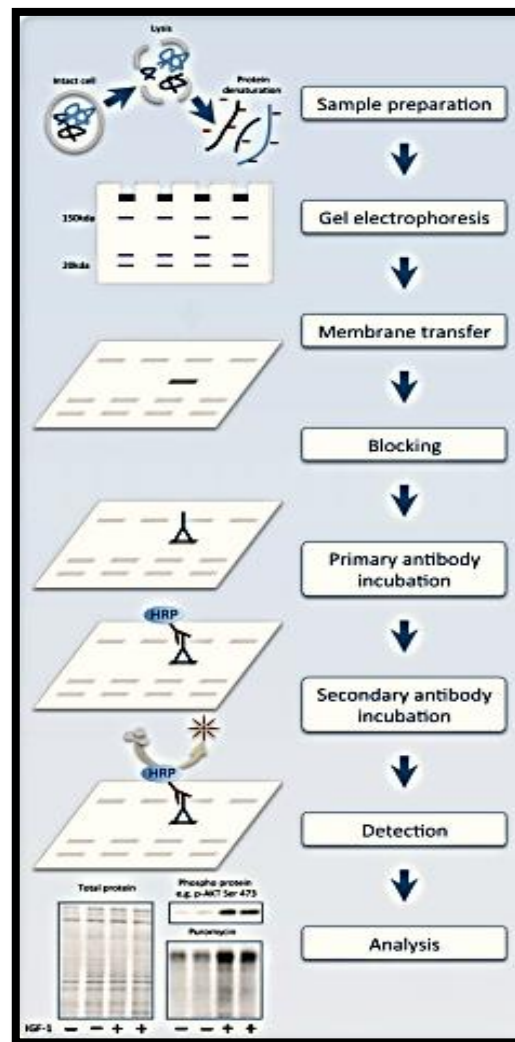


Figure 3.5: Summary of western blot procedure (Bass et al., 2017)

3.2.6.2 Protein isolation and sample preparation

Cells are lysed and proteins are solubilised using different detergents and buffers. To prevent protein degradation proteases and phosphatase inhibitors are used during this process. Following protein extraction, the bicinchoninic acid (BCA) assay is used to quantify the protein concentration. This is carried out to ensure that enough protein is available and allows for protein concentration to be adjusted to ensure even concentrations between samples during separation. The BCA assay relies on the conversion of cupric (Cu^{2+}) to cuprous ion (Cu^+). The produced Cu^+ reacts with the BCA reagent and this allows for detection. The reaction yields an intense purple colour that is detected at a wavelength of 562 nm using a spectrophotometer. The intensity of colour produced is directly proportional to protein concentration. Considering that the Cu^+ formed is a function of protein concentration, unknown protein concentrations may be determined by comparison with known standard proteins (Walker, 2009).

Once proteins have been quantified and standardised they are diluted with Laemmli buffer. The buffer comprised of the following components with specific functions (Gavini and Parameshwaran, 2019):

- Glycerol- increases the viscosity of the sample allowing it to sink into wells
- Sodium dodecyl sulphate (SDS) - causes the protein sample to have a homogenous negative charge and ensure linearization of protein samples.
- Tris- maintains the pH of the sample
- B-mercaptoethanol- breaks down disulphide bridges and bond in proteins promoting linearity.
- Bromophenol blue- acts as a tracking dye for proteins during electrophoresis

3.2.6.3 SDS-PAGE

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) is used to separate proteins after which the relative molecular mass, relative concentration and purity of proteins may be determined. SDS-PAGE allows for the movement of charged molecules through a gel matrix following application of an electric current (Manns, 2011).

SDS is a negatively charged detergent that can denature proteins and give them an overall negative charge. The proteins solubilised by SDS possess a larger negative charge in proportion to mass and this influences the rate at which they move towards the anode under the influence of an electric field (Manns, 2011).

Polyacrylamide creates a gel matrix, which acts as a filter allowing for small molecules to migrate at a faster rate than larger molecules. A greater percentage of acrylamide results in smaller pores, while a lower percentage of acrylamide results in larger pores. The gel has two sections namely the resolving and stacking gel. The stacking gel has a lower percentage of acrylamide and forms part of the starting point of migration allowing the proteins to form thin defined bands. The resolving gel has a higher percentage of acrylamide and is more important for protein separation by size (Manns, 2011).

The rate of migration is influenced by gel pore size, protein size, charge and shape. Proteins migrate after a current is applied and differentially migrate with larger proteins staying closer to the origin and smaller proteins migrating further down the gel. Molecular weight markers are used to aid in determination of the sizes of unknown proteins (Manns, 2011).

3.2.6.4 Transfer of proteins

Following protein separation, the proteins are transferred to a nitrocellulose membrane. This is performed using an electric field that is perpendicular to the gel allowing proteins to migrate from the gel onto the membrane. This is created by placing the membrane between the gel and a positive electrode creating a sandwich that is protected by fibre pads at each end. The process is known as electrophoretic transfer (Bass et al., 2017).

3.2.6.5 Immuno-blotting

Prior to immune blotting, membranes with protein are incubated in a diluted protein such as bovine serum albumin (BSA) or non-fat dry milk in a blocking step. This prevents non-specific binding of antibody to the membrane and reduces background to yield more accurate results (Gavini and Parameshwaran, 2019).

Following this step, a non-labelled primary antibody is used to bind to the protein of interest followed by a labelled secondary antibody to bind to the primary antibody. The labelled secondary antibody is conjugated to an enzyme commonly horseradish peroxidase (HRP) or alkaline phosphatase and aids in signal amplification. The enzyme is able to oxidise the chemiluminescent detection reagent (luminol) to produce light. The light intensity is directly proportional to expression of the protein of interest (Gavini and Parameshwaran, 2019).

3.2.6.6 Detection

The resultant signals produced are detected using a camera based imager, generally the image shows one or more protein bands and comparison of the band to the molecular weight marker can be used to determine the size of the proteins. Quantity of the protein is determined in relation to band intensity. A housekeeping protein is

used to normalise protein expression in order to determine accurate protein levels. Housekeeping proteins are internal reference proteins that are expressed constitutively and are used as a corrective measure to minimise errors occurring during loading and varying protein concentrations (Gavini and Parameshwaran, 2019).

3.2.6.7 Protocol

Protein expression of regulatory mitochondrial proteins was assessed by western blots. Proteins were isolated in a single experiment prior to western blots. Following treatment for 24 hr, cells were incubated with 150 μ l of Cytobuster™ Reagent (Novagen, San Diego, CA, USA, catalogue no. 71009) on ice for 30 mins. Cells were lysed mechanically via scraping. Lysed cells were transferred to 1.5 ml microcentrifuge tubes and centrifuged (400 x g, 10 mins, 4 °C). The supernatant containing crude protein isolates were aspirated and quantified. The BCA assay was used for quantification; protein samples were standardised to a concentration of 1.5 mg/ml. Protein samples were boiled in Laemmli Buffer containing distilled water, glycerol, 10% SDS, β -mercaptoethanol, 0.5M Tris-HCl (pH 6.8), 1% bromophenol blue and glycerol (5 mins; 100 °C).

A Bio-Rad compact supply was used to electrophorese 25 μ l samples (1hr at 150V) in sodium dodecyl sulphate (SDS) polyacrylamide gels (4% stacking, 10% resolving). A Bio-Rad Trans-Blot® Turbo Transfer system was used to transfer proteins from the gel onto nitrocellulose membranes using a pre-programmed protocol for mixed molecular weight. All membranes were blocked using 5 ml of 5% Bovine Serum Albumin (BSA) (1 g BSA in 100 ml Tris-buffer saline [TTBS-0.5% Tween20, dH₂O, KCl, NaCl, Tris, pH 7.5]).

