Deoxynivalenol Downregulates NRF2-induced Cytoprotective Response in Human Hepatocellular Carcinoma (HepG2) Cells.

By

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PREFACE

The experimental work described in this dissertation was carried out in the School of Life Sciences, Department of Medical Biochemistry, University of KwaZulu-Natal, Howard College, under the supervision of Prof. A.A. Chuturgoon and Dr S. Nagiah.

These studies represent original work by the author and have not otherwise been submitted in any form for any degree or diploma to any University. Where use has been made of the work of others it is duly acknowledged within the text.

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DECLARATION

I, Siqiniseko Sinikiwe Ndlovu declare that:

1. The research provided in this dissertation is my original research, unless stated otherwise.

2. This dissertation has not been submitted for any examination or degree at any other university.

3. This dissertation does not contain other person's writing, unless specifically acknowledged, and the source being detailed in the dissertation and reference section.

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Siqiniseko Sinikiwe Ndlovu

ABSTRACT

Deoxynivalenol (DON) is a mycotoxin produced by *Fusarium* species that commonly infect agricultural foods. DON exhibits multiple toxic effects in both animals and humans, binding to the A site of the 28S ribosome and inhibits peptidyl transferase and protein elongation. It induces cytotoxicity through oxidative stress and inhibition of protein synthesis. Liver cells possess the antioxidant signalling mediator - Nuclear erythroid-2-Related factor (NRF2) that is activated in response to oxidative stress. There is no sufficient work done to show if the HepG2cells have an ability to withstand the molecular modifications induced by DON. The aim of the study was to investigate the cytotoxicity of DON and its effect on the NRF2 antioxidant response in HepG2 cells. The MTT assay was used to determine a dose response of DON (72 hr) on cell viability and to generate an IC_{50} value to use in subsequent assays. The intracellular concentration of GSH and ATP was determined using Luminometry. Lipid peroxidation and membrane damage were assessed by TBARS and LDH cytotoxicity assays respectively. Protein expression of NRF2, phosphorylated (p-)NRF2, catalase (CAT), superoxide dismutase (SOD)2, and Sirtuin (Sirt)3 was quantified by Western Blotting. The mRNA expressions of *GPx, CAT* and *SOD2* were quantified using qPCR. DON decreased cell viability in a dose-dependent manner with an IC_{50} value of 26.17 µM. DON caused a significant decrease in the intracellular GSH concentration (1.77-fold, p= 0.0005). There was a significant decrease in the intracellular ATP content (1.92-fold, p= 0.0002).The study shows an induced lipid peroxidation and membrane damage in HepG2 cells by DON, as there was a significant increase in extracellular levels of both MDA (1.89-fold, p=0.0020) and LDH (1.35-fold, p=0.0207). DON reduced total NRF2 expression (0.30-fold, p= 0.0017), however activated p-NRF2 was significantly up-regulated (3.54-fold, p= 0.0085). There was a downregulation in the NRF2 target antioxidant proteins: CAT (0.33-fold, p= 0.005) with a concomitant decrease in *CAT* mRNA levels (0.02-fold, p= 0.0003), SOD2 (0.02-fold, p= 0.0137), with a parallel trend in the levels of *SOD2* mRNA (0.06-fold, p= 0.0020) by DON. This toxin also significantly decreased the mRNA expression of *GPx* levels (0.03-fold, p= 0.0006). The expression of a mitochondrial stress response Sirt3 was significantly decreased (0.14-fold, p= 0.0058). Taken together, the data shows that DON causes oxidative stress and downregulates the NRF2-induced cytoprotection in HepG2 cells.

Keywords:

Deoxynivalenol

Antioxidant response

NRF-2

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PRESENTATION

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LIST OF ABBREVIATIONS ANS UNITS

ADP_ Adenosine diphosphate

AMPK_ Adenosine monophosphate activated protein kinase

ATP_ Adenosine triphosphate

BBB _Blood-Brain Barrier

BCA _Bicinchoninic acid

BHT _Butylated hydroxytoluene

c DNA _Complementary DNA

CCM _Complete culture medium

CREB _Cyclic AMP (cAMP) response binding element protein

Ct _Cycle time

Cu+ _Cuprous ion

Cu2+ _Cupric ion

DAS _Diacetoxyscirpenol

DON _Deoxynivalenol

DBH _Dopamine-β-hydroxylase

DMSO _Dimethyl sulphoxide

dNTPs _Deoxynucleotide triphosphates

ds _Double stranded

ETC _Electron transport chain

FADH2 _Flavin adenine dinucleotide

Fe2+ _Ferrous iron

G _Gravitational force

GIT _Gastrointestinal tract

GPx _Glutathione peroxidase

GR _Glutathione reductase

GSH _Glutathione

GSSG _Glutathione disulfide

GST _Glutathione S-transferase

GSTA2 _Glutathione S-transferase A2

hr _Hour(s)

H_2O_2 _Hydrogen peroxide

HCC _Hepatocellular carcinoma

Hck _hematopoietic cell kinase

HRP _Horseradish peroxidase

IC_{50} _Median inhibition concentration

ICDH _Isocitrate dehydrogenase

IFNγ _Interferon γ

Keap1 _Kelch-like ECH-associated protein 1

LDH _Lactate dehydrogenase assay

LON _Lon protease xv

LPO _Lipid peroxidation

MFO_ Mixed function Oxidase

Mn-SOD2 _Manganese superoxide dismutase

MDA _Melandialdehyde

Min _Minute(s)

MIP _Macrophage inflammatory proteins

mRNA _messenger RNA

mtDNA _Mitochondrial DNA

NAD _Nicotinamide adenine dinucleotide

nDNA _Nuclear DNA

NFDM _Non-fat dry milk

NK _Natural Killer

NMN _Nicotinamide mononucleotide

NOS _Nitric oxide synthase

NRF2 _Nuclear factor-erythroid 2-related factor 2

OH^- _Hydroxyl radical

OXPHOS _Oxidative phosphorylation

PBS _Phosphate buffer saline

PCR _Polymerase chain reaction

PGC-1 _Peroxisome proliferator-activated receptor gamma co- activator

PUFA _Polyunsaturated Fatty Acids

RBD _Relative band density

RLU _Relative light units

RNA _Ribose nucleic acid

ROS _Reactive oxygen species

RT Room temperature

SDH _Succinate dehydrogenase

Sec _Second(s)

SDS-PAGE _Sodium dodecyl sulphate polyacrylamide gel

SIRT _Sirtuins

SGLT-1 _Sodium-Glucose Dependent Transporter

Smac/DIABLO _Second mitochondria-derived activator caspases/DIABLO

SOD _Superoxide dismutase

SOCS _Silencers of Cytokine Signalling

ss _Single stranded

TBA _Thiobarbituric acid

TBARS _Thiobarbituric acid reactive substances

tRNA _transfer RNA

Tfam _Mitochondrial transcription factor A

TTBS _Tris-buffer saline

CHAPTER ONE

GENERAL INTRODUCTION

1.1 Background

Mycotoxins are toxic substances, produced by fungal species as secondary metabolites. They are a family of mostly small molecular weight and structurally diverse compounds (Marin et al., 2013a). Mycotoxins contaminate several agricultural foods, especially cereal grains (maize, wheat, rice and oats), but are also found to contaminate other agricultural food products. They are mainly produced by *Aspergillus*, *Penicillium* and *Fusarium* species during pre-harvest, harvest or storage. When ingested these compounds exhibit toxic effects, inducing significant health complications in both humans and animals post consumption (Shephard, 2008), depending on the dosage, period of exposure and the type of mycotoxin. These toxins are considered to be carcinogenic, mutagenic, teratogenic, oestrogenic, neurotoxic, hepatotoxic, nephrotoxic, cytotoxic and promote immunosuppression in humans and animals (Edite Bezerra da Rocha et al., 2014, Marin et al., 2013b).

Mycotoxins are thus major food contaminants and present a global health concern. The African continent is described as the most susceptible continent to hazards of mycotoxins (Darwish et al., 2014). Acute mycotoxicosis (an illness triggered by a natural fungal produced toxin) outbreaks have been reported frequently in Africa (De Ruyck et al., 2015). It has been reported that a chronic exposure, to even low concentrations of various mycotoxins is a risk factor for human diseases such as cancer and childhood growth retardation (Kimanya, 2015, Katerere et al., 2008). In most developing countries, including South Africa, rural populations are heavily dependent on grains especially maize, as a staple source of nutrition. Many of these communities lack appropriate storage facilities for harvested grains, while hot and humid conditions promote fungal growth and subsequent mycotoxin production in these products (Misihairabgwi et al., 2017).

