AFLATOXIN B₁ MODULATES OXIDATIVE STRESS AND APOPTOSIS IN HUMAN EMBRYONIC KIDNEY CELLS

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Submitted in fulfilment of the requirements for the degree of Master of Medical Science in the Discipline of Medical Biochemistry and Chemical Pathology
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Durban, South Africa

2019
DECLARATION

I, Miss Nomali Zanele Dlamini, declare as follows:

1. The research reported in this thesis, except where otherwise indicated, and is my original research.
2. This thesis has not been submitted for any degree or examination at any other university.
3. This thesis does not contain other person’s data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.
4. This thesis does not contain other persons' writing, unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted, then:
   a. Their words have been re-written, but the general information attributed to them has been referenced
   b. Where their exact words have been used, then their writing has been placed in italics, inside quotation marks and referenced.
5. This thesis does not contain text, graphics or tables copied and pasted from the Internet, unless specifically acknowledged, and the source being detailed in the thesis and in the References sections.
6. The research described in this study was carried out in the Discipline of Medical Biochemistry and Chemical Pathology, School of Laboratory Medicine and Medical Sciences, College of Health Sciences, University of KwaZulu-Natal, Howard College Campus, Durban, under the supervision of Dr RB Khan.

Signed: ______________________ 
Date: __________
DEDICATION

Myself

My family

In loving memory of my eldest sister
Sthembiso Mbuyisa
3-01-1983 to 23-11-2013
ACKNOWLEDGEMENTS

I would like to express my heartfelt appreciation to GOD almighty and to the following people:

Dr RB Khan
Words are not enough to describe my appreciation for giving this opportunity. I will always be grateful for the patient guidance, advice and encouragement you have provided for me in the course of this study not only as a supervisor but also as an inspiring female mentor. Thank you for believing in me and for the time you invested in me. I would also like to thank you for teaching me the laboratory techniques, allowing me to be hands on and responding to my queries promptly. I have been extremely lucky to have a supervisor like you.

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UKZN - College of Health Sciences
Thank you for financial support as a post graduate student and for running expenses throughout the course of my Masters.
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<tr>
<td>Ab</td>
<td>Antibody</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
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<tr>
<td>AFs</td>
<td>Aflatoxins</td>
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<td>AFAR</td>
<td>Aflatoxin aldehyde reductase</td>
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<td>Aflatoxin M&lt;sub&gt;2&lt;/sub&gt;</td>
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<td>AFBO</td>
<td>Aflatoxin B&lt;sub&gt;1&lt;/sub&gt;-8,9-epoxide</td>
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<td>AIF</td>
<td>Apoptosis inducing factor</td>
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<tr>
<td>Amp</td>
<td>Amperes</td>
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<td>APAF-1</td>
<td>Apoptotic protease activating factor-1</td>
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<td>ATM</td>
<td>Ataxia telangiectasia mutated</td>
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<td>Adenosine triphosphate</td>
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<tr>
<td>Bax</td>
<td>Bcl-2-associated X</td>
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<td>BCA</td>
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<td>BHT</td>
<td>Butylated hydroxytoluene</td>
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<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>Calcium</td>
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<tr>
<td>CAT</td>
<td>Catalase</td>
</tr>
<tr>
<td>CARD</td>
<td>Caspase-recruitment domains</td>
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<tr>
<td>CCM</td>
<td>Complete culture medium</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CHK</td>
<td>Check point kinase</td>
</tr>
<tr>
<td>c-IAP</td>
<td>Cellular inhibitor of apoptosis</td>
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CO₂  Carbon dioxide
COX  Cyclooxygenase
CuSO₄  Copper (II) sulfate solution
Cyt-c  Cytochrome-c
CYP  Cytochrome P₄₅₀
D  Days
DED  Death effector domain
dH₂O  Distilled water
DISC  Death-inducing signaling complex
DMEM  Dulbecco’s Modified Eagles Medium
DMSO  Dimethyl sulfoxide
DNA  Deoxyribonucleic acid
dNTP  Deoxynucleotide
ds-  Double-stranded
e⁻  Electron
EDTA  Ethylenediaminetetraacetic acid
FADD  Fas-associated death domain
FADH₂  Flavin adenine dinucleotide
FasL  Fatty acid synthetase-ligand
FasR  Fatty acid synthetase-receptor
Fe  Iron
Fe²⁺  Ferrous ion
FCS  Foetal calf serum
G  Gram
G6PD  Glucose-6-phosphate dehydrogenase
GAPDH  Glyceraldehyde-3-phosphate dehydrogenase
GGT  γ-glutamyl transpeptidase
GR  Glutathione reductase
GSH  Glutathione
GSSG  Glutathione disulphide
GPx  Glutathione peroxidase
GST  Glutathione -S- transferase
hr/hrs  Hours
H+     Hydrogen ion/proton
H2O    Water
H2O2   Hydrogen peroxide
H3PO4  Phosphoric acid
HBMECs Human brain microvascular endothelial cells
HCC    Hepatocellular carcinoma
HCl    Hydrochloric acid
Hek293 Human embryonic kidney cells
HRP    Horseradish peroxidase
Hsp70  Heat shock protein 70
Huh7   Epithelial cells of human hepatocellular carcinoma
HUVECs Human umbilical vein endothelial cells
IAP    Inhibitory apoptosis protein
IC     Inhibitory concentration
IC50   Half maximal inhibitory concentration
IgG    Immunoglobulin G
KCl    Potassium chloride
kDa    Kilodaltons
LDH    Lactate dehydrogenase
L-glutamine
LMPA   Low Melting Point Agarose
LOOH   Lipid hydroperoxides
LOX    Lipoxygenases
M      Minute
M      Molar
MDA    Malondialdehyde
Mg     Milligram
MgCl   Magnesium chloride
Ml     Millilitre
mM     Millimolar
Mn     Manganese
MOMP  Mitochondrial outer membrane permeability
mRNA  Messenger ribonucleic acid
MTT  3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NaCl  Sodium chloride
Na₂EDTA  Disodium ethylenediaminetetraacetic acid
NADH  Nicotinamide adenine dinucleotide hydrogen
NADPH  Nicotinamide adenine dinucleotide phosphate
NaNO₃  Sodium nitrate
NaOH  Sodium hydroxide
NEDD  N-(1-naphthyl)ethylenediamine
Ng  Nanogram
NFH₂O  Nuclease free water
NF-κB  Nuclear factor kappa-light-chain-enhancer of activated B-cells
Nm  Nanometer
No  Number
NO  Nitric oxide
NO₂⁻  Nitrite
NO₃⁻  Nitrate
Nrf-2  Nuclear factor-erythroid 2-related factor 2
NAT  N-transferase
O₂  Molecular oxygen/ Singlet oxygen
O₂⁻  Superoxide anion
OD  Optical density
OH  Hydroxyl radicals
ONOO⁻  Peroxynitrite
p53  Protein 53
PARP  Poly (ADP-ribose) polymerase
PBS  Phosphate buffer saline
PCR  Polymerase Chain Reaction
pH  Potential hydrogen
PHS  Phosphatidyl serine
PS  Prostaglandin H synthase
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<th>Full Form</th>
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<tr>
<td>PSF</td>
<td>Penicillin-streptomycin-fungizone</td>
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<tr>
<td>PUFA</td>
<td>Polyunsaturated fatty acids</td>
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<tr>
<td>qPCR</td>
<td>Quantitative polymerase chain reaction</td>
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<tr>
<td>RBI</td>
<td>Relative band intensity</td>
</tr>
<tr>
<td>RFU</td>
<td>Relative fluorescence units</td>
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<tr>
<td>RIP</td>
<td>Receptor-interacting protein</td>
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<tr>
<td>RLU</td>
<td>Relative light units</td>
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<td>ROS</td>
<td>Reactive Oxygen Species</td>
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<tr>
<td>RNS</td>
<td>Reactive nitrogen species</td>
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<tr>
<td>Rpm</td>
<td>Revolutions per minute</td>
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<td>Room temperature</td>
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<td>Seconds</td>
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<td>ss-</td>
<td>Single-stranded</td>
</tr>
<tr>
<td>SA</td>
<td>South Africa</td>
</tr>
<tr>
<td>SCGE</td>
<td>Single Cell Gel Electrophoresis</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
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<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
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<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate polyacrylamide gel electrophoresis</td>
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<td>SEM</td>
<td>Standard error of the mean</td>
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<td>SOD</td>
<td>Superoxide dismutase</td>
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<td>SULF</td>
<td>Sulfanilamide</td>
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<tr>
<td>TBA</td>
<td>Thiobarbituric acid</td>
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<tr>
<td>TBARS</td>
<td>Thiobarbituric acid reactive substances</td>
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<tr>
<td>TCA</td>
<td>Tricarboxylic acid</td>
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<tr>
<td>TEMED</td>
<td>Tetremethylethlenediamine</td>
</tr>
<tr>
<td>Tris</td>
<td>Trisaminomethane</td>
</tr>
<tr>
<td>Tris-HCl</td>
<td>Trisaminomethane hydrochloride</td>
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<tr>
<td>TRAIL</td>
<td>TNF-Related Apoptosis-Inducing Ligand</td>
</tr>
<tr>
<td>TRADD</td>
<td>TNF receptor-associated death domain</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>TNFL</td>
<td>TNF-ligand</td>
</tr>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>TNFR</td>
<td>TNF- receptors</td>
</tr>
<tr>
<td>TTBS</td>
<td>Tris-buffer Saline Tween-20</td>
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<tr>
<td>UKZN</td>
<td>University of KwaZulu-Natal</td>
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<tr>
<td>V</td>
<td>Voltage</td>
</tr>
<tr>
<td>VCl₃</td>
<td>Vanadium (III) chloride</td>
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<td>Zn</td>
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ABSTRACT

Introduction: Aflatoxin B₁ (AFB₁) is produced by filamentous fungal strains of *Aspergillus flavus* and *Aspergillus parasiticus* that infect field crops, therefore AFB₁ is a frequent contaminant of dietary staples such as rice, maize and peanuts. Humans and animals are exposed to AFB₁ through consumption of contaminated foods, predisposing them to various diseases. AFB₁ is a potent hepatotoxin that has been classified by the International Agency of Research on cancer (IARC) as a group 1 carcinogen. The carcinogenic effects of AFB₁ have been attributed to the metabolism of this toxin to an epoxide that promotes the production of free radicals, mitochondrial toxicity and induction of cell death. With the increasing prevalence of kidney associated diseases in humans, and the AFB₁-associated kidney toxicity observed in animals, this study investigated the cytotoxic effects/mechanism of AFB₁ in human embryonic kidney (Hek293) cells.

Methods: Hek293 cells were exposed to AFB₁ (0-100 µM) for 24hrs. The effect on cell viability was assessed using the methylthiazol tetrazolium (MTT) assay, which also produced the half maximal inhibitory concentration (IC₅₀) used in subsequent assays. Free radical production was evaluated by quantifying malondialdehyde (MDA) and nitrate concentration, while DNA fragmentation was determined using the single cell gel electrophoresis (SCGE) assay and DNA gel electrophoresis. Damage to cell membranes was ascertained using the lactate dehydrogenase (LDH) assay. The concentration of ATP, reduced glutathione (GSH), necrosis, annexin V and caspase activity was measured by luminometry. Western blotting and quantitative PCR was used to assess the expression of proteins and genes associated with apoptosis and oxidative stress.

Results and discussion: The MTT assay revealed a reduction in cell viability of Hek293 cells as the AFB₁ concentration was increased, with a half maximum inhibitory concentration (IC₅₀) of 32.60 µM. The decreased viability corresponded to decreased ATP concentration. The upregulation of Hsp70 indicated that oxidative stress was induced in the AFB₁-treated cells. While this implies an increased production of free radicals, the accompanying upregulation of the antioxidant system indicates the activation of defense mechanisms to prevent cellular damage. Thus, membrane damage associated with increased radical formation was prevented as indicated by the reduced LDH release and necrosis. In addition, cytotoxic effects were evident as AFB₁ activated the intrinsic pathway of apoptosis with corresponding increased DNA fragmentation, p53 and Bax upregulation and increased caspase activity, but externalisation of phosphatidylserine (PS), a major hallmark of apoptosis, did not occur in AFB₁ treated Hek293 cells.
Conclusion: The results suggest that AFB\textsubscript{1} induced oxidative stress leading to cell death by the intrinsic pathway of apoptosis in Hek293 cells.

Keywords: Aflatoxin B\textsubscript{1} (AFB\textsubscript{1}), oxidative stress, apoptosis, Hek293 cells
CHAPTER 1 : INTRODUCTION

1.1 BACKGROUND

Fungi belonging to *Aspergillus*, *Penicillium* and *Fusarium* genera are frequent contaminants of a variety of agricultural commodities that form part of the South African staple diet (da Rocha *et al.*, 2014, Alshannaq and Yu, 2017, Misihairabgwi *et al.*, 2019). These contaminating fungi produce toxic low molecular weight secondary metabolites (mycotoxins) due to compromised environmental conditions such as high temperatures and increased humidity (Liu and Wang, 2016). Mycotoxins persist in food despite cooking and processing, and consumption of contaminated food commodities can cause diseases and death in humans and animals (da Rocha *et al.*, 2014).

Aflatoxins are mycotoxins that are produced by the filamentous fungi *Aspergillus flavus* and *Aspergillus parasiticus* that are found on peanuts, wheat, corn, beans and rice products (de Oliveira and Corassin, 2014, Alshannaq and Yu, 2017). It is postulated that aflatoxin B$_1$ (AFB$_1$) is the most potent member of the aflatoxin family, and has been classified as a group 1 carcinogen by the International Agency for Research on cancer (IARC) (Chen *et al.*, 2016). The carcinogenic effects of AFB$_1$ are based on its metabolism; AFB$_1$ is bioactivated to a potent AFB$_1$-8,9-epoxide by cytochrome P$_{450}$ (CYP), but this can be detoxified by the phase 2 glutathione (GSH) pathway (Kumar *et al.*, 2017, Deng *et al.*, 2018). The AFB$_1$-8,9-epoxide targets guanine residues of DNA forming adducts that result in well-known mutations associated with adduct formation, most notably the Ras oncogene and p53. The epoxide also forms adducts at lysine residues of proteins, as observed with albumin, the albumin-lysine adduct is used as a biomarker for AFB$_1$ exposure (Rushing and Selim, 2018).

Aflatoxin B$_1$ exposure is implicated in hepatocellular carcinoma and increased lung cancer risk. Cytotoxic effects related to these organs have been demonstrated in human hepatocellular carcinoma (HepG2) cells (Liu *et al.*, 2014, Li *et al.*, 2017) and human bronchial epithelial (BEAS-2B) cells (Zhang *et al.*, 2014). Oxidative stress is one mechanism that may pre-dispose to AFB$_1$-induced cell injury, because free radical damage to DNA and protein are associated with carcinogenesis. AFB$_1$ exposure is also linked to aberrant cell death mechanisms especially apoptosis. In addition to its carcinogenic capability, previous studies have reported that AFB$_1$ is
also mutagenic, genotoxic and immunotoxic to mammals (Kumar et al., 2017). Studies also demonstrate AFB₁ to be nephrotoxic and induce renal damage in rats, and suggests that prolonged exposure has the potential to induce kidney disease (Abdel-Hamid and Firgany, 2015, Kumar et al., 2017, Asa et al., 2018, Yılmaz et al., 2018). However, the biochemical mechanism underlying the mechanism of AFB₁ effects in human kidney has not been elucidated.