Membranes were then immunoprobed with the respective primary antibody (1:1000 dilution in 5% BSA) against phosphorylated (Ser40) Nrf2 (ab76026, Abcam), SIRT3 (ab86671, Abcam), LONP1 (HPA002192, Sigma- Aldrich), PINK1 (a23707, Abcam), p62 (a56416, Abcam) and HSP60 (SAB4501464, Sigma-Aldrich) for 1hr at RT on a shaker and thereafter, overnight at 4 °C. Following incubation, membranes were washed (x5) for 10 mins using 5 ml of TTBS. Membranes were then incubated in HRP conjugated secondary antibodies (Cell signalling Technology- anti-mouse, anti-rabbit) (1:5000 in 5% BSA) for 1hr at RT on a shaker. Membranes were then washed (5 x 10 mins) using 5ml of TTBS and rinsed with distilled water. Clarity Western ECL Substrate detection reagent (400µL) (Bio-Rad) was added to membranes to detect bands and images were captured on a Bio-Rad ChemiDoc™ XRS+ System. Results were analysed using Image Lab™ Software v6.0 (Bio-Rad).

Membranes were quenched using 5% hydrogen peroxide for 30 mins at 37 °C, blocked using 5% BSA and incubated in HRP-conjugated antibody for β -actin (A3854, Sigma-Aldrich) as a house-keeping protein. Results were presented as relative band density (RBD) of protein of interest divided by RBD of respective β -actin.

3.2.7 Quantitative PCR

3.2.7.1 Introduction

Conventional polymerase chain reaction (PCR) is used to amplify a specific DNA sequence from template strands. The method employs the use of primers which are single stranded DNA that flank the targeted gene. Primers are then extended via DNA polymerase (Taq polymerase) which allows for the addition of deoxynucleotide triphosphates (dNTP) to the 3' end of primers. The three main steps in PCR include: (Figure 3.6) (Garibyan and Avashia, 2013).

1. Denaturation (90 °C) - Heating of sample where double stranded DNA (dsDNA) is separated to produce single stranded DNA (ssDNA). These act as templates.
2. Annealing (55 °C -60 °C) - Primers bind to the complementary bases from the 3' end of the template.
3. Extension (72 °C) – Annealed primers have dNTPs added on via DNA polymerase to produce new dsDNA.

The process results in the exponential amplification of DNA however, it does not allow for the quantification of the synthesised products.

Quantitative PCR is used for the quantification of nucleic acids. The method allows for the reliable detection and measurement of products produced during each PCR cycle. Isolated RNA are reverse transcribed into complementary DNA (cDNA). This cDNA acts as a template for conventional PCR to continue. SYBR green intercalates between bases in the dsDNA synthesised. Due to the dye being fluorescent, the fluorescence intensity is measured to quantify amplified DNA. A housekeeping gene is used to normalise gene levels and concentrations of target DNA expressed in relation to the amount of housekeeping gene (Garibyan and Avashia, 2013).

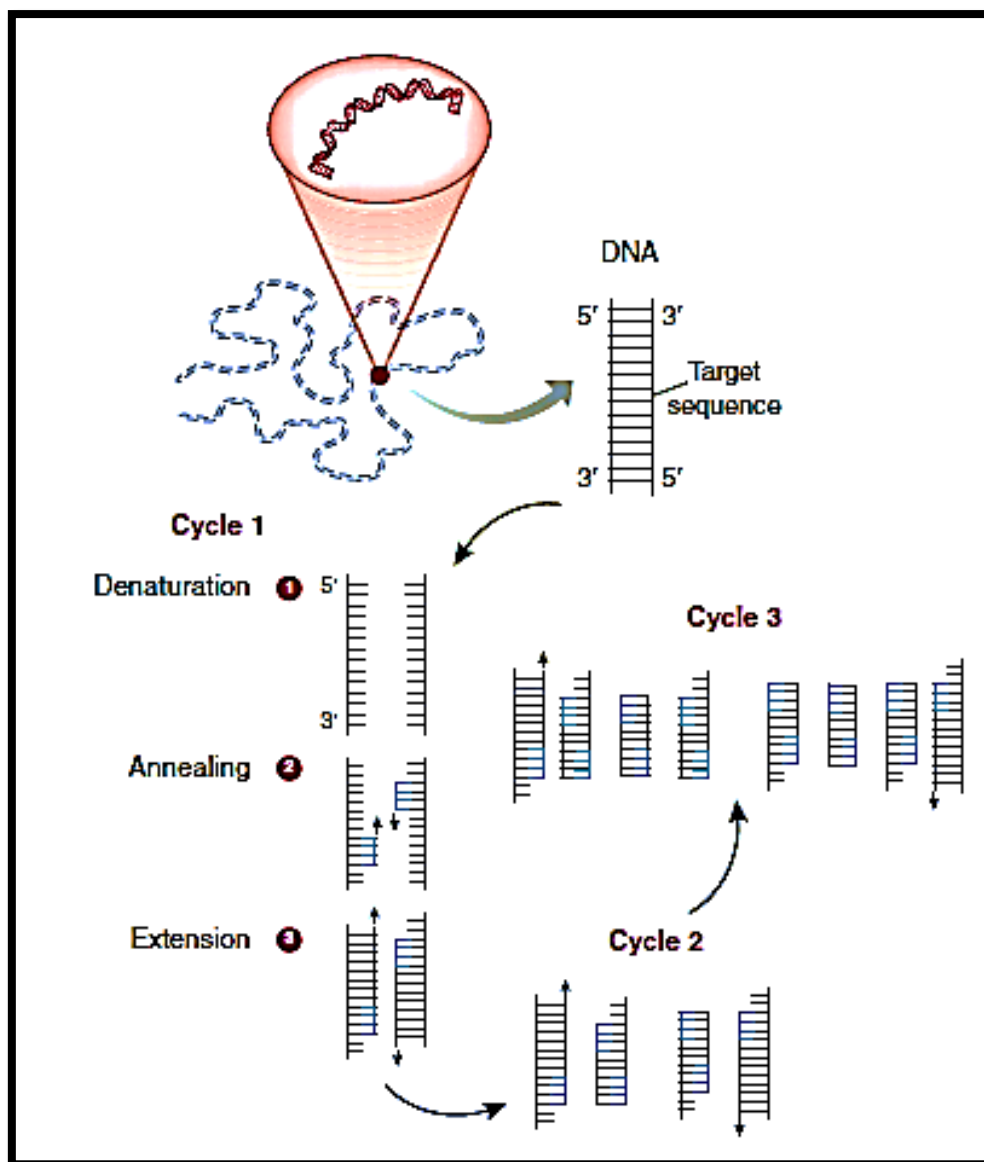


Figure 3.6: Principle of qPCR (Garibyan and Avashia, 2013).

3.2.7.2. Protocol

RNA isolation

Following treatment for 24 hr, cells were incubated with 500 μ l each of Trizol and PBS (5mins; RT). Samples were scraped and transferred to 2 ml microcentrifuge tubes and stored (24 hr, -80 $^{\circ}$ C). The samples were thawed at RT, followed by the addition of 100 μ l of chloroform and centrifugation (12000 g, 10 mins; 4 $^{\circ}$ C). The supernatant was

removed and 250 µl of isopropanol added followed by storage at -80°C overnight. Samples were then thawed at RT and centrifuged (12000 g, 20 mins; 4 °C). The supernatant was discarded and the pellet preserved and washed using 500 µl of ethanol. Following washing samples were centrifuged (7400 g, 15 mins; 4 °C). The ethanol was removed and the pellet left to air dry (20-30 min, 24 °C). Once dry, pellets were resuspended in 15 µl of Nuclease-Free water and samples were stored at -80 °C.