Deoxynivalenol (DON) 12,13-epoxy-3α,7α,15-trihydroxytrichothec-9-en-8-on, ($C_{15}H_{20}O_6$) is a mycotoxin produced mainly by *Fusarium graminearum* and *Fusarium culmorum,* classified under a family of type B trichothecenes (Rotter, 1996). DON's high melting point (151 –153 °C) enables it to withstand high temperatures and it is not deactivated/eliminated during processing and cooking (Turner et al., 2008) . This characteristic makes DON a persistent food contaminant. The 3 free hydroxy groups (-OH) and an epoxy group on C-12 and C-13 are reported essential for its toxicity (Bonnet et al., 2012a, Pestka, 2010). The mechanism of action of DON is it's binding ability to the A site of the 28S ribosomal subunit after entering the

cell membrane, this promotes the inhibition of the enzyme peptidyl transferase and elongation of the growing peptide (Pestka, 2010). DON exerts multiple effects in humans by affecting the liver, intestines, nervous system, reproductive system and immune system (Sobrova et al., 2010). At a cellular and molecular level, DON has been reported to exhibit cellular toxicity such as DNA and/or RNA breakage, inhibition of protein synthesis, lipid peroxidation and membrane breakage and cellular morphology changes (Zhang et al., 2009 Pestka J.J., 2005, Peng et al., 2017).

Studies have reported that DON causes oxidative stress, which is a consequence of high concentrations of reactive oxygen species (ROS), exceeding the antioxidant capacity of the cell (Mishra et al., 2014, Dinu D. et al., 2011, Zhang et al., 2009). The initial cellular defence against oxidative stress is the production and activation of the endogenous antioxidant molecules which neutralise these ROS. Glutathione (GSH) is a physiologically relevant endogenous antioxidant available in high intracellular concentrations and is the first line of defence against ROS. In its reduced form, GSH reacts with O_2^- and H_2O_2 and neutralise these ROS by the activity of glutathione peroxidase (GPx), promoting its oxidation to GSSG (Hwang et al., 1992, Pastore and Piemonte, 2012). Enzymatic antioxidants, catalase (CAT) and superoxide dismutases (SOD) (Dao et al., 2011, Fukai and Ushio-Fukai, 2011) are also involved in primary antioxidant defence.

This cytoprotective mechanism is driven by the transcription factor NRF2 cooperating dependently with a zinc thiol protein known as Kelch-like ECH-associated protein 1 (Keap1) and is referred to as NRF2- Keap1 (Nguyen et al., 2009, Nguyen et al., 2005, Kensler et al., 2007). NRF2 translocates to the nucleus upon activation by ROS, where it binds to the antioxidant response element (ARE), promoting the transcription of the cytoprotective proteins such as CAT, SOD, GPx and glutathione S-transferase A2 (GSTA2). HepG2 cells are derived from the human liver of a male patient. The liver is the main organ mediating the detoxification processes of a variety of toxins in humans. It was hypothesised that DON causes oxidative stress and dysregulate the NRF-2 antioxidant response in HepG2 cells by inhibiting the antioxidant protein translation. This study investigated the cytotoxicity of DON in HepG2 cells, and the induction of the NRF2 cytoprotective response.

1.2 Problem statement

South Africa (SA) is a developing country; the majority of the population in SA is dependent on agricultural foods, particularly maize (which is highly susceptible to DON contamination). Many studies have been conducted on DON but there is limited research on the molecular effects of DON on the NRF2 response in HepG2 cells.

1.3 Aims and objectives

1.3.1 Aim

- The aim of this study was to investigate the cytotoxicity of DON and the DON-NRF2-induced response in HepG2 cells.

1.3.2 Objectives

The objectives of this study were as follows:

- Determine the GSH antioxidant capacity of HepG2 cells following exposure to DON (GSH) and determine the antioxidant capacity of CAT and SOD post treatment.
- Determine the oxidative damage and mitochondrial toxicity of DON in HepG2 cells post treatment through (TBARS, LDH, Sirt).
- Assess the induction of NRF2 response in HepG2 cells treated with DON.

CHAPTER TWO

LITERATURE REVIEW

2.1 Introduction

2.1.1 Mycotoxins

Mycotoxins (secondary metabolites) are a structurally diverse group of mostly small molecular weight compounds that are produced by fungi that frequently contaminates of agricultural food destined for human and animal consumption. Exposure to mycotoxins results in adverse health effects in humans and animals; generally termed mycosis or mycotoxicosis (Pohland, 1993, Marin et al., 2013b). This includes hepatotoxic, genotoxic, immunosuppressive, nephrotoxic, oestrogenic, teratogenic, as well as carcinogenic effects (Edite Bezerra da Rocha et al., 2014). The level of toxicity depends on the type of toxin(s), concentration, a period of exposure, and various other aspects such as species, age, hormonal status, nutrition and concurrent disease of an individual human being (Wild and Gong, 2010).

Mycotoxins are found worldwide as natural contaminants in numerous agricultural foods, especially in cereal grains (maize, wheat, rice and oats). The most commonly occurring fungi associated with mycotoxin production in agricultural commodities are *Aspergillus*, *Fusarium* and *Penicillium* spp. (Figure 2.1).

Mostly, humans are commonly exposed to mycotoxins directly through ingestion, but may also involve dermal, respiratory, and parenteral routes, the last being related to drugs (Nielsen et al., 2009). Animals are directly vulnerable to mycotoxins through consuming mouldy feedstuffs, contacting mould-infected substrates by the skin and inhaling the spore-borne toxins (Zain, 2011). Mycotoxins can therefore also be found in animal-derived foods (meat, eggs, milk and milk derivatives from animals often consuming contaminated feed. Mycotoxin contamination usually occurs in developing and underdeveloped countries and regularly remains unidentified by professionals, until it affects a lot of people (Peraica et al., 1999) . In developing countries, methods of managing and storing food are improper, hence mycotoxin exposure is more likely to occur. Mycotoxin contamination is shown to be a major problem in Africa (Wagacha and Muthomi, 2008), with implications that affect human and animal health and the economy. Furthermore, mycotoxins are incompletely eliminated during food processing procedures and can persist in commercial food products (Bullerman and Bianchini, 2007).

Figure 2.1: An Illustration of the wheat affected by *Fusarium* species. (Salgado et al., 2011)

Trichothecenes are one of the major classes of mycotoxins, causing a significant economic impact on cereal and grain crops each year (McCormick et al., 2011). Trichothecene are a group of tetracyclic sesquiterpenoid compounds mostly produced by *Fusarium* species consist of more than 200 compounds of diverse toxicity. These chemically related compounds exert toxicity through an epoxy group possessed at C-12 and C-13 and are (Qinghua et al., 2013). Trichothecenes are amphipathic and small molecules that are able to pass passively across cell membranes (Wanda and Haschek, 2009). These molecules are absorbed easily via the integumentary and gastrointestinal structures, into living cells where they cause various health complications (McCormick et al., 2011) (Luongo et al., 2010, Nielsen et al., 2009). Trichothecenes are also documented to inhibit synthesis of the mitochondrial proteins and to react with protein sulfhydryl groups (Pace et al., 1988). Trichothecenes in cells, ultimately cause an increase in the levels of oxidative stress due to a production of free radicals (Nielsen et al., 2009).

2.2 Deoxynivalenol

2.2.1 Production and occurrence

DON is classified as a type B trichothecene. This naturally existing mycotoxin and is a toxic and biologically active secondary metabolite produced by several *Fusarium* species mainly *F. culmorum and F. graminearum* (Casteel et al., 2010). DON is found mostly contaminating wheat, barley, and maize (Casteel et al., 2010). It is also referred to as vomitoxin because its effects induce vomiting post-consumption. DON produces adverse effects in both humans and animals (Sobrova et al., 2010, Wang et al., 2014) .

2.2.2 Chemistry of DON

Deoxynivalenol (12, 13-epoxy-3α,7α, 15- trihydroxytrichothec-9-en-8-on) is a polar organic compound that contains 3 free hydroxy groups (-OH) (figure 2.2) that are associated with its toxicity (Sobrova et al., 2010). Physicochemical properties of DON include its capacity to endure higher temperatures and UV light, making it a health hazard. DON is reported to be stable to heat ranging from 170 to 350 °C, with no reduction of DON concentration after 30 min at 170°C (Hazel and Patel, 2004, Sobrova et al., 2010).