Kidney damage in rats is not surprising since the kidney is the principal route for excretion of biotransformed water-soluble metabolites (Pizzorno, 2015). Drugs and metabolites are selectively taken up and concentrated by the renal tubular cells before excretion in the urine, therefore high intracellular concentrations of toxic substances may be retained mainly in the renal medulla (Pizzorno, 2015, Câmara et al., 2017). As a result, direct toxic damage to renal tubules and renal papillae may predispose to kidney disease (Câmara et al., 2017).

Apart from a variety of physiological functions performed by the kidneys, they also possess biotransformation capabilities. The kidneys have been reported to express metabolizing enzymes cytochrome P₄₅₀ (CYP) including CYP2B6 which is involved in the metabolism of diverse environmental chemicals (Zanger and Schwab, 2013). In addition, CYP3A4 and CYP3A5 are also expressed in the kidney; it has been reported that CYP3A4 and CYP3A5 are involved in AFB₁ metabolism (Knights et al., 2013, Zanger and Schwab, 2013).

Toxic phase 1 intermediates require conjugation with GSH. The kidneys are reliant on an adequate GSH supply to function normally. This is based on the exposure to high concentrations of oxidants and reactive electrophiles (Pizzorno, 2015). GSH protects against oxidative stress which is essential for mitochondrial and cellular homeostasis and aids in the detoxification of xenobiotics. Detoxification of chemicals involves conjugation with GSH to form GSH adducts that are considered less toxic and can be excreted from cells via the mercapturic acid pathway (Pizzorno, 2015).

1.2 PROBLEM STATEMENT
Wheat and maize are a major part of the diet, and are considered the most staple foods in South Africa (Mishairabgwi et al., 2019). Maize and its products are highly prone to mycotoxin contamination both in the field and in storage. One of the potent mycotoxins elaborated, AFB₁, is
considered the most toxic amongst mycotoxins and has been reported to possess carcinogenic, mutagenic and hepatoxic effects, which confer major health risks to humans and animals. Moreover, it has been linked to the development of hepatocellular carcinoma (Deng et al., 2018) and nephrotoxic effects in rat kidney (Abdel-Hamid and Firgany, 2015, Asa et al., 2018, Yilmaz et al., 2018). Several studies using human cell lines have indicated aberrant apoptosis and oxidative stress as mechanisms for AFB1-mediated cytotoxicity. Cytotoxicity to human lymphocytes by the induction of apoptosis and necrosis in human lymphocytes has been reported by Al-Hammadi (2014)(Al-Hammadi et al., 2014). Pro-apoptotic activity of AFB1 was also demonstrated in HepG2 and BEAS-2B cells (Liu et al., 2014, Zhang et al., 2014, Li et al., 2017). AFB1 has also demonstrated cytotoxicity to human peripheral blood mononuclear cells (PBMCS) by impairment of oxidative status (Bernabucci et al., 2011). The biochemical mechanisms underlying the effects of AFB1 in kidney cells has not been investigated in humans. Considering the essential role of the kidney as an excretory organ in the body, the effects of AFB1 in kidney cells requires investigation.

1.3 AIM OF THE STUDY
This study aimed to determine the cytotoxic effects of AFB1 on human embryonic kidney (Hek293) cells.

1.4 HYPOTHESIS
Null hypothesis: Acute exposure (single dose in 24 hours) to AFB1 has no effect in Hek293 cells.
Alternate hypothesis: Acute exposure (single dose in 24 hours) to AFB1 induces oxidative stress and apoptosis in Hek293 cells.

1.5 OBJECTIVES OF THE STUDY
This study was conducted to determine the

- changes in cell viability after administration of various AFB1 concentrations in Hek293 cells to derive the IC50
- effect of AFB1 on production of free radicals in Hek293 cells
- antioxidant responses induced by AFB1 in Hek293 cells
- cell death pathways triggered by AFB1 in Hek293 cells
CHAPTER 2 : LITERATURE REVIEW

This study investigated the effects of aflatoxin B$_1$ (AFB$_1$) on Hek293 cells. The literature review established what is known about the mycotoxin AFB$_1$ which colonizes various food commodities and is a health concern worldwide. Given the challenges posed by AFB$_1$ in food toxicology, food security and public health, this chapter covers what has been reported on the effects of AFB$_1$ on human and animal health. It also establishes the gap in literature, since AFB$_1$ effects in the human kidney have not been investigated.

2.1 MYCOTOXINS

Mycotoxins are toxic low molecular weight (below 700kDa) secondary metabolites produced by a variety of fungi or mould. There are over 400 different mycotoxins, with new ones still being classified (da Rocha et al., 2014, Yao et al., 2015). The genera Aspergillus, Fusarium, Penicillium and Alternaria are deemed as the most mycotoxin producing fungi (da Rocha et al., 2014, Carvajal-Moreno, 2015). The synthesis of mycotoxins by these toxigenic species depends extensively on the internal parameters of fungal strains (biochemical, physical and genetic), but also on external factors such as humidity and temperature (Misihairabgwi et al., 2019, Zinedine and El Akhdari, 2019). Therefore, mycotoxins are produced when fungi reach maturity; and their production can occur in the field, before harvest, post-harvest, during processing, packaging, distribution and storage in different grains and agricultural commodities (Liu and Wang, 2016).

The occurrence of mycotoxins in human and animal food is a significant problem adversely affecting food quality in developing countries as well as developed countries (da Rocha et al., 2014, Alshannaq and Yu, 2017). The detrimental effects include a decrease in nutritional value of food and reduced yields in crop and livestock. It has been reported that approximately 25-50% of products produced worldwide, particularly basic foodstuffs are contaminated with mycotoxins (da Rocha et al., 2014, Misihairabgwi et al., 2019). Most mycotoxins are not affected by food processing; for example cooking and they can be converted to toxic intermediates by metabolism in humans and animals (Alshannaq and Yu, 2017). In addition, the metabolism of mycotoxins to their toxic form can potentially pose risks to human health, resulting in disease and death in mammals.
Exposure to mycotoxins occurs when fungal infected crops are directly consumed by livestock, for example wheat, corn, oats and sorghum (Alshannaq and Yu, 2017). However, indirect contamination may occur when the toxigenic fungus has been eradicated during processing, but the mycotoxins remain in the final product. The main route of exposure to mycotoxins in humans is through the ingestion of contaminated plant and animal products, including food derived from these products such as cheese, milk and meat (Figure 2.1) (da Rocha et al., 2014, Alshannaq and Yu, 2017). However, inhalation and dermal contact may also be important routes of exposure. Direct effects of mycotoxins range from acute disease where exposure to the toxin may result in severe health conditions prior to death, these conditions are expected after exposure to high levels of mycotoxin (da Rocha et al., 2014, Alshannaq and Yu, 2017, Zinedine and El Akhdari, 2019). Chronic disease manifestations may result from prolonged exposure to small quantities of the toxin. The diseases resulting from mycotoxin exposure are known as mycotoxicosis. Mycotoxicosis usually remain unrecognized except when large numbers of people are affected with no apparent connection to a known aetiological agent (Alshannaq and Yu, 2017, Zinedine and El Akhdari, 2019).

**Figure 2.1** : The main route of exposure to mycotoxins humans and animals (Prepared by author).
‘Turkey-X disease’ marked a breakthrough in the association of mycotoxin-contamination with disease in animals. The plague occurred in the early 1960’s, and was marked by the death of turkey poults in England after consumption of a peanut meal that originated from Brazil and Africa (Alshannaq and Yu, 2017). The secondary metabolite responsible for the death of the birds was produced by *Aspergillus flavus* and studies of these toxins ensued. The genus *Aspergillus*, first described in 1729 by Pier Antino Micheli, is more common in warmer tropical and subtropical areas than in moderate areas of the world. *Aspergillus* species are filamentous saprophytic fungi that can produce active compounds like aflatoxins, penicillic acid, ochratoxins, citrinin, sterigmatocystin and other important metabolites (Yao *et al*., 2015, Misihairabgwi *et al*., 2019).

### 2.2 AFLATOXINS

Aflatoxins (AFs) are a class of structurally related toxic metabolites produced by the fungal strains *Aspergillus flavus* and *Aspergillus parasiticus* that are normally found in soil and organic materials (Kumar *et al*., 2017). There are approximately 20 known aflatoxins that are frequently found as contaminants in maize grains, cereals, peanuts, rice and oil seeds (de Oliveira and Corassin, 2014). There are favourable conditions for aflatoxin production, these include high moisture during harvest, dry weather near crop maturity and insufficient drying and storage of crops (Figure 2.2). Post harvesting conditions such as storage (long periods of storage, excess heat and moisture, pest related damage) and food processing also influences the production of aflatoxins (Figure 2.2). The identification of aflatoxins was linked to the discovery that a ground nut contaminated with *Aspergillus flavus* lead to a turkey disease that killed approximately 100 000 turkey poultry birds in England (da Rocha *et al*., 2014, Yao *et al*., 2015). Aflatoxins therefore pose a threat to human and animal health, their toxicity can result in kidney and liver damage, cause carcinogenic, mutagenic and immunosuppressive effects (Carvajal-Moreno, 2015).
2.2.1 Physical and chemical properties of aflatoxins

Aflatoxins are colourless to pale yellow crystalline solids with low molecular weight and display fluorescence under UV light. Aflatoxins are unstable under UV light in the presence of oxygen and extreme pH levels (<3 or >10). Under alkaline conditions the lactone ring opens and aflatoxins are destroyed, but this is a reversible reaction upon acidification (Kumar, 2018). Ammoniation causes irreversible opening of the lactone ring at high temperatures and pressure resulting in decarboxylation of aflatoxins (Kumar, 2018). Aflatoxins have high decomposition temperatures ranging from 237°C to 320°C; this makes them stable at temperatures present when cooking or boiling food, milk and alcoholic fermentation (Bbosa et al., 2013c) and their destruction is not easily achieved. They are slightly soluble in water and easily soluble in moderately polar solvents such as methanol, chloroform and dimethyl sulfoxide (de Oliveira and Corassin, 2014, Kumar, 2018). Due to the lipid solubility, aflatoxins are readily absorbed from the site of exposure, across the cell membranes into the blood stream. They are then distributed to various tissues or organs especially the liver where they are biotransformed (Kumar, 2018).
2.2.2 Types of aflatoxins

Of the approximately 20 known aflatoxins, only aflatoxin B₁ (AFB₁), AFB₂, AFG₁, AFG₂, AFM₁ and AFM₂ (Figure 2.3) are usually found in foods. The B and G denotes the blue and green fluorescent colours produced under ultraviolet light, while the subscript numbers 1 and 2 indicate the major and minor compounds respectively (Deng et al., 2018, Kumar, 2018). Less toxic oxidative metabolites of aflatoxin produced by farm animals after ingestion of contaminated feed include AFM₁, AFM₂, AFG₁ and AFG₂ (Figure 2.3). Even though the metabolites are less toxic than the parent compound, AFM₁ in dairy milk can cause liver illness in humans especially among infants and children (Kumar, 2018). The toxicity of aflatoxins decreases in the order B₁>G₁>B₂>G₂.

![Figure 2.3: Different types of aflatoxins](image)

Figure 2.3: Different types of aflatoxins, AFB₁ (Mw=312.27g/mol), AFB₂ (Mw=314.29g/mol), AFG₁ (Mw=328.276g/mol), AFM₁ (Mw=328.276g/mol), AFM₂ (Mw=330.29g/mol), AFG₂ (Mw=330.29g/mol) (Musleh et al., 2017).

2.3 AFLATOXIN B₁

Aflatoxin B₁ (AFB₁) is found as a contaminant in peanuts and grains such as maize, sorghum and rice. It is the most potent of the aflatoxin family and has been reported to have mutagenic, hepatocarcinogenic, teratogenic and genotoxic abilities, it causes immunosuppression in animals and can influence how cells are produced in the body (Liu and Wang, 2016). It has been classified
as a group 1 carcinogen in humans by the WHO-international agency for research on cancer (Al-Ouqaili, 2018). Although AFB₁ mainly targets the liver, it also affects other organs and tissue, including lungs and kidney.

2.3.1 Structure
Aflatoxin B₁ (C₁₇H₁₂O₆) has a difuro-coumaro-cyclo pentenone structure fused with a lactone ring of the coumarin structure with oxygen (O₂) at positions 1, 4 and 11 (Marchese et al., 2018). The difuro terminus of this compound has a 2,3 double bond which is essential for the carcinogenicity of this toxin (Figure 2.4). On condition that the bond is single, the carcinogenic properties of AFB₁ are lost but toxicity is still retained (Kumar, 2018, Marchese et al., 2018).

![Chemical Structure of Aflatoxin B₁](image)

Figure 2.4 : The chemical structure of aflatoxin B₁ (C₁₇H₁₂O₆) (Hedström and Mattiasson, 2015).

2.3.2 Metabolism

2.3.2.1 The cytochrome P450 (CYP) enzyme system
Cytochrome P₄₅₀ (CYP) enzymes are so named because they are bound to membranes within a cell (cyto) and comprise of a haem pigment (chrome and P) that absorbs light at a wavelength of 450nm when exposed to carbon monoxide (Guengerich, 2015). Each CYP gene is assigned a number that relates it to a specific group, a letter that represents the gene’s subgroup and a number given to the specific gene within the group (Guengerich, 2015). For example, the cytochrome P₄₅₀ gene found in group 3, subgroup A and gene 1 will be written as CYP3A1. There are about 60 CYP genes in
humans (Raunio et al., 2015). The CYP family play a role in the synthesis and metabolism of numerous endogenous molecules and chemicals including cholesterol, steroid hormones and bile acids. They also break down xenobiotics like ingested medication and toxins found within cells (Knights et al., 2013, Raunio et al., 2015).

2.3.2.2 Metabolism of AFB1

The bioactivation and detoxification of AFB1 in the body requires the activity of metabolizing enzymes (Figure 2.3). The CYP family is responsible for AFB1 bioactivation in the liver and lipoxygenases (LOX) and prostaglandin H synthase (PHS) are necessary for its biotransformation in the lung (Bbosa et al., 2013c, Deng et al., 2018). In poultry species the CYP2A6, CYP3A37, CYP1A1 and CYP1A5 are responsible for AFB1 bioactivation. Human CYP1A2 and CYP3A4 in the liver and CYP3A5 and CYP2B6 in the kidney play a role in the activation of AFB1 to a reactive toxic metabolite, aflatoxin B1-8,9-epoxide (AFBO) which facilitates its carcinogenic and cytotoxic effects (Knights et al., 2013, Liu and Wang, 2016).

The conjugation of AFBO with GSH is a major route of detoxification leading to the excretion of the epoxide (Figure 2.5). Conjugation with GSH disrupts AFBO ability to bind to DNA and proteins forming an inert metabolite that is then converted to a mercapturic acid adduct through a series of enzymatic reactions including γ-glutamyl transpeptidase (GGT), dipeptidase (PEP) and N-acetyltransferase (NAT) and then can be excreted in urine (Rushing and Selim, 2018). This is an important pathway of for detoxification of AFB1 as a carcinogen even though depletion of GSH results in high levels of reactive oxygen species (ROS) causing oxidative damage (Marchese et al., 2018, Rushing and Selim, 2018).