RNA quantification

Quantification of the crude RNA product was carried out using the Nanodrop2000 Spectrophotometer (Thermo-Fisher Scientific). RNA quality was determined using A260/A280 ratio. All RNA samples were standardised to 500 ng/µl.

cDNA synthesis

i. cDNA synthesis for mRNA

The cDNA was synthesized from the crude RNA samples using the iScript™ cDNA Synthesis kit (Bio-Rad, catalogue no 107-8890). A master mix was created using reagents from the kit and standardised RNA (Table 3.1).

Table 3.1: cDNA synthesis (iScript cDNA synthesis kit, Bio-Rad).

Component	1 x Reaction (µl)
5x iScript Reaction Mix	2
iScript Reverse Transcriptase	0.5
Nuclease-Free Water	5.5
RNA Template (500ng/µl)	2
Total	10

The 10 µl Master Mix was used to synthesis cDNA and incubated in a thermocycler (GeneAmp® PCR System 9700, Applied Biosciences, California, USA) [25 °C for 5 min, 42 °C for 30 min, 85 °C for 5 min, 4°C hold]. Following synthesis, cDNA was stored at -20 °C until further use.

ii. cDNA synthesis for miRNA

The cDNA was synthesized from crude RNA samples using the miScript® II RT Kit (Qiagen, Hilden, Germany; catalogue number 218160). A master mix was created using reagents from the kit and standardised RNA (Table 3.2).

Table 3.2: cDNA synthesis (miScript® II RT kit, Qiagen).

Component	1 x Reaction (µl)
5x miScript HiFlex Buffer	4
10x miScript nucleics mix	2
Nuclease-Free Water	10
miScript RT mix	2
RNA template (500ng/µl)	2
Total	20

The 20 µl Master Mix was used to synthesis cDNA and incubated in a thermocycler (GeneAmp® PCR System 9700, Applied Biosciences, California, USA) [37 °C for 60 min, 95 °C for 5 min and 4 °C for 5 min]. Following synthesis, cDNA was stored at -20 °C until further use.

Gene expression

i. mRNA gene expression

Gene expression of *SIRT3* and *PINK1* (Table 3.3) was assessed using the iQ™ SYBR® Green PCR kit and the CFX Touch™ Real Time PCR Detection System (Bio-Rad, Hercules, CA, USA). A master mix was created using the cDNA and reagents from the kit (Table 3.3).

Table 3.3: Reaction mix for qPCR (iQ™ SYBR® Green PCR kit)

Component	1x Reaction (μl)
SYBR® Green	5
Forward primer	1
Reverse primer	1
Nuclease-Free Water	2
cDNA template	1
Total	10

The thermocycler conditions for each gene were as follows: initial denaturation (8 mins, 95 °C), followed by 40 cycles of denaturation (15 sec, 95 °C), annealing (Table 3.4), extension (30 sec; 72 °C) and final extension (30sec; 72 °C). Data was normalized against housekeeping gene *GAPDH*. Results were analysed using the Livak and Schmittgen (2001) method and represented as fold change relative to the housekeeping gene ($2^{-\Delta\Delta C_t}$) (Livak and Schmittgen, 2001).

Table 3.4: Primers sequences with respective annealing temperatures for genes assessed

Gene		Sequence (5'-3')	Annealing temperature (°C)
<i>SIRT3</i>	Sense	GAGCGGCCTCTACAGCAAC	60
	Anti-sense	GAGTAGTGAGTGACATTGGG	
<i>PINK1</i>	Sense	AAGCGAGGCTTTCCCCTAC	56
	Anti-sense	GCACTACATTGACCACCGATTT	
<i>GAPDH</i>	Sense	TCCACCACCCTGTTGCTGTA	-
	Anti-sense	ACCACAGTCCATGCCATCAC	

ii. MiRNA gene expression

MiRNA expression for *miR-27b* was assessed using the MiRNA SYBR® Green PCR Kit and CFX Touch™ Real Time PCR Detection System (Bio-Rad, Hercules, CA, USA). A master mix was created using the cDNA and reagents from the kit (Table 3.5).

Table 3.5: Reaction mix for qPCR (MiRNA SYBR® Green PCR Kit).

Component	1x Reaction (µl)
2x Quantitect	6
SYBR® Green mix	
10x miScript	1.25
universal primer	
10x miScript primer	1.25
assay	
RNase-Free Water	2.25
cDNA template	1
Total	12

The thermocycler conditions were as follows: initial denaturation (15 mins, 95 °C), followed by 40 cycles of denaturation (15 sec, 94 °C), annealing (30 sec; 55 °C) and extension (30 sec; 70 °C). Data was normalized against housekeeping gene, RNU6 (218300, MS00033740). Results were analysed using the Livak and Schmittgen (2001) method and represented as fold change relative to the housekeeping gene ($2^{-\Delta\Delta C_t}$) (Livak and Schmittgen, 2001).

3.2.8 Statistical analysis

All assays were carried out in duplicates with a minimum of three replicates for each treatment unless stated otherwise. Data was analysed using One-way analysis of variance (ANOVA) followed by a Bonferroni test for multiple group comparison (GraphPad Prism V5.0 Software). Data was considered significant with $p < 0.05$.

Chapter 4: Results

4.1 Cell Viability

Cytotoxicity was determined using serially diluted concentrations of FB₂ (0-500 μ M) in Hek293 cells over 24 hr. Analysis of the curve indicated that 317.4 μ M of FB₂ was sufficient to induce 50% cell death in Hek293 cells (IC₅₀) (Figure. 4.1). Furthermore, 100 μ M (128.45% viability) showed the highest increase in cell viability and 500 μ M (67.05% viability) showed the second highest inhibition of cell viability. These concentrations were used for treatment in subsequent assays to achieve a broader toxic profile.

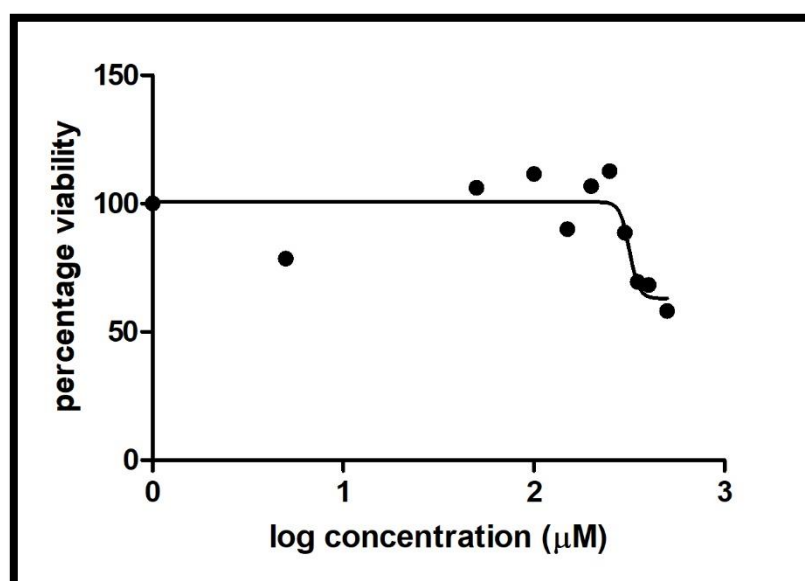


Figure 4.1: FB₂ is cytotoxic to Hek293 cells. A dose dependent decline in viability of the cell occurred after treatment with varying concentrations (0 -500 μ M) of FB₂.