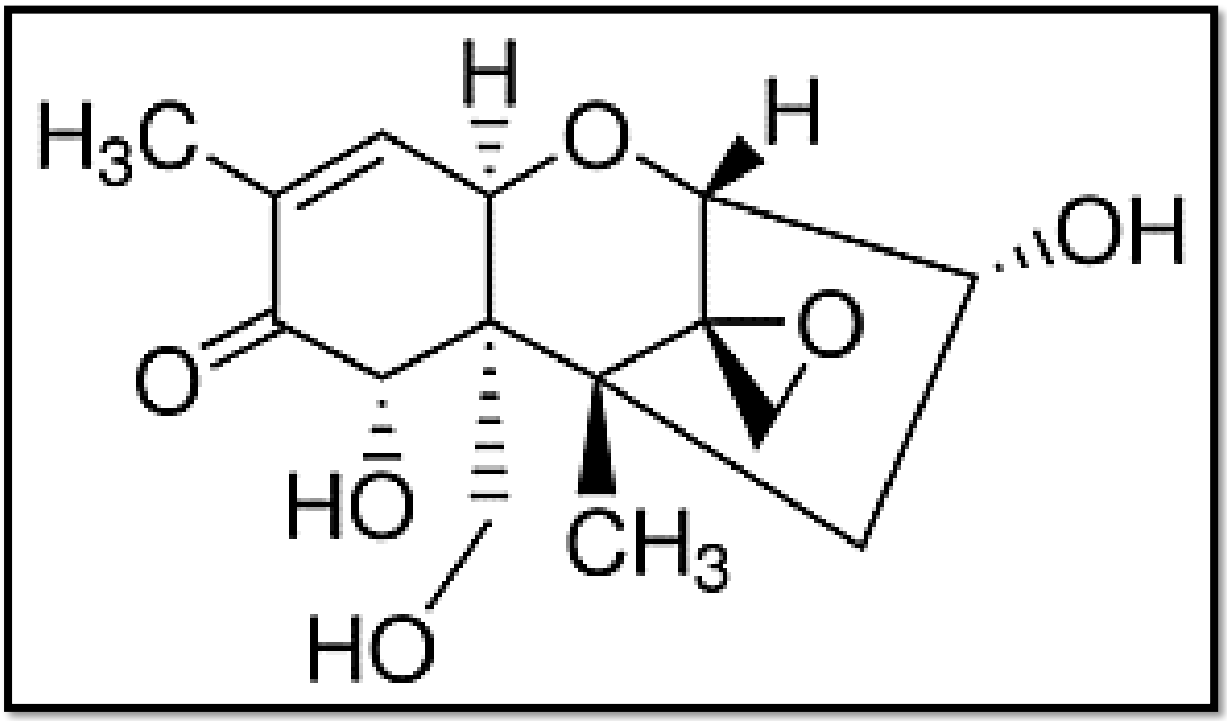

Figure 2.2: The chemical structure of Deoxynivalenol (Awad et al., 2013).

2.2.3 DON absorption, metabolism and distribution

Following the ingestion of DON, it is absorbed through the digestive system where it crosses the intestinal mucosa by the paracellular pathways through the tight junctions. DON is then absorbed into the blood stream and enters other cells through membrane diffusion using the transporters of the cell membrane. Once DON is inside the cell it is metabolised by the intracellular carboxyl-transferase (UDP-glucuronyl transferase) which converts DON to a glucuronide metabolite called glucuronide-DON (Warth et al., 2013). Following glucuronidation, the liver cells are activated to detoxify the DON metabolite (figure 2.3). The detoxification pathway is catalysed by Cytochrome P_{450}, which catalyses the oxidation of DON.

This pathway promotes the cleavage of fresh hydroxyl groups of the DON metabolite, forming DON radicals which are more reactive and dangerous (Sobrova et al., 2012). These radicals can then be scavenged by GPx, SODs or CATs. Phase 1 can be followed by Phase 2 where DON is detoxified by Glutathione-S-transferase (SST) forming a conjugate with GSH in the process (Maresca, 2013).

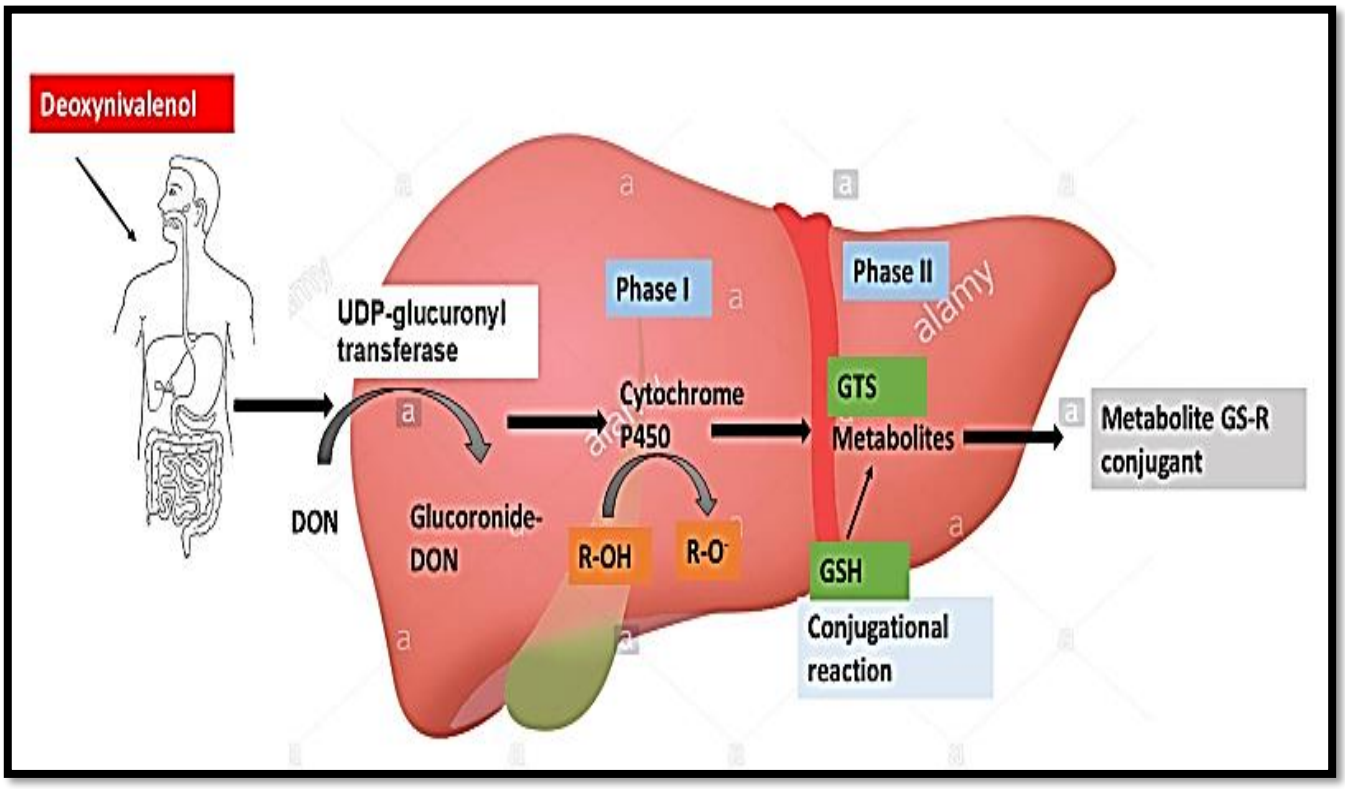

Figure 2.3: The illustration of DON absorption and metabolism. Prepared by Author.

2.2.4 Mechanism of action

Protein synthesis is a process in a cell that enables it to produce proteins (a polypeptide chain), mainly with the help of DNA and RNA. This process is extremely important because the proteins produced are necessary to play roles in almost all the activities of the cell (Shaw et al., 2003). Peptidyl transferase is an essential enzyme located at the large ribosomal subunit. This enzyme catalyses the transfer of the peptide from the transfer RNA (tRNA) occupying the P site to the amino acid of the tRNA occupying the A site (figure 2.4A). A peptide bond (a covalent bond between two adjacent amino acids in a growing protein molecule) is formed and the polypeptide chain is elongated by one amino acid (Hansen et al., 2003). DON has an epoxide at position 12 and 13, that is critical for the action on ribosome (Pestka, 2007, Hassan et al., 2015). DON binds to the 28S ribosomal subunit at the A site of the tRNA through the transpetide bond, this leads to inhibition of peptidyl transferase and subsequent protein elongation (figure 2.4B).

An epoxide group possessed by DON enables it to bind to the ribosomes and react with the nucleotides establishing ribosomal (r)RNA. The DON epoxide moiety can react with nucleophilic groups found on the puric/pyrimidic structures of the nucleotides in both DNA and RNA. These nucleophiles include amine groups and/or the side chain of amino acids forming the proteins that are amine, hydroxyl and carboxyl in nature (Bonnet et al., 2012a).