Figure 2.5 : The biotransformation of AFB1 by enzymes CYP3A4/3A5 to form a reactive AFB1-8,9-epoxide with a functional group denoted in red. The epoxide can conjugate with GSH in a reaction catalysed by the enzyme glutathione-S-transferase (GST) for protective effects forming an AFB1 glutathione conjugate that can be detoxified thus minimizing the toxicity of AFB1 (Stewart et al., 1999).
2.3.3 Biochemical effects of AFBO

2.3.3.1 DNA
The detrimental effects of AFB₁ have been attributed to the metabolism of this toxin. The reactive metabolite AFBO reacts with the N7 atom of guanine and forms a pro-mutagenic DNA adduct aflatoxin-N7-guanine which can cause gene mutations resulting in damage to DNA (Figure 2.6) (Bbosa et al., 2013c). The most common mutation found in human hepatocytes exposed to AFB₁ is a G-T transversion on codon 249 of the p53 gene. The tumour suppressor protein p53 regulates various cellular functions including DNA repair, cell cycle progression and apoptosis (Marchese et al., 2018, Rushing and Selim, 2018). Many cancers including hepatocellular carcinoma (HCC) possess mutations in p53 which is thought to alter the tumour suppressive functions of p53 leading to formation of cancerous cells (Marchese et al., 2018). Epidemiological studies in humans in aflatoxin risk regions have revealed a positive correlation between p53 mutations and aflatoxin presence in HCC patients, giving strong evidence that AFB₁ exposure results in HCC development in humans by mutating p53 (Szymańska et al., 2004, Kirk et al., 2005, Rushing and Selim, 2018). In addition, it has been reported that AFBO exerts an inhibitory effect on the biological processes including DNA synthesis, DNA repair, DNA-dependent RNA synthesis and protein synthesis (Deng et al., 2018, Marchese et al., 2018).

Figure 2.6: The reactive metabolite AFBO can react with guanine bases at the N7 atom in DNA to form the AFB₁-N7-dG adduct that can potentially cause gene mutations resulting in DNA damage and can be the onset of many diseases (Stewart et al., 1999).


2.3.3.2 Proteins

The reactive metabolite AFBO has a highly unstable nature, which can cause the metabolite to spontaneously hydrolyse before binding to any macromolecules resulting in the formation of AFB1-dihydrodiol which is in equilibrium with an open ring aldehyde called AFB1 aldehyde (Figure 2.7) (Roebuck et al., 2009, Bbosa et al., 2013c, Rushing and Selim, 2018). The AFB1 aldehyde can form Schiff base derived adducts in cellular proteins by covalently binding to the N-termini and lysine side chains forming AFB1 lysine adducts (AFB1-lys) and these proteins adducts have been implicated in AFB1 acute toxicity (Figure 2.7) (Roebuck et al., 2009, Rushing and Selim, 2018). The AFB1 aldehyde can undergo further metabolism by aflatoxin aldehyde reductase (AFAR) into AFB1 dialcohol eliminating its protein binding potential (Figure 2.7). Protein binding of AFBO and AFB1 aldehyde usually occurs on serum albumin, which has a half of approximately 20 days making it a reliable biomarker of AFB1 exposure (Rushing and Selim, 2018).

![Image]

Figure 2.7 : The mechanism by which the AFB1 epoxide enhances its biochemical effects on proteins by binding to lysine residues resulting in adduct formation, hence protein binding. The reduction enzymatic activity of aflatoxin-aldehyde reductase (AFAR) reducing AFB1-dihydrodiol to AFB1-dialcohol limits binding of the epoxide to proteins reducing it effects (Prepared by author).

2.3.3.3 Lipids

The accumulation of aflatoxins and its metabolites have been reported to induce lipid peroxidation in cells resulting in cell membrane damage (Bbosa et al., 2013c). Lipid peroxidation is described
as a process in which oxidants such as free radicals attack lipids comprising of carbon-carbon double bond(s) especially polyunsaturated fatty acids (PUFAs) (Ayala et al., 2014). This process involves the abstraction of hydrogen from a carbon with an insertion of oxygen resulting in lipid peroxyl radicals and hydroperoxides (Figure 2.8). In response to lipid peroxidation cells may promote survival or induce cell death. Under minimal lipid peroxidation rates cells, stimulate an antioxidant response for their maintenance and survival whereas at high lipid peroxidation rates the extent of oxidative damage overwhelms the cells repair capacity and cells may activate cell death signalling pathways (Ayala et al., 2014). Lipid peroxidation can result in the formation of many oxidation products including malondialdehyde (MDA), lipid hydroperoxides (LOOH), propanal and 4-hydroxynonenal (4-HNE) (Ayala et al., 2014).

![Figure 2.8 : The peroxidation of lipids in cells adapted from (Basu, 2007).](image)

The accumulation of the AFBO metabolite depletes glutathione, an antioxidant which is used as part of the detoxification process. The depletion of GSH leads to the accumulation of abnormally high concentration of free radicals in cells, thus affecting cell membrane integrity (Bbosa et al.,
Deficiency in GSH also enhances damage to essential cellular macromolecules including DNA and proteins (Bbosa et al., 2013b, Rushing and Selim, 2018).

2.4 OXIDATIVE STRESS

Humans and animals require energy to survive. The production of this energy is dependent on mitochondria which utilise the oxygen (O$_2$) breathed in to produce energy in the form of ATP. To facilitate this activity, mitochondria have an electron transport chain (ETC) that passes electrons through a series of complexes, generating a hydrogen gradient that culminates in the reduction of O$_2$ to ATP and water (Yong et al., 2019). This activity can result in the production of highly reactive metabolites known as free radicals within cells. Free radical production can induce oxidative stress when there is an imbalance between the production of reactive oxygen species (ROS) and the biological ability of antioxidants to clear the toxicity of the reactive metabolites (Di Meo et al., 2016). This imbalance has been considered as an important molecular mechanism underlying cell injury that can result in irreparable oxidative injury and even cell death when it gets beyond control.

2.4.1 Production of free radicals

Studies have reported that there are two sources of ROS production; the endogenous such as oxygen leakage from the mitochondria during oxidative phosphorylation and exogenous including xenobiotics, chlorinated compounds, pathogens and inflammatory cytokines. The major endogenous ROS in living cells is the superoxide anion radical (O$_2^-$). Other ROS derived from the metabolism of molecular oxygen include singlet oxygen (O$_2$), hydrogen peroxide (H$_2$O$_2$), peroxynitrite (ONOO$^-$) and the highly reactive hydroxyl radical (·OH) (Figure 2.9) (Ling and Kuo, 2018). Deleterious effects of oxygen result from its metabolic reduction to these highly reactive and toxic species.

Reactive oxygen species are not always harmful metabolic by-products; they normally exist in all aerobic cells as mediators in many biological processes (Liu and Wang, 2016). When tightly regulated they can act as intracellular signalling molecules, but a critical imbalance resulting from excess ROS production, antioxidant depletion or both can result in oxidative stress (Liu and Wang, 2016).
2.4.2 Antioxidant response

Redox balance is required in cells, therefore an antioxidant defence system consisting of antioxidant enzymes is provided. The expression of antioxidant enzymes such as superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPx) is tightly controlled by antioxidant response elements (AREs) and activated nuclear related factor-2 (Nrf-2), which is a redox sensitive transcription factor (Liu and Wang, 2016). The Nrf-2 promotes the induction of target genes such as glutathione-S-transferases (GST), glutathione reductases (GR) and GPx that are responsible for cell survival. When cells are subjected to ROS, Nrf-2 is activated as an adaptive response to protect cells from impending oxidative stress; it translocates to the nucleus where it transactivates genes that encode different antioxidant enzymes, which act to maintain cell homeostasis (Liu and Wang, 2016).

Superoxides are scavenged by SOD in a dismutation reaction that converts $O_2^-$ to $H_2O_2$ and $O_2$ (Figure 2.9, 2.10). Superoxide anions can also react with nitric oxide (NO) to produce ONOO$,\cdot$, the instigator of nitrosative stress (Figure 2.10). Hydrogen peroxide may be converted into water by the enzymes catalase or GPx and inductive changes or variability in the expression of these
enzymes can influence cellular redox potential (Figure 2.10) (Ling and Kuo, 2018). However, Nrf-2 activation by ROS limits the capacity of cells to escape from injury because they also stimulate cell death signals leading to the execution of apoptosis (Liu and Wang, 2016).

Figure 2.10: The scavenging and protective effects of antioxidants against ROS (Sznarkowska et al., 2017)

It has been reported that CYP is a key enzymatic complex in the mitochondria associated with electron leakage, which is one of the important sources of intracellular ROS generation, thus implicating CYP enzymes in the production of ROS. Therefore, bioactivation of AFB₁ by the CYP enzymes results in an increase in ROS production and the generated reactive species can result in lipid peroxidation of membranes and oxidative modification of proteins and DNA (Liu and Wang, 2016). In addition, the accumulation of AFB₁ and its metabolites in the body especially the AFBO depletes the GSH which is an important antioxidant that prevents cellular damage caused by ROS (Carvajal-Moreno, 2015). The depletion of GSH is therefore due to the formation of high amounts of epoxides and ROS in the body resulting in oxidative stress. The expression of GSH could increase protection against AFB₁-induced hepatocarcinogenesis (Liu and Wang, 2016).
Excessive ROS can attack lipids, protein and DNA, and induce oxidative injury that inactivates biological activity of these important cellular macromolecules (Di Meo et al., 2016, Elwahab, 2017). The increase in ROS also reduces the cleaning ability of antioxidants such as SOD, GPx, glutathione reductase (GR) and non-enzymatic antioxidants like GSH resulting in oxidative stress (Elwahab, 2017).

2.5 MECHANISMS OF CELL DEATH

2.5.1 Apoptosis

Apoptosis is a form of programmed cell death that occurs in multicellular organisms in balance with cell division. It is a highly regulated process that serves to eliminate unwanted /unnecessary cells. Apoptosis is carried out by a family of protease enzymes called caspases (cysteine aspartyl-specific proteases) which cleave target proteins; their activity is essential for successful apoptosis (Pfeffer and Singh, 2018). Apoptosis occurs in two main pathways, viz. the intrinsic or mitochondrial pathway, and the extrinsic or death receptor pathway (Figure 2.6). These pathways are linked and the molecules used in one pathway can influence the other (Li et al., 2018).

The extrinsic pathway of apoptosis occurs when a death signal binds to a death receptor; the known death receptors are TNF receptor (TNFR1) and a related protein called Fas (CD95), and their ligands are TNF ligand (TNFL) and Fas ligand (FasL) respectively (Figure 2.11). The death receptors contain intracellular death domains that recruit adaptor proteins, a TNF associated death domain (TRADD) and a Fas-associated death domain (FADD), as well as a cysteine aspartate protease like caspase 8. Binding of the ligand to the death receptor results in the activation of death domains and recruitment of adaptor proteins forming a death inducing signalling index (DISC) complex (Liu and Wang, 2016). The DISC complex initiates the activation of pro-caspase 8 to caspase 8, an initiator caspase that initiates apoptosis by cleaving other downstream executioner caspases (Figure 2.11) (Pfeffer and Singh, 2018).

The intrinsic pathway results when a signal received from signalling molecules such as ataxia telangiectasia mutated (ATM) and check point kinase (CHK) activate the p53 gene. p53 recruits proteins such as Bax/Bak to increase mitochondrial permeability by forming pores that allow the release of pro-apoptotic molecules including cytochrome c into the cytosol (Pfeffer and Singh, 2018). The intrinsic pathway can also be initiated by caspase 8 when it cleaves Bid to t-Bid, thus
facilitating Bax activation (Figure 2.11). Cytochrome c binds to Apaf-1 in the cytosol and forms a wheel-like heptamer that contains caspase activation and recruitment domains (CARDs); pro-caspase 9 binds its respective CARD domain and becomes activated into caspase 9 (Figure 2.11).

Caspase 8 and caspase 9 are both able to cleave and activate pro-caspase 3 to caspase 3, an effector caspase (Figure 2.11). Caspase 3 directs the changes cell morphology that are hallmarks of apoptosis, including cell shrinkage, membrane blebbing, DNA fragmentation and condensation, and formation of apoptotic bodies that are removed by phagocytes (Pfeffer and Singh, 2018).

Figure 2.11: The intrinsic and extrinsic pathways of apoptosis (Rampal et al., 2012)

Apoptosis is a self-managed operation that kills damaged cells and maintains minimal damage to surrounding tissues. Defective apoptotic processes have been reported in various diseases and it
has been reported that AFB$_1$ was able to induce apoptosis in hepatocytes, bone marrow, lung cells or human bronchial epithelial cells (Chen et al., 2016).

2.5.2 Necrosis

Necrosis is a form of cell death that is considered accidental, unprogrammed or due to cell injury and occurs when ATP is depleted (Jog and Caricchio, 2014). This form of cell death results from different stimuli such as cellular stress, heat, osmotic shock, high concentrations of H$_2$O$_2$ and toxin exposure that initiate changes at the cellular level. Under pathological conditions an increase in ROS produced by the mitochondria can induce damage in biomolecules which can lead cells towards necrosis.

The morphological characteristics of necrosis include swelling of the cell membrane, chromatin condensation and irregular DNA degradation (Figure 2.12) (Escobar et al., 2015). The increased cellular swelling results in the breakdown of plasma membranes which releases cytoplasmic contents into the extracellular space (Figure 2.12). The necrotic process occurs in the absence of phagocytosis and its final stage is mainly characterized by the loss of cell membrane integrity (Escobar et al., 2015).

Figure 2.12 : The morphological characteristics of necrotic cell death (Escobar et al., 2015).
2.6 EFFECTS OF AFB₁

2.6.1 Multiple organs and cell lines
Aflatoxin B₁ (AFB₁) has reported to affect several body organs including the liver, brain, heart skeletal muscle and different body systems. AFB₁ exposure is associated with enlargement of the liver, congestion of the liver parenchyma, necrosis of hepatocytes and liver cancer (Hussain et al., 2008). It has also been reported that AFB₁ induced a decrease in protein content in the liver, heart, and skeletal muscle in animals due to its ability to inhibit metabolic systems such as protein synthesis thus leading to liver and heart damage (Wangikar et al., 2005). People working in food industries get exposed to AFB₁ when they inhale contaminated dust during processing; this may lead to lung cancer (Van Vleet et al., 2002, Zhang et al., 2014). Studies with experimental animals have demonstrated that AFB₁ induces pulmonary adenomas and tumours in both the peripheral and central nervous system (Coulombe Jr, 1994). AFB₁ is also known to alter neurotransmitters and their precursors in mouse brains (Coulombe Jr, 1994).

It has been reported that the consumption of AFB₁ diets induced an increase in MDA levels and a reduction in GSH levels resulting in oxidative stress in the spleen of broilers, and that excessive apoptosis in splenic lymphocytes correlated with an increase in oxidative stress (Chen et al., 2016). Furthermore, (Yilmaz et al., 2018) also showed that AFB₁ significantly decreased antioxidant activity such as glutathione peroxidase (GPx), GSH, catalases, superoxide dismutase (SOD) and glucose-6-phosphate dehydrogenase (G6PD) in the heart and kidney tissues of AFB₁-treated rats which can lead to cardiac and renal damage (Yilmaz et al., 2018). In a comparative study, (Qureshi et al., 2015) revealed that AFB₁ affected cell viability of human umbilical vein endothelial cells (HUVEC), human brain microvascular endothelial cells (HBMEC) as well as immortalized epithelial cells of human hepatocellular carcinoma (Huh7). It was also observed that at high AFB₁ concentrations, an 85% cell death rate was induced in HBMEC and only 22% in HUVEC, while no cell death was observed in Huh7. It was further revealed that low concentrations of AFB₁ induced DNA adduct formation in HBMECs, while no adduct formation was observed at low and high AFB₁ concentrations in HUVEC (Qureshi et al., 2015). However, high concentrations of AFB₁ induced DNA adduct formation in Huh7. This showed that AFB₁ was converted to its reactive metabolite AFBO in HBMECs and Huh7, which had varying activities of biotransformation enzymes. In addition to the cytotoxic effects of AFB₁ in cell cultures, Al Ouqaili
also showed that high AFB\textsubscript{1} concentrations inhibited growth of hematopoietic stem cells (HSCs) hence decreasing viability of treated cells (Al-Ouqaili, 2018).