4.2 FB₂ induced mitochondrial stress

We next tested the effects of FB₂ on mitochondrial stress - western blot for HSP60 protein and luminometry for ATP quantification (mitochondrial functionality) was carried out. FB₂ induced an increase in HSP60 protein expression suggesting increased mitochondrial stress (Figure 4.2A). Results showed a significant decrease in ATP ($p < 0.001$) in the IC₅₀ treated Hek293 cells when compared to the control whereas the 500 μ M of FB₂ showed a significant increase ($p < 0.001$) in ATP levels when compared to the control (Figure 4.2B). The 100 μ M concentration displayed no significant difference in ATP (Figure 4.2B).

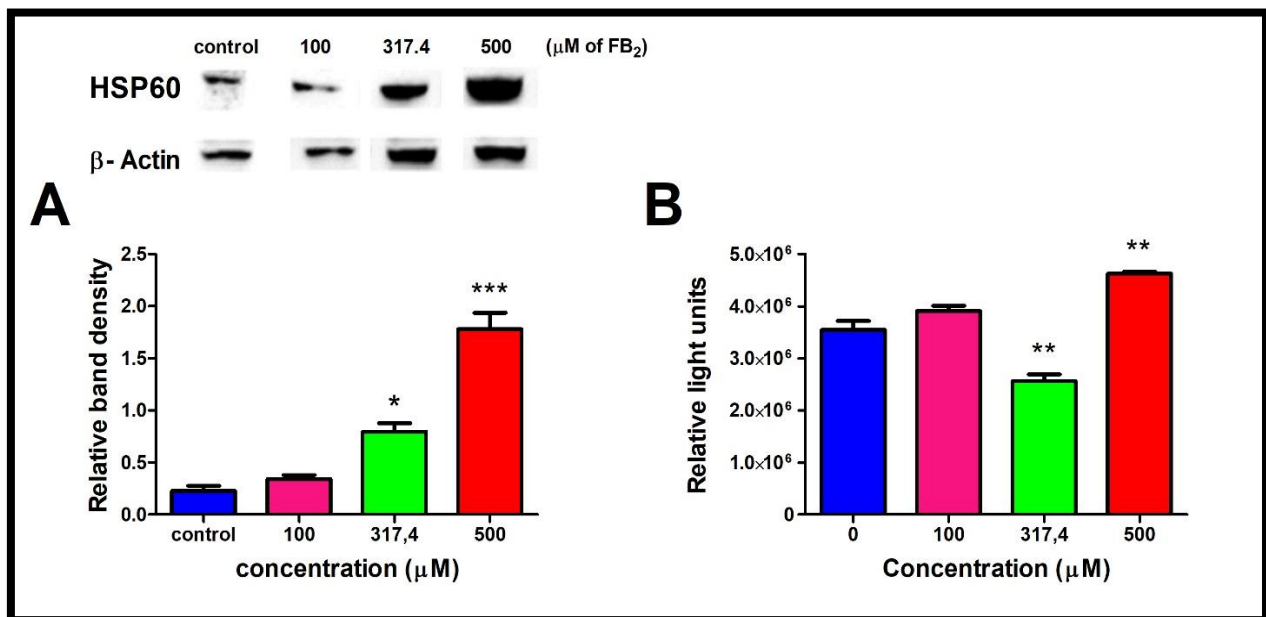


Figure 4.2: FB₂ induced mitochondrial stress. HSP60 protein expression increased in a dose-dependent manner (A). ATP concentrations exhibited a biphasic response following treatment with FB₂ (B). * $p < 0.05$; ** $p < 0.001$; *** $p < 0.0001$ relative to control

4.3 *FB₂ increased ROS production and mitochondrial membrane depolarisation*

Compromised mitochondrial function may result in exacerbated ROS production that further induces mitotoxicity. ROS production increased significantly for higher concentrations of *FB₂* ($p < 0.0001$) whilst the lower concentration showed a significant decrease in ATP concentration ($p < 0.0001$) (Figure 4.3A). Mitochondrial functionality can be observed by quantifying mitochondrial membrane depolarisation. Significant increases in mitochondrial membrane depolarisation were noted at higher concentrations ($p < 0.001$) whilst the percentage of depolarised membranes were lower at the lowest treatment of *FB₂* ($p < 0.0001$) (Figure 4.3B).

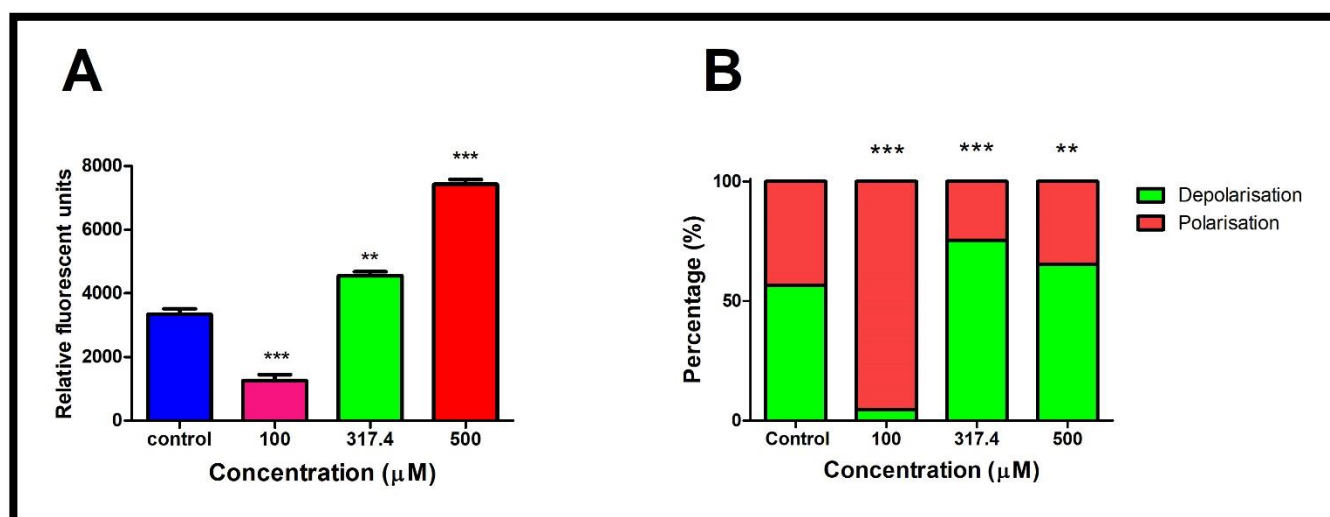


Figure 4.3: *FB₂* increased ROS production and mitochondrial membrane depolarisation. ROS production was suppressed at lower concentrations however, significant increases were noted at higher treatments (A). Mitochondrial membrane depolarisation also significantly increased at higher concentrations (B). A- ** $p < 0.001$; *** $p < 0.0001$ relative to control ; B ** $p < 0.001$; *** $p < 0.0001$ % depolarisation relative to control.