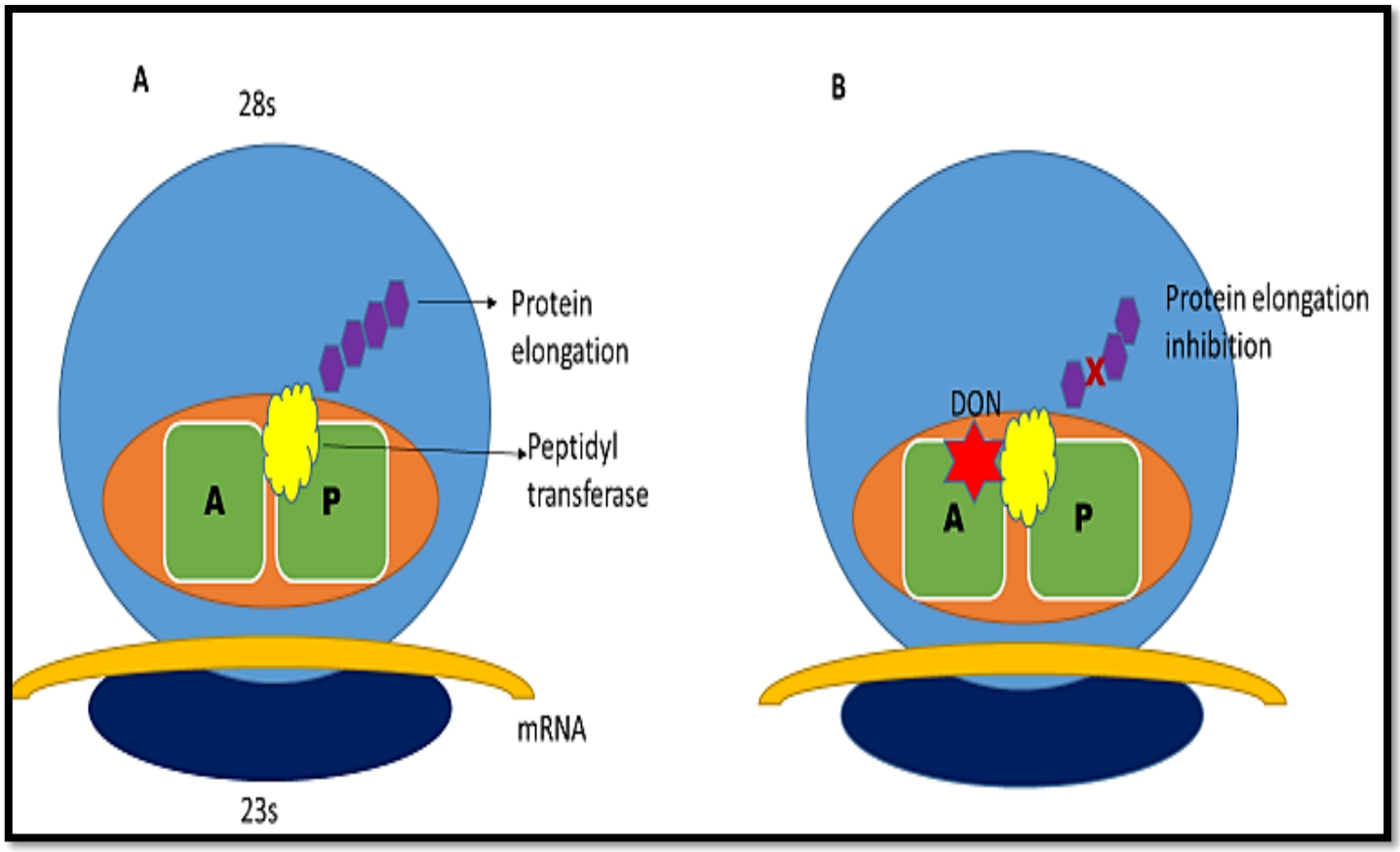

Figure 2.4: The protein translation inhibition, (A) protein translation under normal conditions, (B) protein translation after exposure of cells to DON. DON leads protein translation inhibition. Prepared by Author.

Once DON enters the cell, it binds to rRNA via the interaction of its epoxide group with nucleophile functions on the nucleotides and/or rRNA-linked proteins. This causes the cleavage of proteins and triggers the activation of several cellular signalling pathways eventually disturbing cell functioning and possibly leading to apoptosis (Figure 2.5) (He et al., 2012b). DON activated signal pathways are parallel to the signalling pathways triggered by ribotoxic stress, such as the double-stranded RNA (dsRNA)-activated protein kinase (PKR) and the hematopoietic cell kinase (Hck), which are rRNA associated protein kinases, and the MAP kinases (p38, ERK1/2, JNK), affecting innate immunity and apoptosis involved protein expression (through p53) (He and Pestka, 2010, Pestka, 2007).

Firstly, it was established that DON binds to the rRNA and promotes their cleavage that ends up activating PKR and Hck, hence, the chain activation of MAP kinases, NFκB and apoptosis mechanism (Pestka, 2010). Recent work reported that the induction of apoptosis is not the result, but somewhat the source of the rRNA cleavage upon the activation of caspases and RNases (He et al., 2012a). It has been shown that low concentrations (nM) activate ERK, resulting in cell survival and expression of genes, while high concentrations (μM) trigger p38 resulting in the rRNA cleavage inhibition of protein synthesis and apoptosis (figure 2.5). A long-term relationship of cells and DON triggers the phosphorylation of the MAPKs- Erk1/2, p38 and SAPK/JNK, as well as a decrease of the transepithelial resistance in cells.

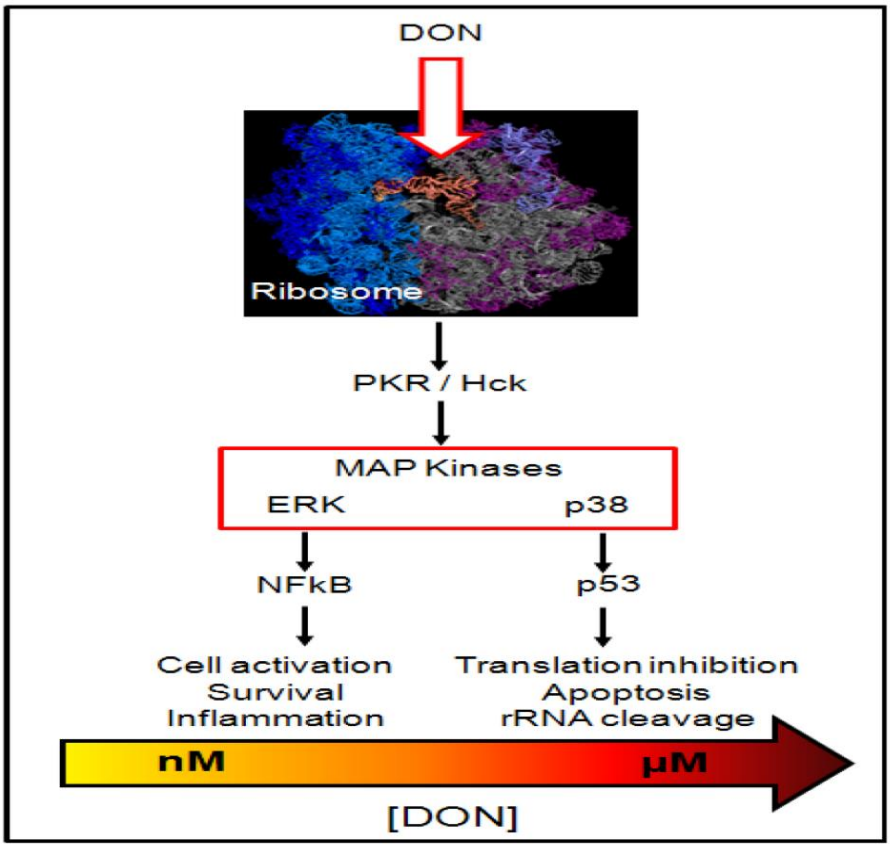

Figure 2.5: DON effects on cell signalling pathways in macrophages, (Bonnet et al., 2012a)

2.2.4 Physiological effects of DON

Deoxynivalenol generally exhibits the toxic effect on both animal and human cells. DON affects different organs including the liver, brain, heart, and intestine. It further affects the immune system and the nervous system. DON induces cellular effects by its capability to promote ribotoxic stress through targeting the ribosome (Zhou et al., 2005).

Usually, the primary target for DON after its consumption is the intestinal tract (figure 2.6). DON affects intestinal digestion and nutrient absorption. The activity of sodium-glucose dependent transporter (SGLT-1) found in the intestinal mucosa is sensitive to DON (Pinton and Oswald, 2014, Pierron et al., 2016). When DON passes through the intestinal epithelium, it attacks the immune system as the second organ system. For the immune system as the second organ system, DON modifies serum IgA concentrations, IgA-related nephropathy immune cells [counting macrophages, T and B lymphocytes and natural killer (NK) cells] are highly susceptible and sensitive to DON. Exposure to DON leads to either immunosuppressive or immunostimulatory/inflammatory effects (Bonnet et al., 2012a).