While AFB\textsubscript{1} mainly targets the liver, it was recently reported to induce myocardial toxicity in rats. Ge et al. (2017) revealed that the consumption of AFB\textsubscript{1} by rats lead to disruption of the mitochondrial membrane and disorganisation of the cristae, an indication of mitochondrial damage (Ge et al., 2017). It was found that myocardial apoptosis was significantly higher after treatment with AFB\textsubscript{1} relative to the control as the activities of caspase 3, Bax and BCl\textsubscript{2} were upregulated in the cardiac tissue. Due to AFB\textsubscript{1} lipophilicity, its distribution in blood and the excretion by kidney, the kidney may also be susceptible to its toxic effects. Various studies have also reported that AFB\textsubscript{1} is nephrotoxic and induces renal damage in rats (Abdel-Hamid and Firgany, 2015, Asa et al., 2018, Yilmaz et al., 2018).

2.7 TOXIC EFFECTS ON THE KIDNEY

2.7.1 Kidney structure and function

The kidneys are a pair of bean-shaped organs located on either side of the spine, below the ribs and behind the belly that play a vital role in the body because they remove wastes and excess fluid from the body. The kidneys also remove acid produced by cells of the body and maintain a healthy balance of water, salts and minerals such as calcium, sodium, and potassium in the blood (Lawrence et al., 2018). Without this balance, heart, muscles, nerves and other tissues in the body won’t be able to function properly, as all blood in the body passes through the kidney properly. Blood enters the kidney, waste gets removed and salt, water and minerals are adjusted if necessary. The filtered blood is transferred to different parts of the body and waste is turned into urine, which collects in the pelvis, a structure that drains down a tube called the ureter to the bladder.

The most important part of each kidney are the nephrons (Figure 2.13) (Lawrence et al., 2018). Each kidney is made up of approximately 1 million nephrons. Nephrons are tiny filtering units and each nephron includes a filter called a glomerulus and a tubule (Figure 2.13). Nephrons work in a two-step process; the glomerulus filters blood and the tubule restore the needed substances to the blood and removes waste (Figure 2.13). If blood stops flowing into a kidney that could result in kidney failure (Lawrence et al., 2018).
Due to their critical role as an excretory organ, the kidney receives a disproportionate percentage of blood flow, thus exposing the kidneys to high concentrations of circulating toxins. In addition, the workload imposed increases their nutrient, oxygen and energy requirements (Moosa et al., 2015). The kidneys not only facilitate many vital functions in the body, but it has been reported that they possess a significant metabolizing capacity. The kidney expresses a variety of enzymes involved in metabolism including CYP2B6, CYP3A4, CYP3A5, CYP4A11, CYP4F2, CYP4F8, CYP4F11 and CYP4F12 (Knights et al., 2013).

2.7.2 The Human embryonic kidney (Hek293) cell line
The Hek 293 cell line was derived from primary human embryonic kidney cells that were obtained from a health aborted foetus and is an adherent cell line. The Hek 293 cell line is understood to be of epithelial origin and has been transformed with sheared adenovirus 5 DNA (Stepanenko and Dmitrenko, 2015). Due to its high metabolic nature, easy reproduction and maintenance makes its one of the most used cell lines in biotechnology and cell biology studies. These cells are used as models of human renal cells in invitro studies (Stepanenko and Dmitrenko, 2015).

2.7.3 Effects of AFB₁ on the kidney
Different parts of the nephron are exposed to aflatoxins before they are excreted, thus AFB₁ and its metabolites may lead to nephrotoxicity (Coulombe Jr, 1994, Sharma et al., 2011, Bbosa et al., 2013a). The reduction in protein induced by aflatoxin has been attributed to increased necrosis of
the kidney (Sharma et al., 2011, Bbosa et al., 2013a). AFB₁ has been implicated in kidney tumour formation in experimental animals and a mixture of aflatoxin B and aflatoxin G was reported to cause approximately 80% of renal and hepatic tumours in hamsters (Coulombe Jr, 1994, Bbosa et al., 2013a). There were also renal lesions with features of megalocytosis in the proximal renal tubes.

In Africa, AFB₁ exposure lead to the development of fatty and hemorrhagic kidney syndrome abnormal development of glomerular epithelial cells, thickening of the glomerular basement membrane and degenerative changes in renal tubular cells in birds (Hussain et al., 2008, Bbosa et al., 2013a). In other animals, a reduction in the glomerular filtration rate, glucose reabsorption and tubular transport of electrolytes and organic anions, reduced glutamate-oxaloacetate and pyruvate transaminases was observed in rats. These changes were attributed to aflatoxins and other metabolites as well as the produced ROS. In cultured kidney cell lines, AFB₁ was implicated in the induction of aggregated and condensed chromatin, mitochondrial degeneration and loss of microvilli (Bbosa et al., 2013a).

The reported information clarifies that AFB₁ poses a great challenge to human health and even though the main target organ for this toxin is the liver, it can also be toxic to other parts of the body including the kidney which is one of the important body organs. Studies have reported the effects of AFB₁ on other organ systems like the liver, and studies have been done on mouse/rat kidneys but a gap in literature came to light as studies on the effects of AFB₁ in the human kidney have not been have been reported. The methodology employed to evaluate the effects of AFB₁ on human embryonic kidney (Hek293) cells will be discussed in the following chapter.
CHAPTER 3 : MATERIALS AND METHODS

This chapter describes the protocol principles and procedures followed to evaluate the effects of aflatoxin B₁ (AFB₁) on Hek293 cells. It provides information on the materials used for this particular study, how the cells were maintained prior to treatment and how cell treatments were prepared. The instruments used to collect data and statistical tests used to analyse the data collected are also described.

3.1 MATERIALS

The mycotoxin AFB₁ was purchased from Sigma Aldrich (Johannesburg South Africa (S.A)), the human embryonic kidney (Hek293) cell line was purchased from Highveld Biological in Johannesburg, South Africa (SA). Tissue culture reagents were purchased from Whitehead Scientific (Johannesburg, SA). The methylthiazol tetrazolium (MTT) salt, phosphate buffered saline (PBS) tablets were purchased from Capital Laboratory Supplies, SA. Reagents for the thiobarbituric acid (TBA) reactive substances assay (TBARS) and nitric oxide synthase (NOS) assay were procured from Capital Laboratory Supplies, SA. The lactate dehydrogenase (LDH) cytotoxicity detection kit was purchased from Roche (Manheim, Germany). DNA fragmentation kit was purchased from Thermo fisher Scientific (Johannesburg, S.A) All western blotting reagents were purchased from Bio-Rad (Hercules, CA). The Cell Signalling Technology (CST) antibodies and Promega luminometry kits were purchased from Anatech (SA). Other reagents such as salts and solvents used for preparation of buffers were purchased from Merck (Darmstadt, Germany).

3.2 CELL CULTURE

3.2.1 Background

Cell culture is a technique in which cells are grown outside of the body in an artificial environment that mimics physiological conditions (Jedrzejczak-Silikić, 2017). Under appropriate conditions of temperature, pH, a solid support, fluid medium and aseptic conditions the cells can live and grow abundantly. This technique was developed by an American embryologist Ross Granville Harrison (1870-1959) in the first decade of the 20th century and was first practised in 1907 at the Yale university (Jedrzejczak-Silikić, 2017). The development of cell culture techniques shed light to many possibilities in cell and tissue culture and as has been used as a tool in many applications.
3.2.2 Protocol
A vial of cryopreserved Hek293 cells was thawed at 37°C and reconstituted in a 25 cm³ flask, in 10 ml of complete culture medium (CCM) comprising of Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% foetal calf serum (FCS), 1% L-glutamine, 1% penicillin streptomycin fungizone and 1.25 mM HEPES. The flask was placed in an incubator containing 5% CO₂ at 37°C for 4 hours (4hrs). After 4hrs the CCM was discarded, the flask was replenished with 5 ml of CCM and incubated overnight (5% CO₂, 37°C). The cells were maintained and monitored, the CCM was changed frequently until cells reached confluency. Confluent cells were counted using the trypan blue method for use in consecutive assays.

3.3 PREPARATION OF THE TREATMENTS
The aflatoxin B₁ stock solution (640 µM) was prepared by dissolving 1 mg AFB₁ 5 ml of CCM. The AFB₁ stock was stored at 4°C and diluted in CCM to obtain different AFB₁ concentrations required for the different treatments in subsequent assays. Untreated cells served as the control in all experiments.

3.4 MTT ASSAY

3.4.1 Background
The MTT (3- (4,5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide) assay is a colorimetric assay used to evaluate cell viability or cytotoxicity (Adan et al., 2016, Riss et al., 2016). The determination of cell viability is principally guided by ascertaining mitochondrial function of cells by measuring the activity of mitochondrial enzymes such as succinate dehydrogenase (Adan et al., 2016, Riss et al., 2016). The MTT salt is a water-soluble tetrazolium dye that is reduced to a water insoluble purple formazan product in metabolically active cells using succinate dehydrogenase and NADH (Figure 3.1) (Adan et al., 2016, Riss et al., 2016). The intensity of the formazan product is directly proportional to the number of viable cells which can be measured at a specific wavelength using a spectrophotometer.
3.4.2 Protocol

The toxicity and half inhibitory concentration of AFB₁ was evaluated using the MTT assay. The Hek293 cells were seeded at a density of 20000 cell/well (200 µl) in a 96 well microtiter plate in triplicates and incubated overnight (5% CO₂, 37°C) for attachment. Treatments with different AFB₁ concentrations (0-100 µM) were prepared and the cells were treated with 300 µl of each treatment in triplicates and incubated (5% CO₂, 37°C) for 24hrs.

Following incubation, the treatments were discarded, and the cells were incubated (37°C, 5% CO₂) with 120 µl/ well of the MTT salt solution (4mg in 800 µl of 0.1M PBS and 4 ml of CCM) for 4hrs. The MTT salt solution was replaced with 100µl of the solubilising agent dimethyl sulfoxide (DMSO) for 1 hr at 37°C to dissolve the formazan crystals. The absorbance of the samples was ascertained using the Bio-Tek µQuant plate reader (USA) at a wavelength of 570 nm and reference wavelength of 690 nm. The absorbance values were used to calculate % cell viability as follows: \[
\frac{av\ abs\ of\ control}{av\ abs\ of\ treatment} \times 100.
\]

The half maximum inhibitory concentration (IC₅₀) was obtained using GraphPad Prism v5.0 (GraphPad Software Inc., La Jolla, USA) by plotting a concentration response curve (log concentration vs cell viability of samples). The calculated IC₅₀ was used to extrapolate a concentration lower (IC₂₅) and one concentration higher (IC₇₅) for use in subsequent assays.
3.5 ATP ASSAY

3.5.1 Background

The main energy source for all cellular activities is ATP, a nucleotide triphosphate produced mainly by mitochondria in all living cells that functions to store and supply cells with energy when necessary (Yong et al., 2019). Mitochondrial toxicity and loss of membrane integrity impacts on the ability to synthesize ATP, the ATP levels of cells drastically decrease, and cells may die (Yong et al., 2019).

The ATP assay is based on the luciferin and oxyluciferin reaction. During the reaction the enzyme luciferase catalyses the interaction between luciferin and oxygen to form oxyluciferin in the presence of Mg$^{2+}$ and ATP, yielding a luminescent signal released in the form of light (Figure 3.2) which can be measured using a luminometer (Riss et al., 2016). Since ATP is necessary for the reaction, there is a linear relationship between the intensity of luminescent signal produced and ATP concentration (Adan et al., 2016, Riss et al., 2016).

![Figure 3.2: Luciferase catalysed bioluminescence reaction in the presence magnesium ion (Mg$^{2+}$) and ATP (Prepared by author).](image)

\[
\text{D-luciferin} + \text{Mg}^{2+} + \text{ATP} + \text{O}_2 \rightarrow \text{Oxyluciferin} + \text{PPi} + \text{AMP} + \text{CO}_2 + \text{Light}
\]
### 3.5.2 Protocol

The intracellular activity of ATP was quantified using the Promega Cell Titre-Glo® (#G7570) luminescent assay. The reagents were prepared according to the manufacturer’s instructions. Hek293 cells were plated in a 96 well white luminometer plate at volume of 20000 cells/well (200 µl) and incubated overnight for adherence (5% CO₂, 37°C). The cells were then treated with 300µl of AFB₁ concentrations (IC₂₅, IC₅₀, IC₇₅) in duplicates for 24hrs, the untreated cells served as the control. Following treatment, the treatment media was discarded, and the cells were gently washed 1x with 300 µl of 0.1 M PBS. The wells were replenished with 50 µl of 0.1 M PBS and 25 µl of Cell Titre-Glo® ATP reagent was added to each well. The plate was incubated at room temperature in the dark for 30 minutes. Luminescence was measured using a Modulus™ microplate luminometer (Turner Bio-Systems, Sunnyvale, California, USA) and data was expressed as relative light units (RLU).

### 3.6 TBARS ASSAY

#### 3.6.1 Background

Lipid peroxidation occurs when hydrogen atoms in PUFAs containing a single electron are removed by free radicals leaving behind an unpaired electron on the carbon atom. The molecule undergoes molecular rearrangement, followed by a reaction with O₂ to generate a peroxyl radical. The peroxyl radicals may then attack membrane proteins, but are also capable of abstracting hydrogen atom from an adjacent fatty acid side chain thus propagating the chain of lipid peroxidation (Ayala et al., 2014).

Lipid peroxidation results in the formation of highly reactive and unstable lipid peroxides. The decomposition of these peroxides results in malondialdehyde (MDA) formation. This intermediate can be quantified following its reaction with thiobarbituric acid (TBA) (Zeb and Ullah, 2016). The reaction between MDA and thiobarbituric acid (TBA) results in the formation of a pink chromogen which can be detected using a spectrophotometer at 532nm (Figure 3.3).
Figure 3.3: The principle of the TBARS assay showing that thiobarbituric acid (TBA) reacts with MDA in the presence of heat or acid to produce a coloured product that is proportional to lipid peroxidation levels in samples (Prepared by author).

3.6.2 Protocol

Treatment media (200 µl) from the control and treatments were added to four clean labelled glass test tubes. Two additional tubes were prepared; viz. the negative (200 µl CCM) and positive (199 µl CCM + 1 µl MDA) controls. Each tube then received 200 µl of 2% and 7% phosphoric acid (H₃PO₄) each, followed by the addition of 400 µl of TBA/butylated hydroxytoluene (BHT) solution to every sample except the blank, which received 400 µl of 3 mM HCL. The tubes were briefly vortexed and the mixture in each tube was acidified by adding 200 µl of 1 M HCl. The tubes were boiled in a water bath for 15 minutes to allow for the hydrolysis of MDA adducts. The samples were left to cool to room temperature and 1500 µl of butanol was added to each tube. After vortexing each tube for 30 seconds, the tubes were allowed to stand so that samples can settle, and the two distinct phases were visualised. Thereafter, 200 µl of the upper phase of each sample was pipetted in triplicate in a 96-well plate and the plate was read using Bio-Tek µQuant spectrophotometer at 532 nm, with a reference wavelength of 600 nm. The average of 3 replicates
was calculated and divided by the absorption coefficient, 156 mM\(^{-1}\) and multiplied by 1000 to determine the average concentration of MDA (µM).