4.4 *FB₂* induced mitochondrial stress responses

To confirm the induction of mitochondrial stress, protein (western blots) and mRNA (qPCR) levels of SIRT3 were analysed. Additionally, protein expression of LONP1 (activated by SIRT3) was measured. Biphasic changes in the protein expression of SIRT3 were observed (Figure 4.4A). Gene expression of *SIRT3* was significantly downregulated at the IC₅₀ ($p < 0.05$) (Figure 4.4C). Protein expression of LONP1 was significantly upregulated at the lowest treatment ($p < 0.0001$) (Figure 4.4B) correlating to increase in SIRT3 protein expression (Figure 4.4A)

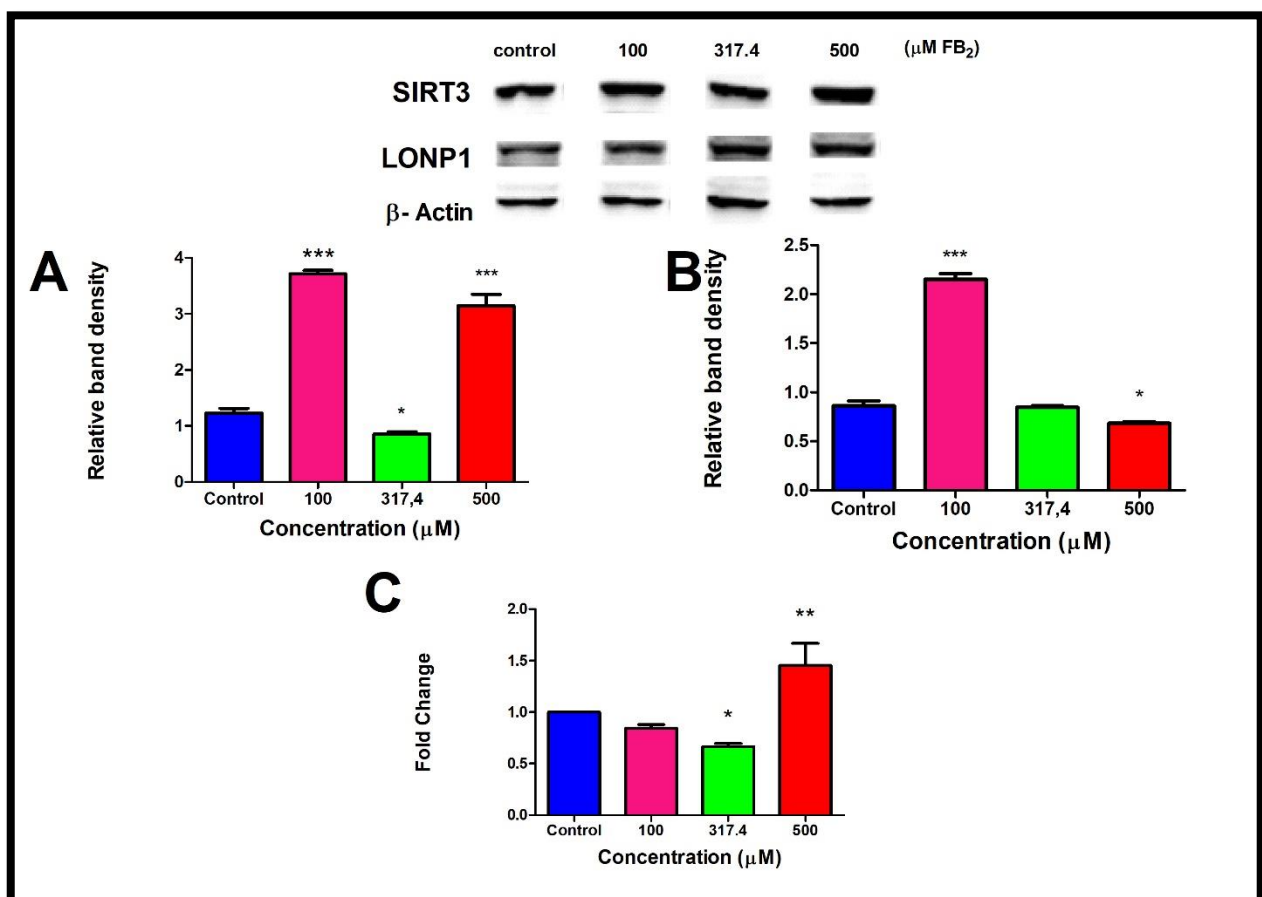


Figure 4.4: *FB₂* induced a biphasic mitochondrial stress responses. SIRT3 protein expression was up-regulated at 100 μM and 500 μM , but down-regulated at IC₅₀ when compared to the control (A). *SIRT3* gene expression is suppressed at the IC₅₀ (C).

LONP1 expression increases at lower concentrations but decreases at higher concentrations (B). * $p<0.05$; ** $p<0.001$; *** $p<0.0001$ relative to control.

4.5 FB_2 promoted mitophagy via Nrf2 activation

Following FB_2 exposure, significant increases in phosphorylated Nrf2 (Ser40) were noted for all treatment concentrations (Figure 4.5). Phosphorylated Nrf2 (Ser40) is the activated form of Nrf2 that has dissociated from KEAP1 allowing it to transcribe for proteins. The lowest concentration exhibited the most significant increase in phosphorylated Nrf2 (Ser40) ($p<0.0001$).

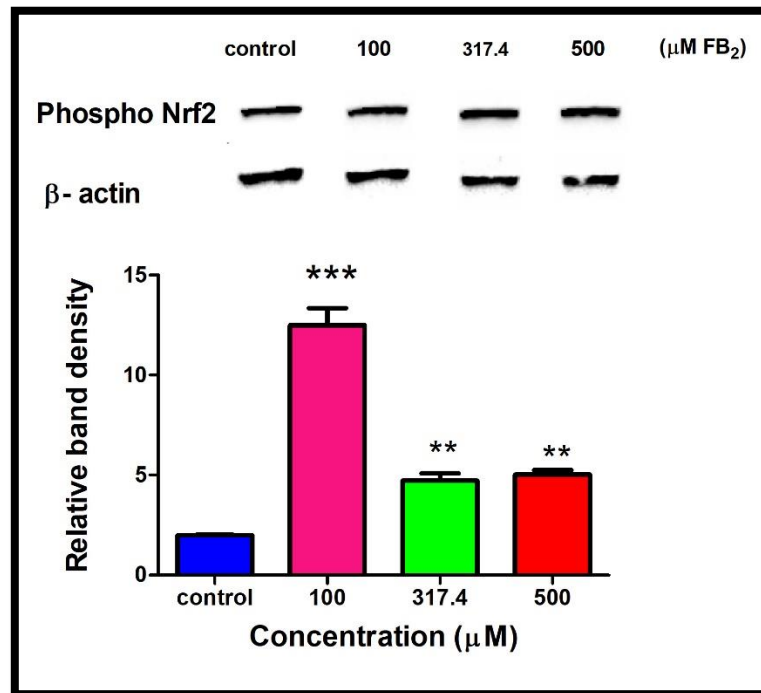


Figure 4.5: FB_2 promoted mitophagy. Significant upregulations in phosphorylated Nrf2 (Ser40) expression following FB_2 treatment. ** $p<0.001$; *** $p<0.0001$ relative to the control.

4.6 *FB₂* induced mitophagy

We screened Hek293 cells treated with *FB₂* for mitophagy via analysis of mitophagic proteins and genes. Epigenetic regulation of the markers were assessed via evaluation of a miRNA (*miR-27b*). Protein expression of PINK1 and p62 was quantified to conclude if mitophagic markers were upregulated. Western blots revealed that an increase in PINK1 transcript levels (Figure 4.6C) resulted in an increase in protein expression (Figure 4.6B) with concomitant decreases at lower concentrations. *FB₂* correspondingly increased expression of p62 (Figure 4.6D) ($p < 0.0001$). Gene expression of *miR-27b* is suppressed for exposure to all concentrations of *FB₂*.