DON has been shown to negatively affect the endocrine and the nervous systems (figure 2.6). It stimulates the activation of silencers of cytokine signalling (SOCS) which are capable of inhibiting the induction through the hepatocellular release of IGF-1 and IGF acid labile subunit (IGFALS) of growth hormone, ultimately causing growth retardation in growth (Amuzie and Pestka, 2010, Voss, 2010). In brain cells, DON causes perturbations as it can pass the blood-brain barrier (BBB) and affects neurons and neuroglial cells in the brain (Pestka et al., 2008). Studies have shown that DON also affected the kidney and liver cells (Mishra et al., 2014).

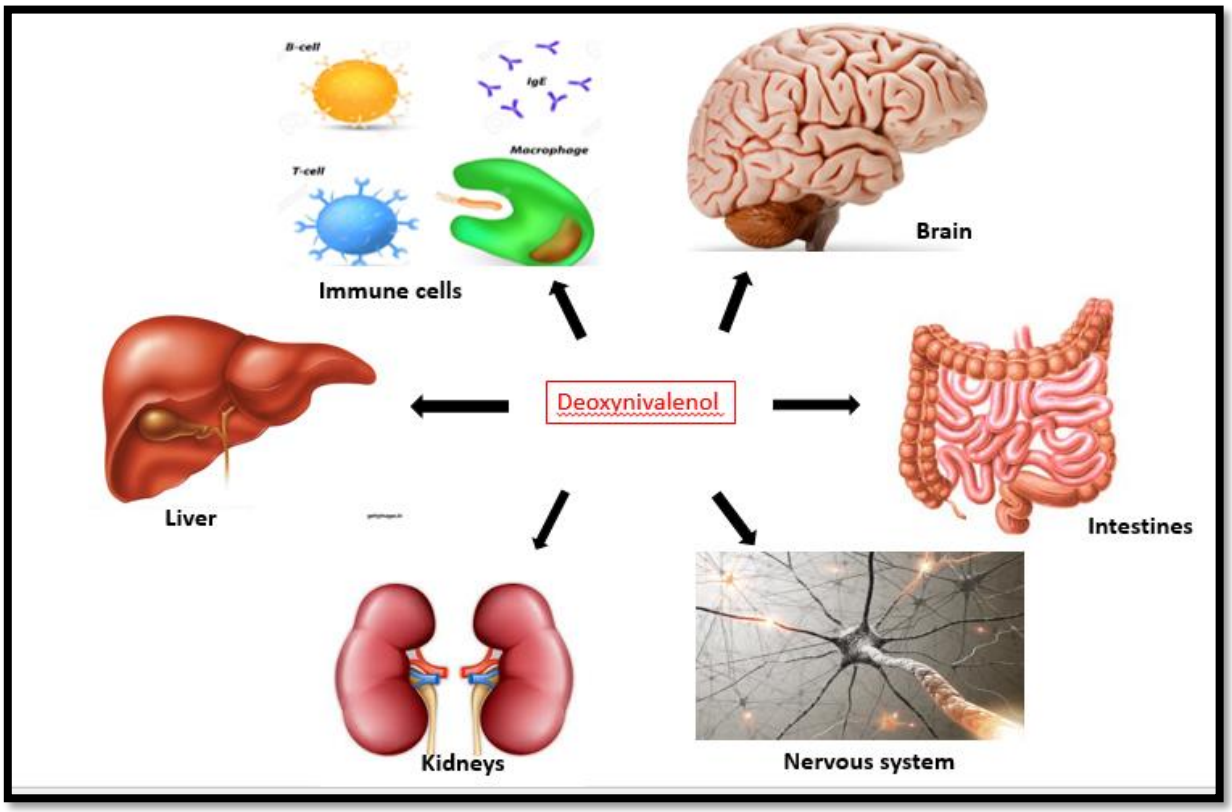

Figure 2.6: The physiological effect of DON, organs affected by the toxicity of DON in human. (Prepared by Author)

2.3 Liver

2.3.1 Liver and Hepatocyte

The liver is the biggest organ (1 500 g) in the body that performs more than 500 essential metabolic functions (Naruse et al., 2007). It plays a major role in the catabolism of glucose from glycogenesis, plasma proteins, coagulation factors and urea secretion; it also regulates amino acid blood levels (Saukkonen et al., 2006). The liver regulates several of chemical species in the blood, at any different concentration. It excretes a bile which aids in the fat breakdown, preparing them for further processes in digestion and absorption. The blood passing the stomach and intestines is processed by the liver, which chemically decomposes and parallel them for the production of nutrients for the body to utilise. Also, it metabolises drugs in the blood stream into forms that are easily utilised by the body. The fundamental role conducted by the liver in the acceptance and conversion of chemicals expose it to toxic injuries in either deactivating or detoxifying them. (Saukkonen et al., 2006).

Liver constitutes approximately 80% of specialized epithelial cells called hepatocytes (figure 2.7), which are highly metabolic and involved in many essential functions such as protein synthesis, detoxification and metabolism of lipids and carbohydrates (Guguen-Guillouzo and Guillouzo, 2010). The liver has a high concentration of mitochondria. Many chronic liver illnesses are related to the gradually damaged mitochondria, responsible for increased concentration of ROS, reduction/depletion of GSH, alkylation of protein, and respiratory complex alterations (Pérez-Carreras et al., 2003, Degli Esposti et al., 2012). Therefore, the huge number of mitochondria are essential for energy and ROS production, the latter being needed for cellular signalling, fine-tuning responses to stress and global adaptations to metabolism (Degli Esposti et al., 2012).

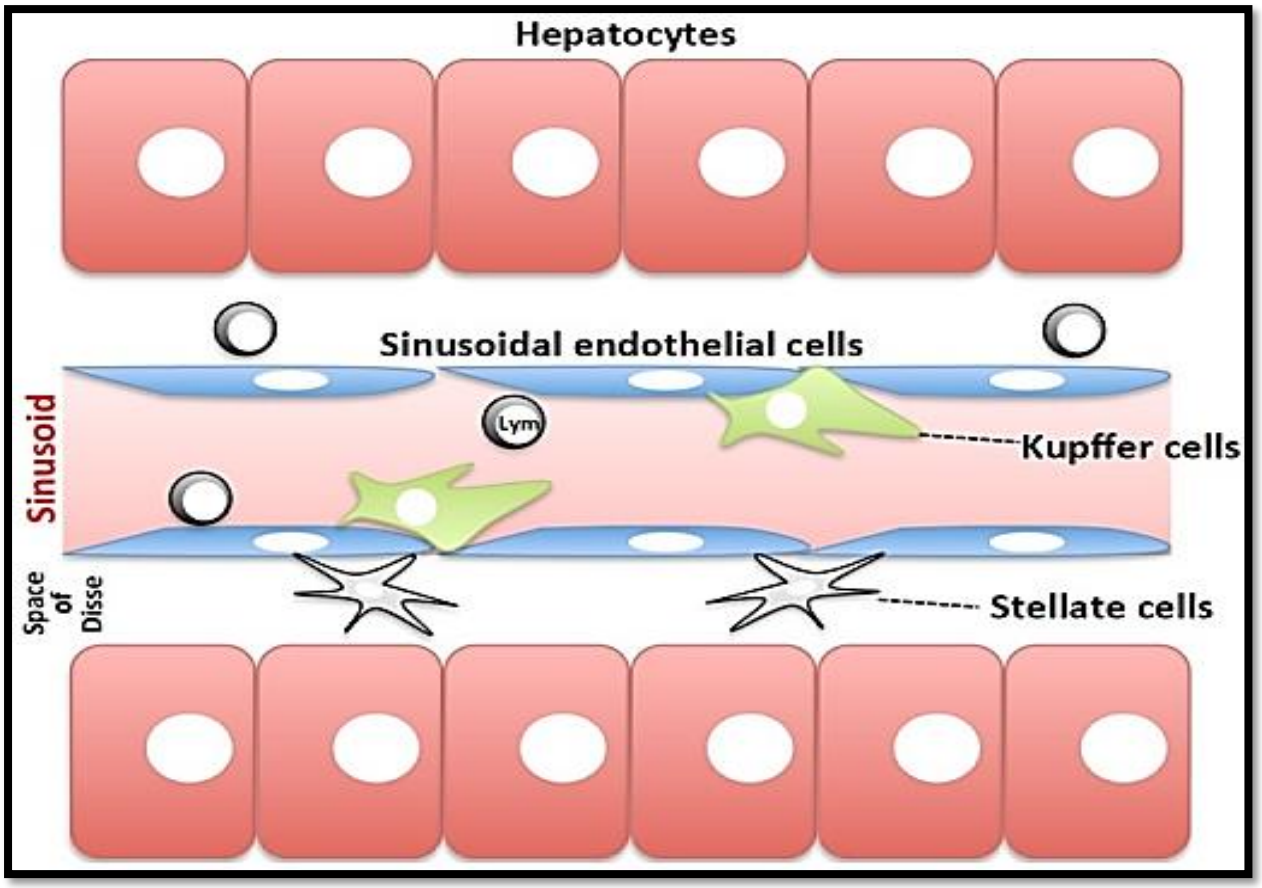

Figure 2.7: Schematic basic structure of the liver, consisting of the hepatocytes cells adjacent to sinusoids and Kupffer cells which are tissue macrophages, as well as the stellate cells in the space between hepatocytes and endothelial cells Prepared by Author.