### 3.7 NITRIC OXIDE SYNTHASE (NOS) ASSAY

#### 3.7.1 Background

Reactive nitrogen species are produced in all eukaryotic cells and are involved in the regulation of different physiological processes including differentiation, proliferation and metabolism. Nitric oxide (NO) is an unstable diatomic molecule which contains 1 unpaired electron which makes it a free radical. In mammalian cells, NO is formed from the enzymatic oxidation of L-arginine by nitric oxide synthases (Csonka et al., 2015). NO is a highly reactive molecule with a short half-life in biological environments. NO undergoes oxidative degradation to form stable metabolites products, nitrates (NO\(_3^-\)) and nitrites (NO\(_2^-\)) (Csonka et al., 2015). The NOS assay is based on the conversion of nitrate to nitrite by nitrate reductase, which is accomplished by vanadium chloride (VCl\(_3\)) in this assay. This is followed by the acidic Griess reaction (Figure 3.4). Under acidic conditions, nitrates react with sulfanilimide (SULF) to form a diazo compound which couples with N-(1-naphthyl) ethylenediamine (NEDD) to form a purple chromophoric azo-derivative that can be measured at 540/690 nm (Csonka et al., 2015).

![Diagram of NOS assay](image)

**Figure 3.4**: 1. Nitrates (NO\(_3^-\)) and nitrites (NO\(_2^-\)) are formed nitric oxide (NO) in a reaction catalysed by the nitric oxide synthase (NOS) 2. The quantification of nitrites uses the GRIESS reaction to form a purple azo dye product that can be read spectrophotometrically at 540/690 nm (Prepared by author).
3.7.2 Protocol
Sodium nitrate standards (0 - 200 µM) were prepared and the retained supernatants from treatments with different AFB1 concentrations, including the control samples were used. In a 96 well microtiter plate, 50 µl of samples/standard solution were pipetted into each well in duplicate. Sequentially, 50 µl of VCl₃, 25 µl of SULF, 50 µl of NEDD were added in quick succession to each well, and the plate was incubated at 37˚C for 30 minutes. The absorbances were read using Bio-Tek µQuant plate reader at a wavelength of 540 nm and a reference wavelength of 690 nm. A standard curve was constructed to extrapolate sample nitrate concentrations.

3.8 GSH ASSAY

3.8.1 Background
Reduced glutathione (GSH) is a tripeptide (L-γ-glutamyl-L-cysteinyl glycine) that serves as a key antioxidant to tissues and cells by providing a free thiol group (Salbitani et al., 2017). The reduced form (GSH) provides reducing equivalents for the glutathione peroxidase (GPx) catalysed reduction reaction of lipid peroxides to their corresponding alcohols and hydrogen peroxide to water. (Salbitani et al., 2017). During the GPx catalysed reaction the formation of a disulphide bond between two GSH molecules gives rise to oxidised glutathione (GSSG). The enzyme glutathione reductase (GR) recycles GSSG to GSH with simultaneous oxidation of NADPH₂ resulting in a decrease in GSH:GSSG ratio (Salbitani et al., 2017).

The quantification of GSH is based on a reaction catalysed by glutathione-S-transferase whereby a luciferin derivative is converted to luciferin in the presence of GSH. The light emitted is proportional to the amount of luciferin formed, which depends on the amount of GSH present (Elwahab, 2017). This makes the luminescent signal produced directly proportional to the amount of GSH present in a sample (Figure 3.5).
3.8.2 Protocol

The levels of intracellular GSH were quantified using the GSH-Glo™ assay (#V6911, Promega, Madison, USA). Reagents were prepared according to the manufacturer’s guidelines. A volume of 200 µl (20000 cells/well) were seeded into a white 96 well luminometer plate and left to attach overnight (5% CO₂, 37°C). Following attachment, 24hr treatments with different concentrations of AFB₁ were done in duplicates and the plate was incubated at 37°C in a humidified incubator containing 5% CO₂. The treatment media was then removed and the in the wells were replenished with 50 µl of 0.1 M PBS. Thereafter, 25 µl of 1x GSH-Glo reagent was added to each well and the plate was incubated at room temperature, away from light. After 30 minutes, 50 µl of reconstituted luciferin detection reagent was added to each well and the plate was incubated in the dark for 15 minutes. Luminescence was measured using the Modulus™ microplate luminometer (Turner Bio-Systems, Sunnyvale, California, USA) and the data was presented as RLU.

3.9 THE SINGLE CELL GEL ELECTROPHORESIS (SCGE) ASSAY

3.9.1 Background

The comet assay is commonly known as the single cell gel electrophoresis (SCGE) assay. It is a simple technique used to detect DNA fragmentation resulting from DNA damage in individual cells. The comet assay is based on the quantification of DNA fragments migrating from the nucleus.
of the cell during electrophoresis (Nandhakumar et al., 2011). The comet assay was initially used to detect double stranded DNA breaks, however in 1988 Singh and his colleagues optimised the protocol in alkaline conditions thus allowing for the detection of DNA single strand breaks. The alkaline comet assay involved embedding cells in agarose gel on a microscopic slide and immersing the slides in lysis solution. The undamaged DNA in the nucleus remains intact within the comet head and does not migrate. However, when DNA is damaged the damaged DNA strands unwind and electrophoretically migrates out of the nucleus into the agarose suspension due to attraction of negatively charged DNA towards the positively charged anode and yield a comet tail (Nandhakumar et al., 2011). Jointly, the DNA tail and undamaged DNA resemble a comet (Figure 3.6). The longer and brighter the comets tails become, the more extensive is the damage to DNA (Nandhakumar et al., 2011).

**Figure 3.6** : Schematic representation of the SCGE assay procedure (Prepared by author).
3.9.2 Protocol
The comet/SCGE assay was used to detect DNA fragmentation in AFB1-treated Hek293 cells. Slides with frosted ends were used and labelled in duplicates for each treatment (C, IC_{25}, IC_{50}, IC_{75}). Resuspended control and treated Hek293 cells were encompassed in 1% low melting point agarose (LMPA) sandwiched between a base layer comprising 2% LMPA and a top layer comprising 1% LMPA as each slide consisted of 3 gel layers. For the first gel layer, 800 µl of 2% LMPA was pipetted onto each slide, covered with a coverslip and left to set at 4°C for 10 minutes. The coverslip was removed and the second layer consisting of cell suspension (20000 cells in 400 µl of 1% LMPA and 1 µl gel red) was pipetted onto the slide, overlayed with a coverslip and left to set at 4°C for 10 minutes. Following removal of the coverslip, the third layer consisting of 400 µl of 1% LMPA was added, covered with a coverslip and left to set at 4°C for 10 minutes.

After all the layers were set the slides were immersed in cell lysis solution that was prepared fresh beforehand (100 mM EDTA, 2.5 M NaCl, 1% Triton X-100, 10% DMSO, and 10 mM Tris (pH 10) for an hour at 4°C. The slides were then allowed to equilibrate by immersing them in electrophoresis buffer (1mM Na₂EDTA and 300 mM NaOH, pH 13) for 20 minutes preceding electrophoresis (25V, 35 minutes at 37°C). The electrophoresed gels were rinsed 3 times with neutralising buffer (0.4 M Tris, pH 7.4) at 5-minute time intervals. The slides were then viewed under a under a fluorescent microscope [Olympus IXSI inverted microscope] with 510-560nm excitation and 590nm emission filters. Images were captured using Soft imaging system (Life Science - ©Olympus Soft Imaging Solutions v5) and 50 measurements of comet-tail length in µm were done for each treatment.

3.10 CASPASE ACTIVITY

3.10.1 Background
Cysteine aspartate proteases (caspases) are a family of protease enzymes that play an essential role in the initiation and execution of programmed cell death. Caspases are expressed in all cell types and are initially expressed as inactivezymogens and are only activated once an apoptotic signal is received (McIlwain et al., 2013). This assay is based on the cleavage of conjugated aminoluciferin by caspases (Figure 3.7). Upon release of aminoluciferin, it reacts with luciferase in the presence
of ATP and molecular oxygen generating a luminescent signal that is proportional to the levels of caspase activity present in cells (Figure 3.7) (McIlwain et al., 2013).

![Diagram of proteasomal cleavage of conjugated aminoluciferin to aminoluciferin thereby producing light as a product which is directly proportional to caspase activity.](image)

**Figure 3.7**: Representation of proteasomal cleavage of conjugated aminoluciferin to aminoluciferin thereby producing light as a product which is directly proportional to caspase activity (Prepared by author).

### 3.10.2 Protocol

The activity of caspases 3/7, 8 and 9 were assessed using the Promega Caspase-Glo® assay [ #G8090, #G8200, #G8210 (Madison, USA) ] respectively. The reagents were prepared according to the manufacturer’s directions. The cells were plated at a density of 20000/well in a 96-well white luminometer plate and incubated overnight (5% CO₂, 37°C) and were then treated in duplicates with 300 µl of various concentrations of AFB₁ (IC₂₅, IC₅₀, IC₇₅) for 24hrs with untreated cells serving as the control. Following treatment, the cells were rinsed gently with 300 µl of 0.1 M PBS. Thereafter, 50 µl of 0.1 M PBS and 25 µl of Caspase-Glo®-3/7 reagent was added into each well. The plate was incubated away from light for 30 minutes at room temperature. This procedure was repeated for caspase-8 and caspase-9 respectively. Luminescence was detected using the Modulus™ microplate luminometer (Turner Bio-Systems, Sunnyvale, California, USA) and the data was expressed as RLU.
3.11 ANNEXIN V ASSAY

3.11.1 Background

The annexin V assay was used to quantify the percentage of cells that are actively undergoing apoptosis and necrotic cell death within the cell population. This assay is guided by the principle that normal cells express the membrane phospholipid phosphatidylserine (PS) on the inner membrane (Figure 3.8) (Lakshmanan and Batra, 2013). When cells are damaged or undergo apoptosis, PS is exposed by flipping from the inner membrane to the outer membrane. The exposed PS is recognised by annexin V (Lakshmanan and Batra, 2013). Damaged membranes allow for a fluorescent DNA binding dye to enter the cells; the fluorescence is indicative of cells undergoing necrosis (Figure 3.8).

![Diagram showing annexin V assay principle](image)

**Figure 3.8**: The annexin V assay principle in the detection of apoptotic and necrotic cells (Prepared by author).

3.11.2 Protocol

Human embryonic kidney cells (Hek 293 cells) were seeded at a volume of 20000 cells/ml (200 µl) in a 96-well white luminometer plate, and incubated overnight to adhere (5% CO₂, 37°C). The
2x detection reagent was prepared according to the manufacturer’s instructions; 1µl of 1000x annexin NanoBit, 1 µl 1000x CaCl₂, 1 µl 1000x Annexin V-SmBiT and 1µl 1000x Annexin V-LgBiT. After incubation the cells were treated with different concentrations of AFB₁ (C, IC₂⁵, IC₅₀, IC₇₅) for 24 hrs, then treatment media was removed, and the cells were washed gently with 300 µl of 0.1M PBS and replenished with 50 µl of 0.1M PBS, then 25 µl of the detection reagent was loaded into each well and the plate was incubated at room temperature protected from light for 30 minutes. The luminescence and fluorescence were detected using Modulus™ microplate luminometer (Turner Biosystems) for apoptosis and necrosis and expressed in RLU and relative fluorescence units (RFU) respectively.

3.12 LDH ASSAY

3.12.1 Background
Lactate dehydrogenase (LDH) is a stable, cytosolic enzyme found in cells that can be released from damaged cells (Fiume et al., 2014). When cell viability decreases, an increase in plasma membrane leakage occurs resulting in the release of LDH into the cell culture medium. The activity of LDH can be quantified using NADH produced during the conversion of lactate to pyruvate for the reduction of a yellow tetrazolium salt to form a red water-soluble formazan product (Figure 3.9) with quantifiable properties (Kumar et al., 2018). The amount of formazan produced is directly proportional to the amount of LDH in the culture which in turn is proportional to the number of damaged cells (Kumar et al., 2018). LDH release is considered as an early event in necrosis, but a late event in apoptosis.
Figure 3.9: Representation of LDH release from a damaged cell and its conversion to measurable red formazan by enzyme diaphorase (Prepared by author).

3.12.2 Protocol

Hek293 cells were treated with various concentrations of AFB₁ and the treatment supernatants were retained in eppendorf tubes labelled according to the different AFB₁ treatments. In a 96 well plate, 100 µl of the positive control (CCM only) and treatment supernatants (C, IC₂₅, IC₅₀, IC₇₅) were pipetted into each well, followed by the addition of 50 µl of the reaction mix (25 µl blue cap catalyst, 0.563 ml LDH reagent- 2). The plate was incubated in the dark at 37°C for 30 minutes. Following incubation, 25 µl of stop solution was added and the absorbances were read at 490/690 using a Bio-Tek µQuant plate reader.

3.13 WESTERN BLOTTING

3.13.1 Background

Western blotting is a technique used for the isolation and quantification of proteins in biological samples. This is achieved by separating proteins through a gel and allowing them to migrate under the influence of and electric field (Figure 3.10) (Taylor and Posch, 2014). The rate of migration is
based on their shape and molecular mass. The proteins are transferred to a nitrocellulose membrane which is incubated with specific antibodies that bind to the protein of interest (Figure 3.10, 3.11, 3.12). The unbound antibody is washed off leaving only the bound antibody to the protein of interest (Taylor and Posch, 2014). Chemiluminescence is then used to detect the bound antibodies. Antibodies only bind to the protein of interest and only one band is visible. The thickness of the band corresponds to the amount of protein present.

**Figure 3.10:** Short overview presentation of the western blotting procedure (Prepared by author).

### 3.13.2 Protein isolation

Flasks of Hek293 cells were treated with IC_{25}, IC_{50}, IC_{75} concentrations of AFB_{1} and the untreated flask served as the control. Cytobuster™ (Roche, Johannesburg, SA) reagent supplemented with protease and phosphatase inhibitors as per manufacture’s guidelines was used for the isolation of proteins from cells. The treated cells were washed once with 5 ml of 0.1 M PBS and 300 µl of the cytobuster reagent was added to each flask, then the flasks were left on ice for 15 minutes. The cells were scraped and transferred to 1.5 ml eppendorf tubes and centrifuged (2000 rpm, 4°C, 5 minutes) for the separation of pellet and crude protein. After centrifugation the supernatant was
transferred into an eppendorf tube and was used for protein quantification using the bicinchoninic acid assay (BCA) assay.

3.13.3 Protein quantification and sample preparation

Bovine serum albumin (BSA) standards were prepared (0-1 mg/ml) and 25 µl per standard solution/sample was pipetted into a 96 well plate in duplicate. The BCA working solution was prepared (198 µl BCA:4 µl CuSO₄) and 200 µl of this solution was added to each well and the plate was incubated at 37˚C, away from light for 30 minutes. Following incubation, the absorbances were read on a Bio-Tek spectrophotometer (USA) at 562 nm and a standard curve was constructed. Sample protein concentrations were calculated, and standardised to 1 mg/ml.