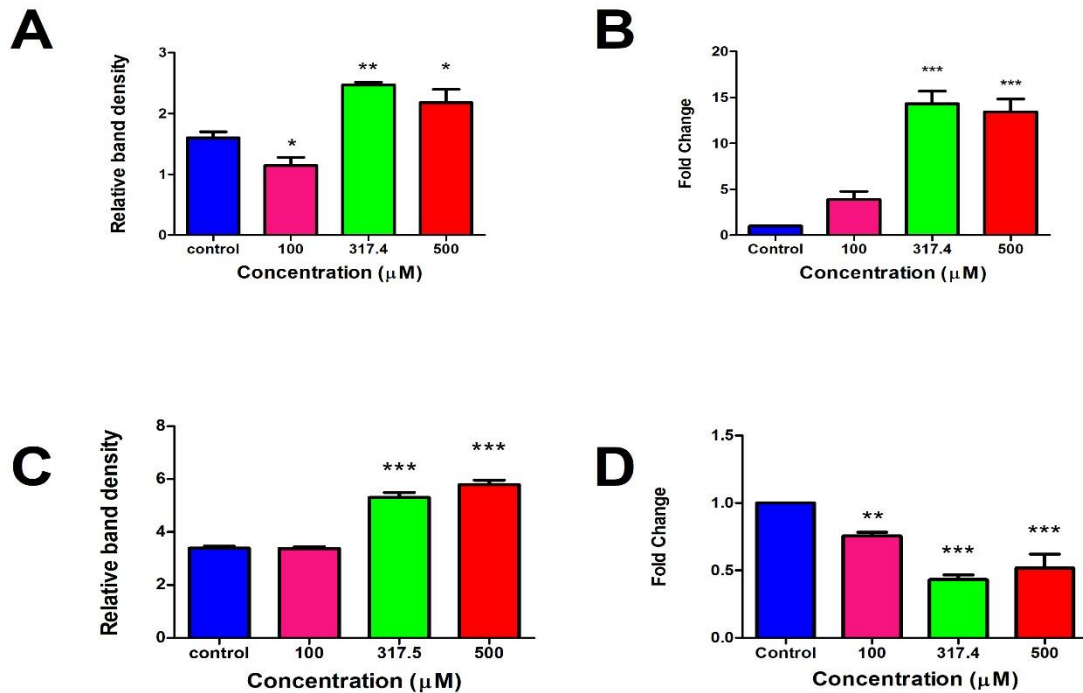
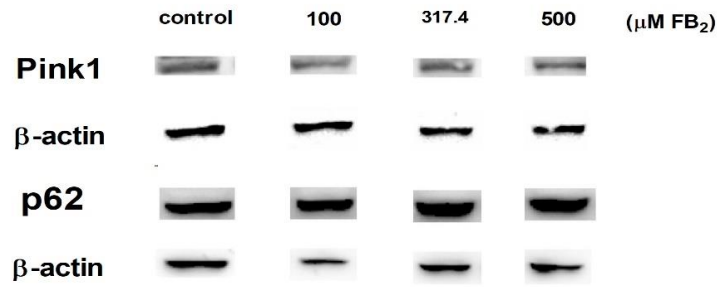


Figure 4.6: FB₂ increased mitophagy markers in Hek293 cells. PINK1 protein (A) and gene expression (B) was significantly up-regulated at the higher concentrations of FB₂. Significant up-regulation in p62 protein as compared to the control was noted at high treatment concentrations (C). *MiR-27b* expression exhibited downregulation in comparison to the control (D) * $p < 0.05$; ** $p < 0.001$; *** $p < 0.0001$ relative to control.

Chapter 5: Discussion

The toxicity exhibited by FB₁ through inhibition of sphingolipid metabolism is well established (Wang et al., 1991). FB₂ being the structural analog of FB₁ has shown to mimic toxicity leading to diseases such as ELEM (Thiel et al., 1991). However, minor differences in the structure, accounting for increased polarity in FB₂ and possible greater toxic potential. Aside from the mechanism of sphingolipid metabolism disruption, FB₁ has been shown to induce mitochondrial toxicity. The toxin possesses the ability to inhibit complex I of the mitochondrial respiratory chain resulting in a decrease in mitochondrial respiration, increase in mitochondrial membrane depolarisation and oxidative stress as well as disturbance in calcium signalling (Domijan and Abramov, 2011, Arumugam et al., 2019). The present study supports the hypothesis that FB₂ has similar mechanisms of toxicity as FB₁ in relation to interference of mitochondrial function.

FB₂ suppressed mitochondrial stress responses, induced ROS production and increased mitophagy markers at high dose exposure. These results illustrate the toxic potential of FB₂ in inducing cell death via induction of mitochondrial dysfunction. Exposure to low doses of the compound proved to upregulate mitochondrial stress responses, reducing mitochondrial stress and mitophagy accounting for the proliferative and probable oncogenic properties of the toxin (Ueno et al., 1997). The concentration dependent effects of FB₂ suggest that exposure to both low and high concentrations of the compound may induce severe side-effects on the consumer.

The MTT assay was used to select concentrations causing cell death and proliferation to achieve a broader toxic profile. The WHO has reported that numerous countries exceed the tolerable intake limit of 2 µg/kg FB₂ per day with some countries being

exposed to more than 15 µg/kg body weight (Shephard et al., 2007). Based on this data, concentrations were selected within close proximity to these values to ensure accurate representation of data.

A common marker for compromised mitochondrial activity is reduced ATP production and an increase in mitochondrial stress markers. Previous studies have shown a direct correlation in the upregulation of HSP60 protein expression in response to mitochondrial stress making it a suitable bio-marker for the phenomenon (Pellegrino et al., 2013). Alternatively in excessively high stress conditions, cells are able to increase the rate of cytoplasmic glycolysis resulting in greater ATP production. This increase in ATP is annotated to certain mitochondrial stress mediators such as LONP1 and HSP60 being ATP-dependent proteins (Pellegrino et al., 2013). Figure 4.2 shows that both phenomena were observed. The IC₅₀ significantly increased HSP60 protein expression and reduced ATP production. FB₂ is structurally similar to FB₁ having an amino group that has been suggested as an imperative moiety for FB₁ toxicity (Gelderblom et al., 1993). Previous studies have implicated FB₁ in the inhibition of complex I in the ETC. The mechanism was compared to that of rotenone in inhibition of complex I, thus suppressing its function and inducing perturbations in the ETC. Rotenone is able to bind to sites on complex I via structures similar to that of quinone (recognised by complex I) (Heikkila et al., 1985, Domijan and Abramov, 2011). In similar ways, FB₁ is able to bind to sites on complex I that recognise the functional groups (Domijan and Abramov, 2011). The structural similarities between FB₂ and FB₁ may allow the toxin to bind to complex I and cause inhibition in mitochondrial respiration. The reduction observed in ATP production (Figure 4.2B) may have resulted due to inhibition of complex I in the ETC suggesting a similar mechanism of toxicity of FB₂ as FB₁ in human neuroblastoma (50 µM, 24 hr) (Domijan and Abramov,

2011). The highest concentration increased both HSP60 protein expression and ATP production as a survival mechanism (Figure 4.2). No significant changes occurring in ATP levels for the lowest concentration (Figure 4.2B) accounts for the proliferation observed (Figure 4.1) suggesting the presence of healthy mitochondria. This is further verified by minimal mitochondrial stress (Figure 4.2A).

A common consequence of dysregulation of mitochondrial dynamics is depolarisation of the mitochondrial membrane (Domijan and Abramov, 2011). An increase in ROS production contributes greatly to both depolarisation and stress of the mitochondria. The ETC has various sites for ROS production and complications in the process induce inefficient control of ROS production and ultimately oxidative stress. Consequently, the increase in ROS production causes decreases in mitochondrial membrane potential and mitochondrial production of ATP (Small et al., 2012). Toxins such as FB₁ have the ability to inhibit complexes in the ETC thus exacerbating ROS production and reducing the membrane potential in mitochondria (Domijan and Abramov, 2011). The present study suggests that FB₂ acts in a similar way with increased production in ROS and an increase in mitochondrial membrane depolarisation at high concentration exposure (Figure 4.3). This is attributed to inhibition of complex I by FB₂ suggested due to the reduction in ATP observed (Figure 4.2B) correlating with the investigation carried out using FB₁ in human neuroblastoma (50 µM, 24 hr) (Domijan and Abramov, 2011). Consequences of altered mitochondrial membrane potential include modification in proliferation and cell death as seen during viability testing (Figure 4.1) (Zorova et al., 2018). Alternatively, it was noted that FB₂ increased the percentage of polarised mitochondria at the lowest concentration (Figure 4.3B). This data correlates with minimal ROS production (Figure 4.3A) and

unaltered ATP production (Figure 4.2B) proposing that at low concentrations, the functional groups of FB₂ do not bind to complex I of the ETC.