2.3.2 Detoxification in liver

Liver cells have advanced mechanisms that have evolved to break down toxic substances. The main detoxifying mechanisms of the liver cells are categorised as the Phase 1 and Phase 2 detoxification pathways. The first detoxification pathway (Phase 1) contains an oxidation-reduction and hydrolysis. The enzymes called cytochrome P_{450} group catalyse enzyme following this pathway, and the enzymes are also referred to as mixed function oxidase (MFO) enzymes. (Anzenbacher and Anzenbacherová, 2001). The cytochrome P_{450} enzymes are located on the membrane system of the hepatocytes. Phase 2 detoxification is referred to as the conjugation pathway where the liver cells add a hydrophilic moiety such as glucuronide, sulphate or amino acids (e.g. cysteine, glycine or a sulphur molecule) to a toxic chemical or drug, to render it less harmful (Grant, 1991, Fukao et al., 2004). These reactions modify toxins and allow for their excretion from the body via bile, urine and faeces.

2.3.3 Hepatotoxicity

Hepatotoxicity is damage or injury to the liver as a result of damaging or destructive agents such as drugs, herbal or industrial chemicals which are termed hepatotoxins (Jaeschke et al., 2002). Hepatotoxins gradually damage the liver as the portal vein carries ingested material (which may contain toxins) to the liver in relatively large concentrations. This leads to the gradual injury of the liver by these toxins. Toxins are concentrated in the liver since most detoxification processes take place in this organ (Anita et al., 2011). Depending on the toxin type, toxin concentration and the frequency of exposure, the damage to hepatocytes can be cytotoxic (morphological changes), metabolic (affecting cell metabolism/mitochondria) or genotoxic (DNA damage). The failure of cell survival leads to necrotic hepatocyte death or carcinogenesis (Castell et al., 1997, Anita et al., 2011).

2.3.4 The use of HepG2 cell line

The HepG2 cells are broadly used as an *in vitro* toxicity model, as they are derived from the liver. HepG2 cells show an inducible expression of phase I and phase II enzymes as well as the inducible expression of antioxidant mechanisms toward metabolising xenobiotics (Mersch-Sundermann et al., 2004). This then implies that the HepG2 cells can be employed as an appropriate cell model to determine cellular stress responses.

2.4 Oxidative stress.

ROS are very reactive chemical compounds consisting of several diverse chemical species such as superoxide anion radical (O_2^-), hydroxyl radical ($^{\cdot}OH$), hydrogen peroxide (H_2O_2) and nitric oxide (NO) (Sharma et al., 2012). The regular cellular metabolism in a living organism leads to the generation of ROS, and various environmental factors also contribute to the production of ROS. ROS activity in biological cell processes can be enhanced at a low to average concentrations, but at higher concentrations, they have adverse cellular modifications to molecules including lipids, proteins, and DNA (figure 2.8), Further causing an impaired cellular respiration and ultimately, apoptosis (Valko et al., 2006, Marnett, 1999a). An imbalance in the production of free radicals and the cell's capability to detoxify their detrimental effects through neutralization by antioxidants molecules is called oxidative stress (Cui et al., 2012).

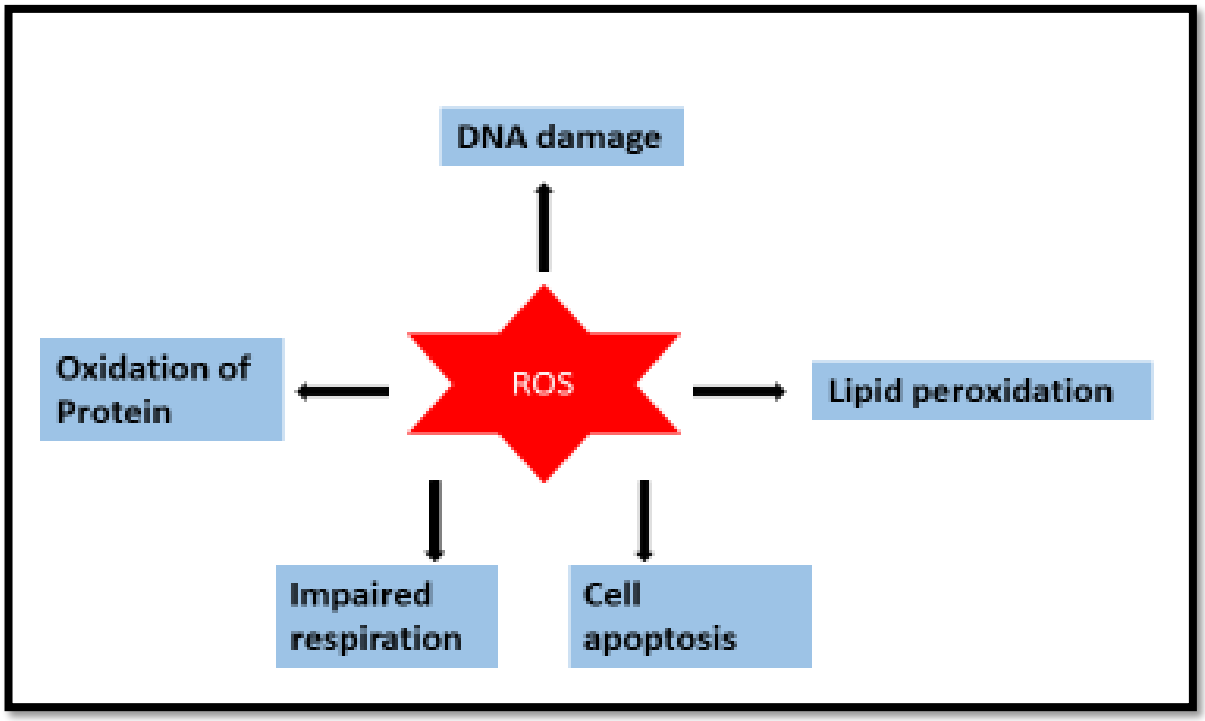

Figure 2.8: ROS effects of on cellular functions. Prepared by Author.

2.4.1 Sources of ROS in a cell.

Mitochondria are the chief sources of intracellular ROS generation in aerobic cells. The electron transport chain (ETC) of mitochondria are the main source of the generation of intracellular ROS (figure 2.9). Also, ETC is an essential target for the detrimental effects of ROS. Mitochondria produce ROS at different sites of ETC (Ott et al., 2007). In mammalian cells, oxygen is reduced to O_2^- in the flavoprotein region of NADH dehydrogenase (complex I) of the respiratory chain (Turrens, 2003). *In vitro* studies on mammalian mitochondria have shown that ubiquinone, a substrate component of the respiratory chain in the mitochondria, linking Complex I with III, and II with III, plays a major role in the formation of O_2^- by Complex III (Liu et al., 2002) . The ubiquinone oxidation occurs during a class of reactions known as the Q-cycle, with semiquinone (unstable) is accounting for O_2^- formation.

Inflammation is also the source of ROS generation. The ROS are the main signalling molecules playing a huge role in the inflammatory disorders progression. Cells like polymorphonuclear neutrophils (PMNs) are involved in the host-defence response and generate ROS, which trigger endothelial impairment by oxidation of important cell signalling proteins such as tyrosine phosphatases. The ROS play a role as either a signalling molecule or a mediator of inflammation (Mittal et al., 2014).

ROS are also generated in the endoplasmic reticulum where NAD(P)H-dependent electron transport requiring Cytochrome P_{450} synthesise $O2^-$. Organic substrate, RH, firstly reacts with Cytochrome P_{450} and reduce it to a radical intermediate (Cytochrome P_{450} R^-) by flavoprotein. In that way, triplet oxygen molecule reacts with this newly formed radical intermediate as each has one unpaired electron. The resulting oxygenated complex (Cytochrome P_{450}-ROO^-) can be reduced by cytochrome b or the complexes may be broken down and release $O_2^{-\cdot}$ (Mittler, 2002).