Standardized proteins were diluted in Laemli buffer (dH₂O, 10% SDS, β-mercaptoethanol, glycerol, 0.5 M Tris, 1% bromophenol blue) and the samples were boiled for 5 minutes at 100˚C for denaturation of protein.

3.13.4 SDS-PAGE and Transfer

The denatured protein samples were separated on SDS-PAGE. A 10% resolving gel and 4% stacking gel [10% SDS, Tris, Bis-acrylamide, dH₂O, 10% ammonium persulfate (APS), TEMED] was prepared and allowed to polymerise for an hour consecutively. Thereafter, the protein samples were subjected to an electric field (150 V) for 1 hr using a Bio-Rad compact power supplier. The electrophoresed gels were placed in transfer buffer (25 mM Tris, 192 mM glycine, 20% v/v methanol, pH 8.3) to allow equilibration between the gel and proteins. The proteins were then electro-transferred from the gel to a nitrocellulose membrane using a Transblot™ turbo programme, the gel was sandwiched between two fibre pads soaked in transfer buffer in a transblot cassette and subjected to a current of (25 V,1.0 Amp, 45 minutes).
3.13.5 Immunoprobing

Following transfer, the membranes were blocked with 5ml of 5% BSA in Tris buffered saline [dH$_2$O, NaCl, KCl, Tris-HCl (pH7.5)] with tween 20 (TTBS) and incubated on the shaker for 2hrs. It was necessary to block the membrane in order to prevent non-specific binding of antibodies. Thereafter the membranes were incubated with specific primary antibodies [SOD2 (#13141S), GPx1 (#3286S), Nrf-2 (#12721S), c-Parp (#9542S), catalase (#12980S), Hsp70 (#46477S), p53 (#48818S), c-IAP (#7065S), Bax (#5023S), NF-κb (#8245S)] diluted in 5 % BSA,1:1000, for 1hour on shaker at room temperature, then overnight at 4°C to allow for the binding of the antibody to its specific protein. Following overnight incubation, the membranes in primary antibodies were equilibrated to room temperature (1 hr on shaker), then the primary antibodies were removed, and membranes were subsequently washed 5 times (10 minutes) with TTBS to remove excess antibodies. This was followed by probing with horse radish peroxidase (HRP) conjugated secondary antibody [anti-mouse IgG (#7076S) for Hsp70and p53, anti-rabbit IgG (7074S) for SOD2, GPx1, Nrf-2, c-Parp, catalase, c-IAP, Bax and NF-κb)] diluted in 5ml of 5%BSA,1:2500, then incubation on shaker for 2 hrs. After incubation the membranes were washed 5 times with TTBS. For viewing, 150 µl of Clarity Western ECL Substrate (Bio-Rad) was added to the
membranes and images were captured using Molecular Imager® Chemidoc™ XRS and Bio-Rad imaging system.

![Diagram of Western Blot Process]

**Figure 3.12** : The binding of primary antibody to the target protein followed by the secondary antibody conjugated enzyme which allows for emission of a chemiluminescent detection signal adapted from (Elab science ® western blot protocols).

To ensure the accuracy of results a housekeeping gene, β-actin was used to normalise the expression of proteins. The membranes were quenched after 1 wash with 5ml of H$_2$O$_2$ (30 minutes). Membranes were washed again (1 wash with dH$_2$O and 1 wash with TTBS; 5 minutes each) before blocking with 5 % BSA for 1hr. Thereafter, the membranes were reprobed with HRP-labelled anti-human β-actin (1:5000 in 5 % BSA) for 1hr. The membranes were then washed 5x with TTBS and viewed as described above. The relative band density was calculated using band intensities of both the proteins of interest and the house keeping proteins and data was expressed as relative band density (RBI).
3.14 QUANTITATIVE PCR (qPCR)

3.14.1 Background

3.14.1.1 Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR, Figure 3.13) is a technique used to amplify specific fragments of DNA in an enzyme reaction (Kralik and Ricchi, 2017). PCR occurs in cycles and in every cycle the number of specific DNA sections is doubled resulting in an exponential amplification of target DNA (Kralik and Ricchi, 2017). Each PCR cycle comprises of steps including denaturation, primer annealing and primer extension. The initial step denatures or separates the double-stranded DNA template by heat (94˚C for 15 seconds to 2 minutes) into single strands for replication by the thermostable DNA polymerase (Jalali et al., 2017). In the next cycle the temperature is reduced to roughly 40-60˚C, allowing the oligonucleotide primers to anneal with the denatured DNA target and serve as primers for the enzyme DNA polymerase this step takes approximately 15-60 seconds. Lastly new DNA is synthesised as the reaction temperature is increased to the ideal temperature suited for the DNA polymerase. Most thermostable DNA polymerases temperatures range from 70-74˚C (Jalali et al., 2017). The extension step allows for the synthesis of new DNA strands by the DNA polymerase that extend the annealed primers. The modification of basic PCR lead to the expansion of its application. The development of quantitative PCR enabled the quantification of target sequences in real time (Jalali et al., 2017).

Figure 3.13: Cycles of conventional PCR within the thermocycler instrument adapted from (Lui et al., 2009)
3.14.1.2 qPCR
Quantitative polymerase reaction (qPCR) also referred to as real time PCR, is a technique that measures and detects PCR products. The process of qPCR occurs in two steps, firstly mRNA is isolated and then reverse transcribed to single stranded complementary DNA (cDNA) (Navarro et al., 2015). Additional characterization of qPCR is based on the addition of a fluorescent dye, SYBR green that can bind to the minor groove of double stranded DNA (dsDNA) amplicons present, resulting in increased fluorescence upon DNA-binding that’s proportional to the amount of PCR product produced at the end of each cycle (Jalali et al., 2017, Kralik and Ricchi, 2017, Navarro et al., 2015).

3.14.2 Protocol

3.14.2.1 RNA isolation
Hek293 cells were treated with different AFB₁ concentrations (IC₂₅, IC₅₀, IC₇₅) for 24hr, untreated cells served as the control. The cells were washed 1x with 0.1 M PBS and 500 µl of Trizol and 500 µl of 0.1 M PBS was added to each flask and the flasks were incubated at room temperature (RT) for 5 minutes. Following incubation, the flasks were scrapped, and the contents were transferred to 1.5 ml eppendorf tubes and stored at -80ºC.

Thereafter, to thawed samples 100 µl of chloroform was added, mixed vigorously for 15 seconds and samples were incubated at RT for 2-3 minutes. The samples were then centrifuged (12000 x g, 4°C, 15 minutes). While working on ice, the upper aqueous phase was transferred to new 1.5 ml eppendorf tube and 250 µl of isopropanol was added to each tube, then the samples were stored overnight (-80°C) overnight. Thawed samples were centrifuged (12000 x g, 4°C, 20 minutes) and the supernatant was removed leaving only the pellet containing total RNA. The pellet was then washed with 500 µl of 75% cold ethanol and flicked to loosen the pellet. The tubes were then centrifuged again (7400 xg, 4°C,15 minutes) and thereafter all ethanol was discarded, and the samples were air dried for 1-1.5hrs.

Thereafter the pellets were resuspended in 15 µl of nuclease free water (NFH₂O) and samples were incubated at RT for 2-3 minutes on ice and isolated RNA was quantified using a nanodrop 2000 (Thermo Fisher Scientific, Johannesburg, S.A.).The purity of RNA was determined using an
absorbance ratio of 260/280 nm, samples between 1.8-2 were considered as pure. RNA samples were standardized to 5000 ng/µl with nuclease free water (NFH2O) and stored at -80°C.

### 3.14.2.2 cDNA synthesis

Following the manufacturer’s guidelines, RNA was reverse transcribed to cDNA using the iScript™ CDNA synthesis kit. A 20 µl reaction mixture (4 µl of 5x iScript reaction mix, 1 µl iScript reverse transcriptase, 11 µl NFH2O and 4 µl RNA sample) was prepared for each sample. The prepared samples were placed in a thermocycler and reverse transcribed as follows: 25 °C for 5 minutes, 42 °C for 30 minutes, 85°C for 5 minutes and final hold at 4°C. The cDNA was diluted with 80 µl of NFH2O to obtain a final volume of 100 µl and stored at -80 °C for further use.

### 3.14.2.3 qPCR

Working stock solutions of forward and reverse primers were prepared separately according to manufacture guidelines (6.25 µl SYBR green, 0.5 µl forward primer, 0.5 µl reverse primer, 3.75 µl of NFH2O and 1.5 µl of cDNA) for both the gene of interest and housekeeping gene, this made up to a reaction volume of 12.5 µl. The gene of interest was 8-oxoguanine DNA glycosylase (OGG1) (Forward primer : 5’-GCATCGTACTCTAGCCTCCAC-3’, Reverse primer : 5’-AGGACTTTGCTCCCTCCAC-3’, 60°C). The house keeping gene was glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Forward primer : 5’-TCCCTGAGCTGAACGGGAAG-3’, Reverse primer : 5’-GGAGGAGTGGGTGTCGCTGT-3’). Each treatment was done in triplicates in a plate that was covered with plastic and centrifuged (200 rpm, 37°C, 3 minutes) then placed in the CFX Touch™ Real Time PCR Detection System (Bio-Rad; Hercules, California, United States). The reaction was subjected to initial denaturation (95°C, 4 minutes), followed by an annealing phase (60°C, 40 seconds) which can be altered to accommodate different genes, and finally the extension phase (72°C for 30 seconds). To determine the initial amount of DNA present in samples a curve was plotted with fluorescent emission over time. The expression of the house keeping gene (GAPDH) was also quantified for normalization of the target gene expression. The data acquired was analysed using the Livak & Schmittgen (2001) method and presented as relative fold change in mRNA expression.
3.15 DNA fragmentation

3.15.1 Background
Deoxyribonucleic acid (DNA) is a negatively charged nucleic acid that when subjected to an electric field migrates from negative (anode) to positive (cathode). This assay is based on the fragmentation of DNA separated on an agarose gel (Figure 3.14). The visualization of DNA migration on the gel is facilitated by the use of intercalating dyes that bind and stain DNA. These dyes have been used to indicate DNA quantity, size and quality (Haines et al., 2015).

![Diagram of DNA separation on agarose gel](image)

**Figure 3.14**: The separation of DNA fragments bases on their size and charge on an agarose gel (Prepared by author).

3.15.2 Protocol
Hek293 cells were exposed to various AFB1 concentrations for 24hrs, with untreated cells serving as the control. Following treatment, the supernatants were discarded, and cells were rinsed 1x with 5 ml 0.1 M PBS. Thereafter, 600 µl cell lysis solution (EDTA, Tris-Cl, 0.1% SDS) was added to the flasks of treated cells and left at room temperature (RT) for 5 minutes. The flasks were then scraped, and the constituents were transferred to 1.5 ml eppendorf tubes then stored at -80°C overnight. To thawed samples, 600 µl potassium acetate was added and the samples were vortexed, then invert mixed for 8 minutes. The samples were vortexed once more and centrifuged (13000
rpm for 5 min) and the clear supernatant was decanted into eppendorf tubes. To the tubes with the supernatants, 600 µl of isopropanol was added and the tubes were invert mixed for 5 minutes then centrifuged (13000 rpm for 5 min). After centrifugation the supernatant was decanted to a new 1.5 ml eppendorf, leaving behind the pellet and isopropanol was added to the tubes (60 µl supernatant, 200 µl pellet) accordingly. The tubes were invert mixed for 5 minutes and centrifuged (13000 rpm for 5 min). The pellet was noted in each tube, pellets were combined for each treatment and 300 µl of ethanol was added to the final pellet then vigorously vortexed followed by centrifugation (13000 rpm for 5 minutes). The ethanol was removed carefully noting the clean white pellet and the tubes were dried on an absorbent towel for 1hr. Furthermore, 40 µl of DNA hydration solution (1x TE solution) was added to the pellet. Then the samples were vortexed and placed in a water bath (65°C) for 15 minutes. The samples were allowed to cool for 15 minutes and stored at -80°C. The concentration of DNA for each sample was measured using Nanodrop (Nanodrop 2000) and the obtained concentrations were standardized to the lowest concentration (100 ng/µl).

3.15.2.1 Agarose gel
A 2% agarose gel (2 g agarose powder in 100 ml 1x TBE buffer) was prepared. The standardized DNA samples were prepared with loading dye (10 µl : 10 µl) then loaded in to gels, a DNA ladder of 1kilo base pairs (kb) was used as a marker for the migration of DNA samples in the gel and the gels were electrophoresed (120 V) monitoring the migration of the samples. The gels were then viewed using Molecular Imager® Chemidoc™ XRS and Bio-Rad imaging system.

3.16 STATISTICAL ANALYSIS
GraphPad Prism V5 software was used for the analysis of results. Data was presented as mean ± standard deviation (SD). The significant differences among groups were determined using One-way analysis of variance (ANOVA) and Tukey’s multiple comparisons post-test. The unpaired student t-tests with Welch’s correction was also used to measure the significant difference between two sample means. A value of p < 0.05 was accepted as an indication of statistical significance. All the data represented was obtained from at least three replicates of each experiment, and experiments were repeated to confirm the results.
CHAPTER 4: RESULTS

4.1 CELL VIABILITY

4.1.1 MTT assay
The toxicity of AFB₁ in Hek293 cells was assessed using the MTT assay. The results show that at lower AFB₁ concentrations (log 0.097 - 0.699) a slight increase in Hek293 cell viability was observed relative to the control (Figure 4.1). However, a dose dependent decrease was observed at higher AFB₁ concentrations (log 1.0 - 2.0). Analysis of the curve disclosed that 32.60 µM of AFB₁ inhibited cell proliferation by inducing 50% toxicity in Hek293 cells (IC₅₀).

![Figure 4.1: Viability of Hek293 cells after 24 hr treatment with different AFB₁ concentrations. A dose dependent decline in cell viability was observed at high AFB₁ concentrations.](image)

4.1.2 ATP assay
The intracellular levels of ATP were evaluated using the Cell Titre Glo™ assay. AFB₁ induced a decrease in ATP levels in Hek293 cells in a dose dependent manner (Figure 4.2, p < 0.0001 using ANOVA, Tukey’s post-test). Significant decreases in ATP concentration were noted at the IC₅₀ (p = 0.004) and IC₇₅ (p = 0.0143) relative to the control (Figure 4.2).
Figure 4.2: The ATP concentration decreased in Hek293 cells after 24hr treatment with different concentrations of AFB$_1$ (**$p = 0.0044$ and *$p = 0.0143$, unpaired student t-test with Welch’s correction).

4.2 OXIDATIVE STRESS

4.2.1 TBARS assay
The TBARS assay was used to quantify MDA levels, a marker of lipid peroxidation in Hek293 cells treated with different AFB$_1$ concentrations. The dose dependent decline in MDA levels was observed AFB$_1$ treated cells (Figure 4.3, $p < 0.0001$ using ANOVA, Tukey’s post-test). The MDA levels dropped from 2.994±0.03402 µM in the control to 0.6432±0.006586 µM at the IC$_{25}$ ($p < 0.0001$). In addition, the higher concentrations of AFB$_1$ induced a decrease from reported control MDA levels to 0.4829±0.006044 µM at IC$_{50}$ ($p < 0.0001$), and 0.4509±0.007995 µM at IC$_{75}$ ($p < 0.0001$) respectively (Figure 4.3).
Figure 4.3: 24hr treatment with AFB₁ induced in a dose dependent decrease in MDA concentration in Hek293 cells (*** p < 0.0001 for all treatments, unpaired student t-test with Welch’s correction).