Mitochondrial SIRT3, a deacetylase, is a key regulator of mitochondrial homeostasis and antioxidant response to ROS (Huang et al., 2010). Previous studies have shown increases in SIRT3 expression following induction of mitochondrial stress and inhibition in expression has led to mitochondrial toxicity and cell death due to inefficient stress response. Therefore, fluctuations in SIRT3 expression can be considered a biomarker for mitochondrial stress (Weir et al., 2013). Although SIRT3 cannot directly reduce mitochondrial stress, it deacetylates antioxidants such as superoxide dismutase 2 initiating activation and reducing ROS production (Bause and Haigis, 2013). Furthermore, SIRT3 is able to increase NADH concentrations via isocitrate dehydrogenase 2 activation, thus promoting the formation of glutathione, which reduces mitochondrial ROS (Someya et al., 2010). Previously, highly oxidative environments induced by FB₁ (200 μ M, 24 hr) exposure stimulated the upregulation of SIRT3 protein expression in human hepatocellular carcinoma cells (Arumugam et al., 2019). FB₂ showed similar results at the lowest and highest concentrations for both protein and gene expression, suggesting that stress responses were upregulated (Figure 4.4A and C) in response to the excessive ROS production observed (Figure 4.3A). Discrepancies in toxicity is noted at the IC₅₀ where a downregulation in SIRT3 protein and gene expression (Figure 4.4A and C) is observed, which indicates that SIRT3-activated stress responses were inhibited. This result is significant in proposing that FB₂ has the ability to specifically inhibit mitochondrial stress response inducing dysfunction in the organelle and promoting cell death.

Oxidative environments result in damage to proteins. LONP1 is involved in the UPR^{mt} and aids in the degradation of oxidatively damaged and misfolded proteins. The

inhibition of LONP1 results in the accumulation of damaged proteins that induce toxic manifestations within the mitochondria, thus promoting mitophagy. LONP1 is post translationally regulated by SIRT3 whereby LONP1 protein expression is decreased when SIRT3 is present. However, other cases demonstrate that SIRT3 expression had no effect on LONP1 mRNA thus allowing for LONP1 activation in spite of SIRT3 presence (Gibellini et al., 2014, Bota et al., 2005). Previously exposure to FB₁ significantly upregulated LONP1 as a method to remove oxidised proteins. The increase in oxidised proteins was attributed to excessive ROS production caused by FB₁ (Arumugam et al., 2019). Conversely, treatment with high concentrations of FB₂ prevented activation of this protein. Data obtained for the IC₅₀ showed no significant changes in LONP1 indicating that FB₂ prevented an upregulation in mitochondrial stress response (Figure 4.5B). At the highest concentration, SIRT3 expression is elevated, thus causing an inhibition in LONP1 expression (Figure 4.5A and B) in line with previous experiments carried out (Gibellini et al., 2014, Bota et al., 2005).

The inhibition of LONP1 observed indicates that exposure to FB₂ allows for the accumulation of oxidatively damaged proteins in the matrix which may form aggregates and induce mitochondrial toxicity (Ngo et al., 2013). This was prevented in the lowest concentration as an upregulation in LONP1 is noted suggesting an adequate activation of the UPR^{mt} (Figure 4.5B). The upregulation of LONP1 occurred despite SIRT3 activation correlating to the theory that SIRT3 has no effect on LONP1 mRNA expression (Gibellini et al., 2014, Bota et al., 2005).

Nrf2 transcribes for various antioxidant genes in response to unwarranted ROS production (Ma, 2013). Activation of this transcription factor is phosphorylation-dependent (Huang et al., 2002). Studies have implicated excessive ROS production in the activation of phosphorylation pathways, such as the mitogen activated protein

kinase and protein kinase C (Chen et al., 2015). Kinases phosphorylate Nrf2 and triggers dissociation from KEAP1. This promotes the translocation of phosphorylated (Ser40) Nrf2 to the nucleus, wherein genes are transcribed (Huang et al., 2002). More specifically Nrf2 transcriptionally activates PINK1 in response to excessive ROS production. Considering PINK1 is a vital protein in the process of mitophagy, Nrf2 is proposed as a promoter of the mitophagic process (Murata et al., 2015). Additionally, Nrf2 is seen to upregulate p62 protein expression during oxidative stress (Jain et al., 2010). Previously, exposure to FB₁ prompted increases in the expression of phosphorylated (Ser40) Nrf2 as a method to reduce oxidative stress (Arumugam et al., 2019, Khan et al., 2018). This agrees with data obtained for FB₂ (Figure 4.5) however, in the present study, the upregulation noted is attributed to promotion of mitophagy rather than an antioxidant response. The upregulation in Nrf2 is in line with elevated ROS production (Figure 4.3A) and mitochondrial stress noted. Furthermore, increases in Nrf2 protein expression occurs when the mitochondrial respiration chain is disrupted. This coincides with ATP concentrations measured (Figure 4.2B).

MiRNA has been implicated in the epigenetic regulation of various biological processes (Maltby et al., 2016, Hammond et al., 2001). *MiR-27b* is a negative regulator of mitophagy as it has the potential to directly inhibit PINK1 expression. Inhibition is carried out when *miR-27b* binds to the 3'-UTR of PINK1 mRNA, thus preventing its translation. Inactivation of PINK1 results in suppression of downstream mitophagic proteins such as p62 (Kim et al., 2016). At higher concentrations of FB₂, *miR-27b* is suppressed (Figure 4.6D) resulting in increased PINK1 gene and protein expression (Figure 4.6A and B). Furthermore, correlating results are seen in p62 protein expression as PINK1 is known to activate the protein (Figure 4.6C). This phenomenon indicates that at high concentrations of FB₂, epigenetic regulation of

mitophagy occurs. Additionally, the upregulation in PINK1 and p62 coincides with significant increases in Nrf2 protein expression (Figure 4.5). The occurrence of mitophagy post high concentration FB₂ exposure is in agreement with the suppressed mitochondrial stress responses, increased ROS, increased mitochondrial membrane depolarisation and compromised function of the organelle. Furthermore, this suggests that FB₂ like FB₁ induces toxicity via mitochondrial dysregulation.

A new mechanism is noted at the lower concentration where suppression in mitophagic regulators (Figure 4.6A, B and C) are noted regardless of epigenetic stimulation of the proteins (Figure 4.6D). Furthermore, FB₂ was able to prevent the upregulation of PINK1 despite increases in Nrf2 observed (Figure 4.5). The result achieved is ascribed to FB₂ failing to inhibit complexes in the ETC preventing the need for mitophagy. Inhibition of mitophagy is in agreement with the activation of mitochondrial stress responses, decreased ROS production, reduced mitochondrial membrane depolarisation, maintenance of protein clearance and increased proliferation noted. This result has potential consequences in progression of tumour formation and cancer as proliferation is promoted at low concentrations of FB₂.

The overall results suggest that at high concentrations of FB₂, the functional groups are able to act in similar ways as FB₁. This possibly results in inhibition of complexes within the ETC, which causes mitochondrial dysfunction via exacerbated ROS production, derangement in stress responses and ultimately, mitophagy. Lower concentrations however, are unable to do so causing a suppression in mitophagic proteins and thus promoting proliferation.