Peroxisomes were shown to be the major sites for the synthesis of H_2O_2, as a consequence of their oxidative type of metabolism (figure 2.9). In different types of peroxisomes, glycolate oxidase reaction, the fatty acid β-oxidation, the enzymatic reaction of flavin oxidases take place, and the disproportionation of $O2^-$ radicals are the main metabolic processes playing a role in the synthesis of H_2O_2 (del Río et al., 2006).

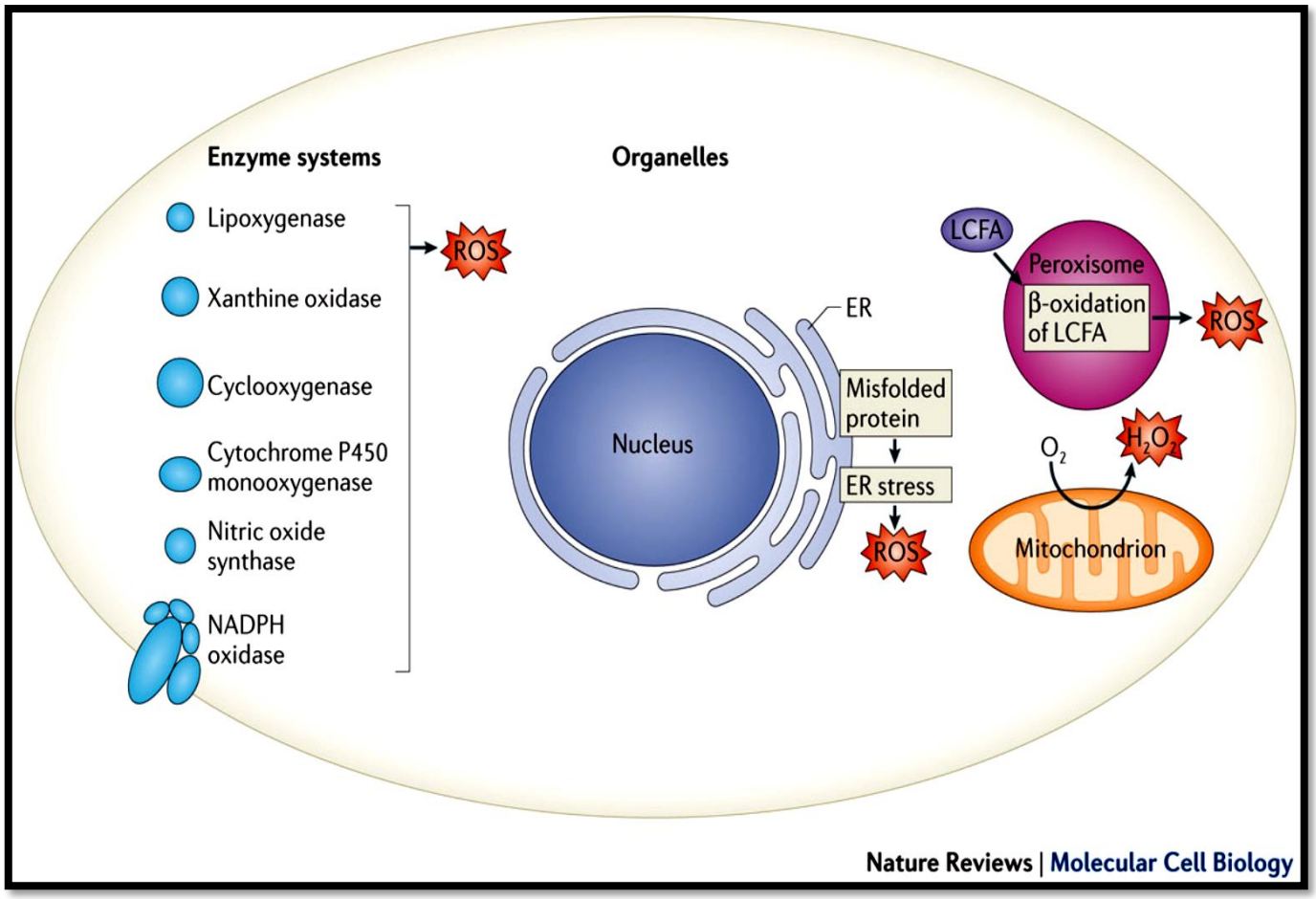

Figure 2.9: Schematic presentation of the various sources of ROS in a cell. (Holmstrom and Finkel, 2014).

2.4.2 Oxidative stress damage in cellular components

When the cell experiences oxidative stress, it strives towards hindering the oxidant effects and re-establish the balanced redox through stimulating or silencing the genes encoding protective enzymes and transcription factors. The ratio of oxidized to reduced glutathione protein (2GSH/GSSG) is the major indicator of oxidative stress (Timothy et al., 1999). The generation of ROS at a higher level in the living organism may modify DNA structure, promoting the alteration of proteins and lipids molecules, activation of numerous transcription factors induced by oxidative stress.

2.4.2.1 Oxidative stress mediated DNA damage.

ROS promotes DNA alterations in numerous ways, which involves degradation of bases, fragmentation of single and/or double-stranded DNA, purine, pyrimidine or modification of sugar-bound, mutations, deletions or translocations, and cross-linking with proteins (Birben et al., 2012). DNA bases oxidation usually includes the addition of •OH to double bonds. The bases impairment is mostly caused by the abstraction of hydrogen from deoxyribose. The hydroxyl radicals are highly reactive towards deoxyribose backbone including its purine and pyrimidine bases (Bohr, 2002). It produces various several by-products from the reaction with DNA bases mostly including 8-oxo-7,8 dehydro-2′- deoxyguanosine from C-8 hydroxylation of guanine, hydroxymethyl urea, urea, thymine glycol, thymine and adenine ring-opened, and saturated products (Tsuboi et al., 1998). Usually, 8-Hydroxyguanine is the primary detected by-product.

Mitochondrial DNA (mtDNA) is one of the important targets by ROS. The mtDNA is very vulnerable to ROS attack because it is adjacent to the respiratory chain, which is the major machine producing free radical, and also, mitochondria lack protecting histones. It has been shown that mitochondrial generated ROS can activate the formation of 8-hydroxydeoxyguanosine, a by-product resulting from of oxidative DNA damage (Ott et al., 2007).

2.4.2.2 Oxidative stress mediated lipid peroxidation.

Higher generation of ROS to above the threshold stimulates lipid peroxidation in either cell and organelle membranes. An increased production of ROS is directly proportional to the elevation of lipid peroxidation under oxidative stresses. The unsaturated fatty acids peroxidation in phospholipids results in the release of malondialdehyde (MDA). MDA is found responsible for the disruption of the cell membrane, and it is used to measure lipid peroxidation in cells (Marnett, 1999b). The development of ROS and activation of lipid peroxidation in mitochondria can result in downregulation of mitochondrial processes. Usually, On phospholipids molecules, ROS attack unstable bond among two carbon atoms and the ester bond between glycerol and a fatty acid. The polyunsaturated fatty acids (glutathione S-transferase) in membrane phospholipids are highly sensitive to ROS attack. It takes a single •OH oxidant to cause the peroxidation of a several PUFAs since the reactions of this process make a cyclic chain reaction (Yin et al., 2011).

Lipid peroxidation process is accomplished in three stages, being the initiation, progression, and termination steps (figure 2.10). The initial step has prooxidants like hydroxyl radical

abstracting the allylic hydrogen resulting in the carbon-centred lipid radical (L•). In the propagation phase, the reaction of the lipid radical L• is facilitated, an oxygen atom reacts with the newly formed lipid radical (L•) rapidly producing a lipid peroxy radical (LOO•), which extracts a hydrogen from another lipid molecule generating a new L• (that proceeds the chain reaction) and lipid hydroperoxide (LOOH) (Yin et al., 2011, Gaschler and Stockwell, 2017, Rådmark et al., 2015). The alkyl, peroxyl, and alkoxyl radicals are created and released in the free radical chain reaction. In the termination reaction, antioxidants like vitamin E donate a hydrogen atom to the LOO• species forming a corresponding vitamin E radical that reacts with another LOO• forming non-radical products.

Under oxidative stress, the rate of the formation of a non-radical product is decreased, hence more radical products are produced (Yin et al., 2011, Ayala et al., 2014b). Peroxidation of poly unsaturated fatty acids (PUFAs) promotes a chain breakage and results in an increase in membrane fluidity and permeability.