4.2.2 NOS assay

The concentration of RNS in AFB₁-treated Hek293 cells was evaluated using the nitrates assay. An increase in nitrates concentration was observed in the IC₂₅ and IC₅₀ treated cells; an increase was also observed at the IC₇₅ relative to the control, but was lower than the IC₅₀ (Figure 4.4, p = 0.0046 using ANOVA, Tukey’s post-test). When compared to the control (2.000±0.3608 µM), a non-significant increase to 2.625 ±0.7217 µM and 3.563±0.5413 µM was observed at IC₂₅ and IC₇₅ respectively, while a significant increase to 6.688±0.9021 µM was observed at IC₅₀ (p = 0.0404, Figure 4.4).

Figure 4.4: Treatment with AFB₁ induced an increase in the production of reactive nitrogen species in Hek293 cells (*p = 0.0444, unpaired student t-test with Welch’s correction).
4.2.3 GSH assay

The GSH assay was used for the quantification of GSH in treated Hek293 cells. The data revealed a concentration dependent increase in GSH levels at all AFB\textsubscript{1} concentrations (Figure 4.5, \( p = 0.0039 \) using ANOVA, Tukey’s post-test). The GSH levels increased from 23200±486.6 RLU in the control to 68160±23650 RLU at IC\textsubscript{25} (\( p = 0.1977 \)), with significant increases to 102300±862.3 RLU at IC\textsubscript{50} (\( p < 0.0001 \)) and 105000 ±716.8 RLU at IC\textsubscript{75} (\( p < 0.0001 \)).

![Figure 4.5: Exposure to AFB\textsubscript{1} induced a dose dependent increase in GSH levels in Hek293 cells (***\( p < 0.0001 \), unpaired student \( t \)-test with Welch’s correction).](image)

4.2.4 Hsp70

The expression of proteins in AFB\textsubscript{1} treated Hek293 cells was determined using western blotting relative to the control. The expression of Hsp70 was upregulated in treated Hek293 cells compared to the control (Figure 4.6, \( p < 0.0001 \) using ANOVA, Tukey’s post-test). The control expression was recorded as 0.8897±0.003612 RBI. An increase to 1.075±0.004250 RBI, 0.9927±0.002614 RBI, 0.9332±0.003711 RBI was observed at IC\textsubscript{25} (\( p < 0.0001 \)), IC\textsubscript{50} (\( p < 0.0001 \)) and at IC\textsubscript{75} (\( p = 0.0005 \)) concentrations respectively, relative to the control.
**Figure 4.6**: Upregulated expression of stress protein Hsp70 after 24hr treatment with AFB$_1$ in Hek293 cells (**$p < 0.0001$, IC$_{25}$ and IC$_{50}$; *** $p = 0.0005$, IC$_{75}$; unpaired student t-test with Welch’s correction).

4.2.5 Antioxidant response

The expression of proteins related to the antioxidant response in AFB$_1$ treated Hek293 cells was established using western blotting (Figure 4.7). Nrf-2 was significantly upregulated in AFB$_1$ treated Hek293 cells, an increase from the control expression of $0.09956\pm0.001364$ RBI to $0.1740\pm0.003141$ RBI was observed at the IC$_{25}$ concentration ($p < 0.0001$). A further increase to $0.3511\pm0.003066$ RBI was observed at IC$_{50}$ concentration ($p < 0.0001$). In addition, an increase to $0.2060\pm0.001476$ RBI at IC$_{75}$ concentration was noted ($p < 0.0001$). SOD2 expression was increased from $1.069\pm0.008645$ RBI in the control to $1.277\pm0.007789$ RBI at the IC$_{25}$ concentration of AFB$_1$ ($p < 0.0001$), a further increase to $1.195\pm0.003530$ RBI and $1.486\pm0.007021$ RBI for the IC$_{50}$ ($p = 0.0009$) and IC$_{75}$ ($p < 0.0001$) concentrations respectively. GPx was upregulated at the IC$_{25}$ and IC$_{50}$ concentration of AFB$_1$ to $1.070\pm0.02179$ RBI ($p = 0.1459$) and $1.055\pm0.01705$ RBI ($p = 0.1846$) respectively compared to the control expression of $1.024\pm0.008110$ RBI; a significant increase to $1.143\pm0.006078$ RBI relative to the control was noted at the IC$_{75}$ ($p < 0.0001$). Catalase was significantly increased in AFB$_1$-treated Hek293 cells relative to the control. Upregulation from control expression ($1.788\pm0.02214$ RBI) to $2.501\pm0.01745$ RBI at IC$_{25}$ ($p < 0.0001$), $1.990\pm0.003122$ RBI was at IC$_{50}$ concentration ($p = 0.0029$) and $3.360\pm0.01842$ RBI at the IC$_{75}$ ($p < 0.0001$) AFB$_1$ concentration comparative to the control.
Figure 4.7: The expression of antioxidant proteins Nrf-2 (***p < 0.0001), SOD2 (***p < 0.0001, IC_{25} and IC_{75}; ***p = 0.0009, IC_{50}), GPx (***p < 0.0001) and catalase (**p < 0.0001, IC_{25} and IC_{75}; ***p = 0.0029, IC_{50}) after 24hrs treatment with AFB_{1} in Hek293 cells, unpaired student t-test with Welch’s correction.
4.3 DNA DAMAGE

4.3.1 SCGE assay

The SCGE assay was performed to detect DNA fragmentation induced by DNA damage in treated Hek293 cells and the untreated cells served as the control. Large intact comet heads indicated no DNA fragmentation and the presence of comet tails lengths is an indication of DNA fragmentation. A significant dose-dependent increase in comet tail length was observed relative to the control (Figure 4.8, \( p < 0.0001 \) using ANOVA, Tukey’s post-test). A significant increase from 6.244±0.1459 µm in the control to 7.046±0.1208 µm, 8.620±0.1370 µm, 9.109±0.1152 µm was observed for IC\(_{25}\) \( (p < 0.0001) \), IC\(_{50}\) \( (p < 0.0001) \) and IC\(_{75}\) \( (p < 0.0001) \) concentration(s) of AFB\(_1\) respectively in treated Hek293 cells.

![Comet tails of Hek293 cells after 24hr treatment with AFB\(_1\)](image)

**Figure 4.8**: Comet tails of Hek293 cells after 24hr treatment with AFB\(_1\). A significant increase in comet tail length was observed in the treated cells relative to the control (\( **\*p < 0.0001 \), unpaired student t-test with Welch’s correction).
4.3.2 DNA fragmentation
To further validate the damage to DNA in AFB1 treated Hek293 cells, DNA fragmentation was assessed by running isolated DNA samples of the treated cells on an agarose gel; untreated cells served as the control. The genomic DNA of AFB1 treated Hek293 cells showed fragmentation in treated cells except the control which indicated that AFB1 exposure induced DNA double strand breaks in Hek293 cells.

![DNA fragmentation](image.png)

**Figure 4.9**: Fragmentation of DNA induced by 24hr exposure to AFB1 in Hek293 cells.

4.3.3 OGG1 expression
The expression of mRNA in Hek293 cells after 24hr exposure to AFB1 was quantified using qPCR. A non-significant concentration dependent increase in OGG1 mRNA expression was observed at IC25 ($p = 0.3837$) and IC50 ($p = 0.7867$) concentrations in AFB1 treated Hek293 cells, and an increase at IC75 ($p = 0.2145$) was observed compared to the control.
Figure 4.10: Increased mRNA levels of OGG1 after 24 hr treatment with AFB1 in Hek293 cells.

4.4 CELL DEATH

4.4.1 Caspase activity

Intracellular activities of caspases 8, 9 and 3/7 were measured using luminometry. Treatment of cells with AFB1 decreased caspase 8 activity (Figure 4.11A) activity in Hek293 cells. There was a decrease to 4329000±724.6 RLU, 2025000±956800 RLU, 3289000±9904 RLU was noted at IC25 (p = 0.6847), IC50 (p = 0.1336) and IC75 (p = 0.0624) AFB1 concentrations sequentially from control activity of 4421000±196700 RLU. Compared to the control (3216000±586800 RLU), only slight but non-significant increases in the activity of caspase 9 were observed (Figure 4.11B). Caspase 9 activity increased to 3337000±401700 RLU at IC25 (p = 0.8928), 3806000±49500 RLU at IC50 (p = 0.5082) and 3432000±90480 at IC75 (p = 0.7773). The increase in caspase 3 activity compared to the control (455900±13440 RLU) was not significant (Figure 4.11C). An increase at IC25 (p = 0.1252) to 617500± 61860 RLU and 541300±46180 RLU IC50 (p = 0.2177) were noted, while caspase 3 activity was similar to the control for the IC75 (p = 0.775) with 450700±9904 RLU in AFB1 treated Hek293 cells.
Figure 4.11: The activity of initiator and executioner caspases in AFB$_1$ treated Hek293 cells, A) caspase 8, B) caspase 9 and C) caspase 3/7.
4.4.2 Apoptosis-related proteins

Evaluation of the expression apoptosis-related protein in AFB1 treated Hek293 cells was ascertained using western blotting. Upregulation in the expressions of p53, Bax and c-Parp was observed in treated Hek293 cells comparative to the control (Figure 4.14). Control expression for p53 was recorded as 1.214±0.01316 RBI and were elevated to 2.046±0.02566 RBI at IC25 (p < 0.0001). In addition, an increase to 1.506±0.01500 RBI and 1.553±0.01318 RBI was observed at IC50 (p < 0.0001) and IC75 (p < 0.0001) concentrations respectively (Figure 4.12A). Regarding the expression of pro-apoptotic Bax protein, a significant concentration dependent increase was observed in AFB1 treated Hek293 cells (Figure 4.12B). An increase from control expression of 0.5447±0.01588 RBI to 0.7113±0.01268 RBI was observed at IC25 (p = 0.0004) concentration with a noted significant increase to 0.8567±0.003298 RBI at IC50 (p = 0.0010) relative to the control, and an observed increase to 1.005±0.01354 RBI was noted at the IC75 (p < 0.0001) concentration. The expression of c-Parp (Figure 4.12C) showed an increase from 0.8430±0.003773 RBI in the control to 1.521±0.004865 RBI at IC25 (p < 0.0001), while an increase to 1.111±0.008022 RBI and 1.225±0.01154 RBI was noted at IC50 (p < 0.0001) and IC75 (p < 0.0001).

The expression of c-IAP was significantly increased in treated cells relative to the control (Figure 4.13A), an increase from 0.7220±0.005607 RBI in the control to 1.300±0.02637 RBI was noted at IC25 concentration (p = 0.0002). An increase from recorded control levels to 0.8199±0.01008 RBI and 0.9026±0.1016 RBI was observed at the IC50 concentration (p = 0.0011) and IC75 concentration (p < 0.0001). A dose dependent increase in the expression of NF-κb (Figure 4.13B) was observed in AFB1 treated Hek293 cells, the control expression of NF-κb was recorded as 1.272±0.009986 RBI, an increase from recorded control expression to 2.413±0.01762 RBI, 2.538±0.02202 RBI and 2.544±0.01727 RBI was noted at the IC25 concentration (p < 0.0001), IC50 concentration (p < 0.0001), and IC75 concentration (p < 0.0001) respectively.
Figure 4.12: The protein expression of p53 (***p < 0.0001), Bax (***p = 0.0004, IC_{25}; **p = 0.0010, IC_{50}; ***p < 0.0001, IC_{75}) and c-Parp IC_{25} (***p < 0.0001) were increased in AFB_{1} treated Hek293 cells (unpaired student t-test with Welch’s correction).
Figure 4.13: Upregulated expression of c-IAP (***p = 0.0002, IC_{25}; **p = 0.0011, IC_{50}; ***p < 0.0001, IC_{75}) and NF-κb (***p < 0.0001) in AFB₁ treated Hek 293 cells (unpaired student t-test with Welch’s correction).

4.4.3 Mode of cell death

The annexin V assay was used to detect apoptotic cells by measuring the exposure of phosphatidylserine (PS) on the outer leaflet of the cell membrane during apoptotic processes. Since annexin binds to PS, the findings indicate that AFB₁ decreased externalization of PS. Thus, annexin binding in treated cells was less compared to the control. Control apoptotic cells were recorded as
886100±21410 RLU (Figure 4.14). A decrease to 695300 ± 40650 RLU was observed at the IC₂₅ ($p = 0.0254$) concentration and to 736800±39000 RLU at the IC₅₀ ($p = 0.0439$) concentration relative to the control (Figure 4.14); phosphatidylserine externalisation at the IC₇₅ was similar to the control ($p = 0.9755$).

**Figure 4.14**: AFB₁ induced a decrease in early apoptotic cells in treated Hek293 cells (*$p = 0.0254$, IC₂₅; *$p = 0.0439$, IC₅₀; unpaired student $t$-test with Welch’s correction).

Necrotic cells were quantified to determine the mode of cell death induced by AFB₁ in treated Hek293 cells. The presence of necrotic cells was detected using the necrosis assay which measured the permeability of the plasma membrane to an impermeable fluorescent dye. Control necrotic cells were recorded as 886100±37080 RFU (Figure 4.15), a decrease to 695300±70410 RFU was observed at the IC₂₅ ($p = 0.2516$) concentration. A further decrease relative to the control to 736800±67550 RFU and 882800±159700 RFU was noted at IC₅₀ ($p = 0.3033$) and IC₇₅ ($p = 0.9873$) concentrations respectively.
Figure 4.15: 24hr treatment with AFB$_1$ decreased levels of necrotic cells in treated Hek293 cells.

Upon membrane damage cells release the enzyme lactate dehydrogenase (LDH) into the extracellular space and its release can indicate membrane damage (Kumar et al., 2018). Extracellular LDH levels were quantified using the LDH assay. A non-significant decrease in LDH concentration occurred for all treatments (Figure 4.12). A decrease from 0.1657±0.02627 OD in the control to 0.1110±0.02078 OD, 0.1347±0.02050 OD, 0.1267±0.02858 OD was noted at the IC$_{25}$ ($p = 0.2012$), IC$_{50}$ ($p = 0.4028$) and IC$_{75}$ ($p = 0.3891$) concentrations respectively (Figure 4.12).

Figure 4.16: Extracellular LDH levels decreased after 24hr treatment with AFB$_1$ in Hek293 cells.
CHAPTER 5 : DISCUSSION

This chapter describes in detail what the obtained results of this study indicate and further outlines the mechanism by which AFB₁ exerts its effects in Hek293 cells. Furthermore, the study limitations and recommendations are also included in this chapter.

Mycotoxins are a group of toxic secondary metabolites produced by fungi that colonise dietary staples. They present a growing health concern associated with mortality and morbidity in animals and humans. AFB₁ is of great concern worldwide because it is considered the most potent mycotoxin and is classified as a group 1 carcinogen (da Rocha et al., 2014, Alshannaq and Yu, 2017, Zinedine and El Akhdari, 2019). Exposure to AFB₁ is mainly through the diet, as AFB₁ is frequently found as a contaminant in maize, peanuts and wheat which are major staple foods especially in South Africa (Alshannaq and Yu, 2017). Carcinogenic, immunotoxic, teratogenic and mutagenic effects have been reported in both humans and animals. Exposure is linked to several diseases including liver cancer in humans (Moosa, 2019) and nephrotoxicity in rats (Abdel-Hamid and Firgany, 2015, Asa et al., 2018, Yilmaz et al., 2018). However, AFB₁ effects on the human kidney have not been fully elucidated. This study therefore investigated the cytotoxic effects of AFB₁ exposure on Hek293 cells.