Mitochondrial dysfunction has been reported as the underlying mechanism for various kidney disorders (Granata et al., 2009). This study reports that FB₂ plays a pivotal role

in inducing dysregulation of mitochondrial function in kidney cells, thus altering the viability of cells. Additionally, FB₂ has been shown to play a role in ROS production. Furthermore, the present study introduces a novel concept of epigenetic regulation in FB₂-induced mitophagy. Despite these findings, further studies need to be carried out to determine the role of FB₂ in mitochondrial dysregulation and ROS production to establish alternative pathways of toxicity.

Chapter 6: Limitations and Recommendations

The current study was a pilot study carried out in an *in vitro* model following 24 hr exposure to varying concentration of FB₂. *In vitro* models generally provide preliminary results, while *in vivo* models provide a more accurate representation of how the toxin would behave in the human body. Furthermore, no data exists to show the effects of FB₂ in humans, thus creating complications in verifying data however, comparisons were drawn to effects of the structural analog FB₁.

Future studies should include longer exposure periods in *in vivo* models to establish pathways of toxicity. Due to comparisons between FB₁ and FB₂, studies should be carried out using both toxins to determine the more toxic compound as well as any synergistic effects that may arise during exposure. This would be particularly interesting as both toxins co-exist in the natural environment increasing the chances of concurrent human exposure.

Chapter 7: Conclusion

The present study exhibiting contrasting results for lower and higher concentrations of FB₂ confirms that the toxin has the ability to selectively promote and inhibit proliferation via interference with mitochondrial stress response function. Therefore, it can be deduced that FB₂ displays biphasic effects (hormesis) characterised by low dose stimulation of cell viability and high dose inhibition of cell viability.

A plethora of mycotoxins have been implicated in the pathogenesis of diseases. It is vital to understand the mechanism by which these fungal secondary metabolites work to induce toxicity especially in humans. Fumonisin are among the most common contaminants of maize however, human studies on the toxic subtype FB₂ are overlooked and limited knowledge is available on the effect of FB₂ on mitochondrial function. Low concentrations of FB₂ (100 µM) induced mitochondrial stress responses therefore maintaining mitochondrial function and preventing mitophagy. This prevented cell death and allowed for cell proliferation alluding to the probable oncogenic properties of the toxin. High concentrations (317.4 µM and 500µM) of FB₂ suppressed mitochondrial stress responses, thus resulting in mitophagy. Furthermore, epigenetic regulation of mitophagy was observed.

FB₂ contamination of maize is highly prevalent. Coincidentally, a large percentage of maize consumers are infected with HIV prompting use of anti-retroviral drugs that induce nephrotoxicity. FB₂ may potentiate nephrotoxicity due to its polarity. The results from this study may provide insights into reducing diet induced nephrotoxicity and may contribute to understanding of the implications of FB₂ toxicity on the health of consumers.

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Appendix A

Ethics Approval- BE336/19



29 May 2019

Ms J Mohan (215024251)
School of Laboratory Medicine and Medical sciences
College of Health Sciences
mxtjivee@gmail.com

Dear Ms Mohan

Protocol: Fumonisin B2 induces mitochondrial stress in Human Embryonic kidney cells (HEK293)
Degree: MMedSc
BREC Ref No: BE336/19

EXPEDITED APPLICATION: APPROVAL LETTER

A sub-committee of the Biomedical Research Ethics Committee has considered and noted your application received 16 April 2019.

Please ensure that site permissions are obtained and forwarded to BREC for approval before commencing research at a site.

This approval is valid for one year from 29 May 2019. To ensure uninterrupted approval of this study beyond the approval expiry date, an application for recertification must be submitted to BREC on the appropriate BREC form 2-3 months before the expiry date.

Any amendments to this study, unless urgently required to ensure safety of participants, must be approved by BREC prior to implementation.

Your acceptance of this approval denotes your compliance with South African National Research Ethics Guidelines (2015), South African National Good Clinical Practice Guidelines (2006) (if applicable) and with UKZN BREC ethics requirements as contained in the UKZN BREC Terms of Reference and Standard Operating Procedures, all available at <http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx>.

BREC is registered with the South African National Health Research Ethics Council (REC-290408-009). BREC has US Office for Human Research Protections (OHRP) Federal-wide Assurance (FWA 678).

The sub-committee's decision will be noted by a full Committee at its next meeting taking place on 11 June 2019.

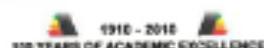
Yours sincerely

Prof V Rambiritch
Chair: Biomedical Research Ethics Committee

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Founding Campuses: Edgewood Howard College Medical School Pietermaritzburg Westville

Appendix B

Standard Curve

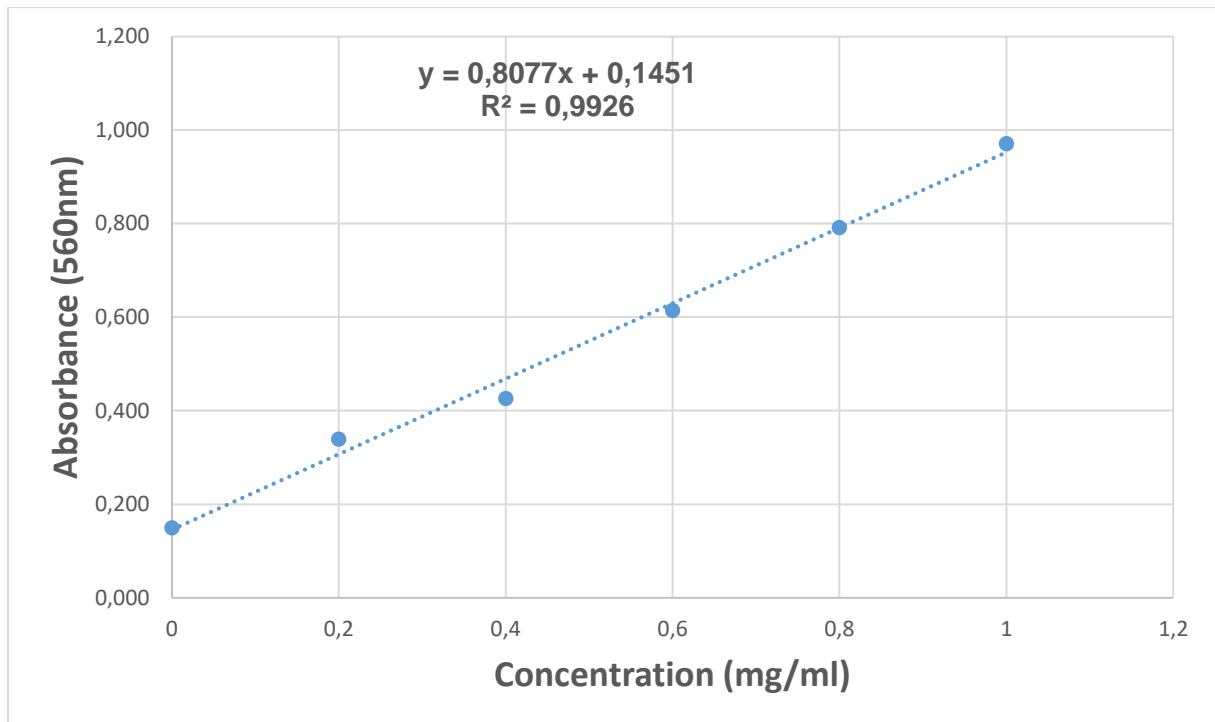


Figure 1: BCA standard curve used for standardising proteins