All living organisms require energy to survive; the energy is provided by the hydrolysis of ATP and is used for various cellular activities (Yong et al., 2019). Mitochondria are the metabolic hub of cells that perform the essential function of oxidative phosphorylation, resulting in the production of ATP necessary for cell viability and survival (Yong et al., 2019). The present study exposed Hek293 cells to different AFB₁ concentrations and cell viability was evaluated using the MTT assay. This study showed that viable Hek293 cells reduced the MTT salt to formazan and decreased cell viability (Figure 4.1). The result corroborates several studies that have demonstrated that AFB₁ decreases viability in human brain microvascular endothelial cells, hematopoietic stem cells, human brain macrovascular endothelial cells, human hepatocellular carcinoma cells and human epithelial colorectal adenocarcinoma cells (Qureshi et al., 2015, Yip et al., 2017, Al-Ouqaili, 2018, Huang et al., 2018, Meneely et al., 2018). The suggested decrease in mitochondrial succinate dehydrogenase activity or NADH (Stockert et al., 2018), both essential components of the electron transfer, would negatively impact oxidative phosphorylation; ATP will be depleted and cell
viability is lost resulting in cell death (Yong et al., 2019). The decrease in Hek293 cell viability corresponded with a dose-dependent decrease in ATP levels (Figure 4.2), and correlated with decreased ATP levels reported following AFB1 administration to HepG2 cells (Liu et al., 2014).

An attempt by the Hek293 cells to enhance the metabolic rate to replenish the depleted ATP could lead to oxidative stress, which is associated with an increased production of ROS (Forrester et al., 2018). Several studies have demonstrated a role for AFB1 in the onset of oxidative stress; AFB1 forms ROS in rat liver, duck liver, mouse lung, chicken liver and the kidney of rats (Shen et al., 1994, Shen et al., 1996, El-Nekeety et al., 2011, Abdel-Hamid and Figuey, 2015). Although mitochondria are a major source of ROS produced as by-products of cellular respiration in the ETC, they are also highly susceptible to ROS molecules and their harmful effects (Milkovic et al., 2019). Specifically, mitochondria produce O$_2^-$ from O$_2$ (Tong et al., 2015, Di Meo et al., 2016, Milkovic et al., 2019). The O$_2^-$ is converted to H$_2$O$_2$ in a dismutation reaction catalysed by SOD or it can react with NO to form peroxynitrite (ONOO$^-$) (Di Meo et al., 2016). In this study, the increased SOD2 (Figure 4.7) and RNS (Figure 4.4) provide evidence of increased O$_2^-$.

Furthermore, the noted increase in SOD2 expression at all AFB1 exposures (Figure 4.7) can be attributed to the correspondingly increased expression of NF-kB (Figure 4.13A), as SOD2 is one of the antioxidant targets of NF-kB (Morgan and Liu, 2011).

H$_2$O$_2$ produced in the dismutation reaction has several fates. It may be converted to the potent OH$^-$ using the Fenton reaction (Di Meo et al., 2016). Both O$_2^-$ and OH$^-$ are able to oxidise cellular macromolecules including proteins and lipids (Di Meo et al., 2016, Milkovic et al., 2019). Decreased MDA levels at all doses of AFB1 (Figure 4.3) is characteristic of decreased lipid peroxidation and implies that membranes were spared from the cytotoxic effects of free radicals (Figure 4.3); the decreased membrane damage is supported by decreased LDH release from all AFB1-treated Hek293 cells (Figure 4.16). This protection could be conferred by the upregulation of another pathway for H$_2$O$_2$, where H$_2$O$_2$ is reduced to water using GSH (Tong et al., 2015). Alternatively, catalase converts H$_2$O$_2$ to water and O$_2$ (Tong et al., 2015, Di Meo et al., 2016). These antioxidants protect cells from the deleterious effects of H$_2$O$_2$ (Dhawan, 2014). Protein expression of both GPx (Figure 4.7) and catalase (Figure 4.7) was increased for all AFB1 treatments, but particularly for the IC$_{75}$ AFB1 treatment. The functioning of GPx is dependent on adequate reduced glutathione (GSH); increased GSH in all AFB1-treated cells (Figure 4.5) was
employed in the reduction of lipid hydroperoxides and thus correlates with the observed decline in MDA levels at all doses of AFB$_1$ (Figure 4.3). Overall, decreased MDA (Figure 4.3) in this study suggests that the antioxidant response in human kidney cells sufficiently keeps ROS within levels suitable to maintain cellular homeostasis. Thus, the Hek293 cells are able to resist oxidative stress; this adaptive advantage is known to permit cancer cells to increase their metabolic rate, proliferate and to escape free radical damage (Sainz et al., 2012) and may indicate a progression to cancer.

The antioxidant response is modulated by the upregulation of Nrf-2 (Figure 4.7) in all treated Hek293 cells. Nrf-2 is a transcription factor involved in the activation of antioxidants and cytoprotective enzymes that protect cells from excess oxidative stress by binding to antioxidant response elements (AREs) (Kavian et al., 2018). Despite an overwhelming antioxidant response, the upregulated expression of the stress response protein, Hsp70 (Figure 4.6) at all AFB$_1$ doses demonstrates that AFB$_1$ induced oxidative stress in Hek293 cells. Several studies also show the involvement of AFB$_1$ in the induction of oxidative stress in rat kidney, splenic lymphocytes of broilers, and heart tissues of rats (Abdel-Hamid and Firgany, 2015, Chen et al., 2016, Yilmaz et al., 2018).

Oxidative damage to DNA is also possible during episodes of oxidative stress. It may manifest as lesions on DNA, breaks in DNA strands or faulty links and base gaps in sequences (Wang et al., 2018). Cells respond to oxidative DNA damage using the base excision repair (BER) pathway that employs enzymes such as OGG1, a DNA glycosylase involved in BER (Wang et al., 2018, Vlahopoulos et al., 2019). OGG1 is a marker of oxidative DNA damage (Wang et al., 2018). The increase in OGG1 (Figure 4.10) may reflect increased strand breaks in DNA that require repair. Strand breaks in this study are indicated by DNA laddering (Figure 4.7) and increased comet tail lengths (Figure 4.8) both of which indicate oxidative damage to DNA. Since OGG1 is transcriptionally regulated by p53, as well as the observed increase in DNA damage, the upregulation of p53 (Figure 4.12) was not surprising. Failure to repair DNA results in activation of the cell death program by p53.

Cell death is mediated by multiple pathways, including apoptosis and necrosis (Jog and Caricchio, 2014). Intact and ruptured plasma membranes are considered as hallmarks of apoptosis and necrotic cell death respectively (Zhang et al., 2018). The results of this study revealed a decrease in necrosis
Evidence for the decreased necrosis is provided by the corresponding decrease in LDH (Figure 4.16) released from treated Hek293 cells at all concentrations of AFB₁; this effect was not dependent on dose. It is further supported by decreased MDA levels and lipid peroxidation (Figure 4.3) indicating reduced membrane damage. Thus, homeostasis is maintained, the influx of water and extracellular ions leading to cell swelling and rupture is prevented, and necrotic cell death is avoided (Zhang et al., 2018). This indicates that treatment of cells with AFB₁ induced a decrease in necrotic cell death at all AFB₁ concentrations relative to the normal untreated cells.

Essential for the occurrence of apoptosis is a group of cysteine proteases (caspases) which become activated in response to pro-apoptotic signals (McIlwain et al., 2013). In the present study, AFB₁ exposure lead to a decrease in caspase 8 activity (Figure 4.11A) in Hek293 cells indicating that the extrinsic pathway of apoptosis was not activated. The intrinsic pathway of apoptosis is controlled by the Bcl-2 family. Bax is a pro-apoptotic member of the Bcl-2 family (Aubrey et al., 2018). The increase in Bax (Figure 4.12B), may have been triggered by upregulation of p53 (Figure 4.12A). The tumour suppressor protein p53, aids in DNA repair and can also initiate cell death, DNA damage was increased as described earlier. p53 activation results in transcription of pro-apoptotic PUMA and NOXA that bind to anti-apoptotic Bcl-2, freeing Bax to dimerize into the mitochondrial membrane forming pores that increase permeabilization of the mitochondrial outer membrane (Peng et al., 2016, Aubrey et al., 2018). This facilitates the release of cytochrome c, apoptosis inducing factor (AIF), endonuclease G, and Smac/DIABLO from the mitochondria (Peng et al., 2016). Subsequently, cytochrome c binds to Apaf-1 forming an apoptosome and facilitating the autocatalytic activation of procaspase 9. Activated caspase 9 (Figure 4.11B) cleaves and activates the executioner caspase 3 (Figure 4.11C) leading to the cleavage of Parp. The cleavage of Parp (Figure 4.12C) renders the enzyme inactive which further explains the observed DNA fragmentation (Figure 4.9) and the progression to apoptosis (Peng et al., 2016). Thus, the mitochondrial pathway of apoptosis was triggered in Hek293 cells after exposure to AFB₁.

Despite the upregulation of p53 (Figure 4.12A) and activation of caspases via the intrinsic pathway (Figure 4.11B), PS was not externalised (Figure 4.14) at all concentrations of AFB₁. Thus, a key hallmark of apoptosis did not occur; this translates to decreased cell death and suggests that the inhibition of the intrinsic pathway apoptosis might be a mechanism by which AFB₁ could cause cancer in kidney cells. Indeed, inhibition of cell death is a hallmark of cancer (Aubrey et al., 2018).
Progression of the intrinsic pathway of apoptosis may also have been hindered by the expression of c-IAP (Figure 4.13A) that was upregulated in this study. Inhibitors of apoptosis (IAPs) have been reported to regulate cell death through caspase inhibition; however, despite their ability to bind to caspases, recent work has reported that cellular IAPs (c-IAPs) do not possess the ability to inhibit caspase activation (Kocab and Duckett, 2016).

It has been demonstrated that AFB$_1$ induces apoptosis in splenic lymphocytes, cardiomyocytes of rats, human bronchial epithelial (B-2A13) cells and human hepatocytes (Liu et al., 2014, Chen et al., 2016, Ge et al., 2017). The induction of apoptosis in broiler hepatocytes as reported by Liu and Wang (2016) revealed that increased caspase 9 and caspase 3 activities and increased Bax expression lead to the activation of the intrinsic apoptotic pathway (Liu and Wang, 2016). In addition, Yang (2012) demonstrated that AFB$_1$ increased the activity of caspase 3, increased the expression of apoptotic Bax and c-Parp in B-2A13 cells (Yang et al., 2012). Moreover, Liu et al. (2014) also discovered that AFB$_1$ induced an increase in the expression of apoptotic proteins p53 and Bax hence activation of caspase 3 in human hepatocytes (Liu et al., 2014). The results of this study correlated with the previous finding as the expression of p53 (Figure 4.12A), Bax (Figure 4.12 B), caspase 9 (Figure 4.11A) and caspase 3 activity (Figure 4.11C), and c-Parp expression was increased resulting in activating the intrinsic pathway of apoptosis.

Even though c-IAPs are poor inhibitors of caspase activity, they have been linked to the regulation of the NF-$\kappa$b signalling pathway, which can contribute to determination of cell fate (Kocab and Duckett, 2016). The results of this study revealed that AFB$_1$ increased the expression of NF-$\kappa$b (Figure 4.13B) in treated Hek293 cells; NF-$\kappa$b is an apoptosis regulator that activates survival genes. This may predispose kidney cells to the potential cancer-causing effects of AFB$_1$. It has been previously reported that AFB$_1$ increases NF-$\kappa$b protein expression in broiler chicks, which correlates with the observed increased expression in Hek293 cells (Rajput et al., 2019).

The results of this study suggested that AFB$_1$ induced a decrease in cell viability through the induction of oxidative stress which lead to cell death through the activation of the intrinsic pathway of apoptosis. Furthermore, no relationship was established between dosage and AFB$_1$ cytotoxicity in Hek293 cells. However, future studies may use concentrations greater than 100 µM and also evaluate the effects of chronic AFB$_1$ exposure as individuals are exposed to AFB$_1$ on a day to day
basis. However, methods of eradicating or minimizing the toxic effects of AFB$_1$ are essential to maintain human health. The Hek293 cell line was an appropriate model to use for evaluating the effects of AFB$_1$ on kidney cells as it exhibits the expression of membrane proteins and optimal biological activity of target proteins.
CHAPTER 6 : CONCLUSION

The results of this study are summarised in Figure 6.1. The present study demonstrated that exposure to high AFB\textsubscript{1} concentrations inhibited Hek293 cell viability evidenced by the noted decrease in ATP concentrations in treated cells. An overwhelming antioxidant response was demonstrated in AFB\textsubscript{1}-treated Hek293 cells by the upregulation of Nrf-2, SOD2, GPx, Catalase and increased GSH levels which correlated with decreased lipid peroxidation and membrane damage in treated cells. Despite the perceived overwhelming antioxidant response, RNS production increased in AFB\textsubscript{1}-treated Hek293 cells, subjecting cells to high levels of free radicals and resulting in oxidative stress, which was confirmed by the increased expression of Hsp70. In response to free radical production oxidative DNA damage was noted in treated Hek293 cells by increased mRNA levels of OGG1, increased comet tail lengths and fragmented DNA. Furthermore, the observed upregulated p53 in response to DNA damage failed to initiate DNA repair mechanisms, therefore apoptotic cell death mechanisms were activated. The upregulated Bax lead to activation of caspase 9 and 3/7, and subsequent execution of the intrinsic pathway of apoptosis. However, the increase in c-IAP expression hindered the extrinsic pathway of apoptosis by inhibiting caspase 8 activation in treated cells. Confirmation that AFB\textsubscript{1} induced apoptotic cell death in Hek293 cells was noted as necrotic cells decreased in treated cells. Survival mechanisms of escaping cell death induced by AFB\textsubscript{1} were observed in treated cells as NF-\kappaB was upregulated and a decrease in externalisation of PS which is considered as a hallmark of apoptosis was observed.

Further studies need to ascertain the effects chronic exposure to AFB\textsubscript{1} as this study only investigated the effects of acute exposure to AFB\textsubscript{1} in human cells. Considering that most of AFB\textsubscript{1} susceptible foods are a major part of the South African diet, future studies might investigate possibilities of ameliorating the toxic effects of AFB\textsubscript{1} in humans using drugs or medicinal plants. Lastly, this study showed the need for the application of safety measures for controlling AFB\textsubscript{1} concentration in commercially produced products.
Figure 6.1: Proposed mechanisms by which AFB₁ exerts its toxic effects on Hek293 cells.
CARVAJAL-MORENO, M. 2015. Metabolic changes of aflatoxin B1 to become an active carcinogen and the control of this toxin. Immune Research, 11, 1.


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APPENDIX

Appendix A

Table A1: MTT assay raw data used to calculate AFB$_1$ IC$_{50}$

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Appendix B: Nitric oxide synthase (NOS) assay

Figure B1: Standard curve generated from NOS standards.
Appendix C : Protein standardisation

Figure C1 : Protein standardisation curve

Appendix D : Quantitative polymerase reaction (qPCR)

Figure D1 : The amplification of OGG1 gene.
Appendix E: Plagiarism report

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