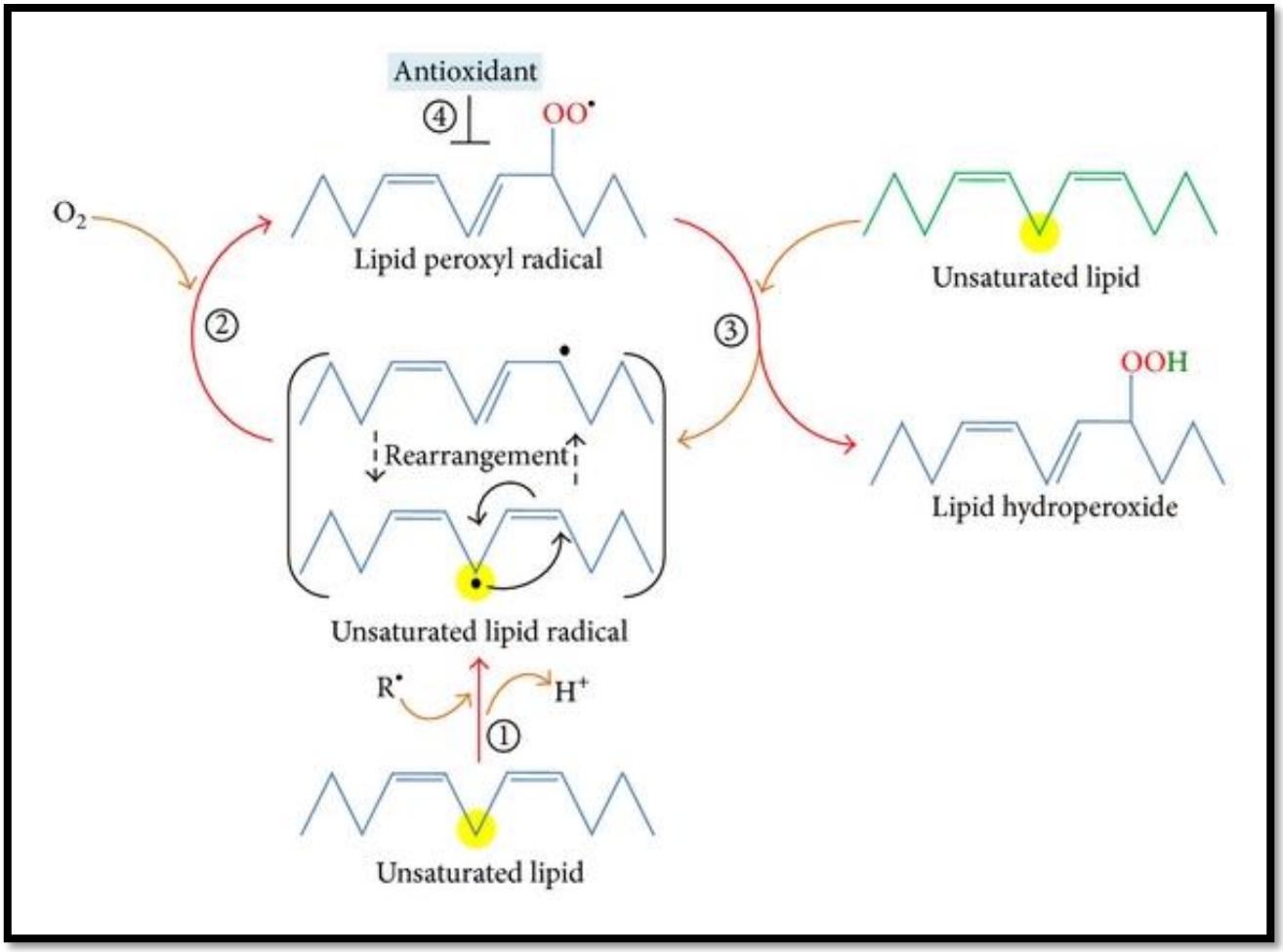

Figure 2.10: Lipid peroxidation process (Ayala et al., 2014b).

2.4.2.3 Oxidative damage to proteins

ROS causes breakage of the polypeptide chain, modification of charges of proteins, cross-linking of proteins, and oxidation of specific amino acids. These increase chances of proteolysis by specified proteases (Kelly and Mudway, 2003). There is a direct and indirect modification of proteins by ROS. The direct modification includes modification of a protein's activity through nitrosylation, carbonylation, disulphide bond formation, and glutathionylation. The indirect modification involves the conjugation with the products, which resulted from peroxidation of fatty acids.

Oxidative stress affected cells generally have a high concentration of carbonylated proteins which are protein oxidation marker. Each amino acid in a peptide varies in its vulnerability to ROS attack (England et al., 2003). ROS highly attack amino acids containing thiol groups and sulphur. Triggered oxygen atom can abstract an H atom from cysteine residues to produce a thiol radical that will cross-link to second thiol radical to form disulphide bridge (figure 2.11) (Davies, 2016).

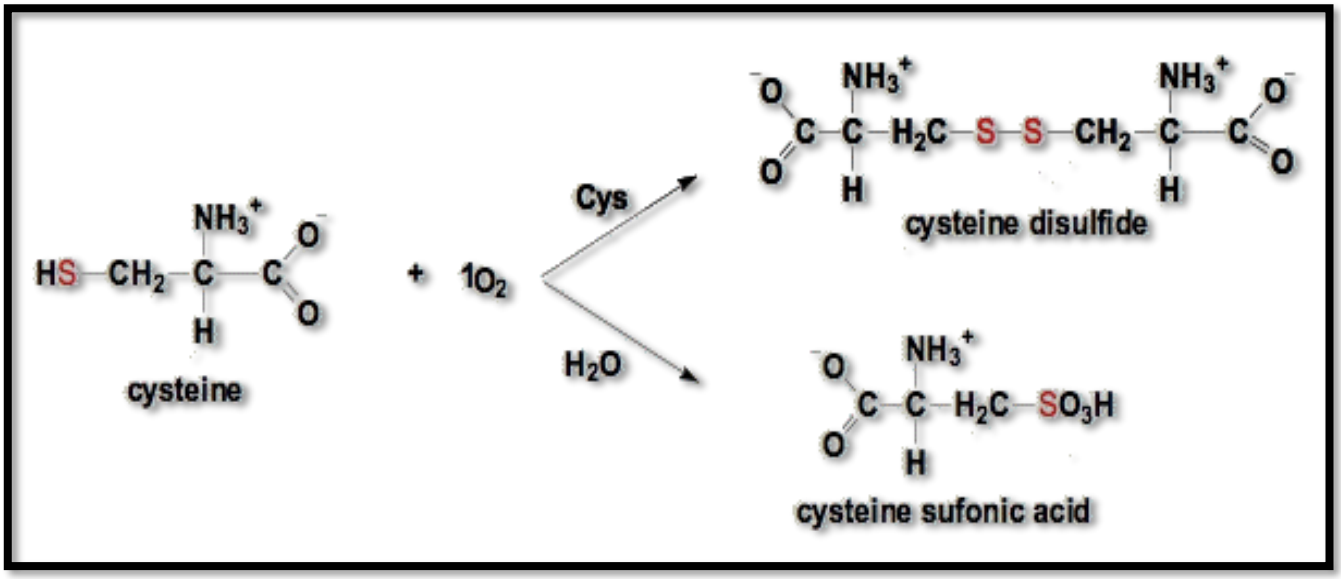

Figure 2.11: The oxidation of protein. The cysteine residue is oxidised by $^{-}O_2$. Prepared by Author.

2.5 Cytoprotective response.

2.5.1 Glutathione (GHS)

Antioxidants are nucleophilic molecules with an ability to radically react with oxidizing agents, which are usually electrophiles, donating a single or two electrons to them. GSH is known as a high-density antioxidant molecule among the cellular endogenous antioxidants. GSH is a peptide that is reduced comprising of three-residues (γ-L-glutamyl-L-cysteinyl glycine) and is able to give an electron to produce its antioxidant effect. This donation results in the oxidation of two-electron donating GSH molecules to form glutathione disulfide (GSSG) (Hwang et al., 1992). GSH is almost exclusively found in a high concentration (1–10 mM) in human. This high concentration enables the easy neutralisation of ROS in either a direct or indirect pathway. It can directly react with O_2^- and some other ROS. Also, it can present an indirect scavenging of ROS, by revitalizing other antioxidants, which are essential; e.g. it can reduce dehydroascorbic acid (Pastore and Piemonte, 2012).

Antioxidant GSH is formed from its essential amino acids, creating a tripeptide thiol under a double ATP-dependent mechanism. The γ-glutamyl-cysteine ligase (GCL) catalyses the first step of the synthesis. GCL is a heterodimeric enzyme composed by a heavy subunit, GCLc (73 kDa) presenting catalytic activity and a smaller one, GCLm (33 kDa) that has a regulatory role on the other subunit (Lu, 2013). The second step is catalysed by GSH synthetase (GS) (figure 2.12).

Cellular GSH is regulated by *de novo* synthesis, and different other aspects like the use, recycling and cellular export, known as the GSH cycle (figure 2.12). The activity of GSH molecule is induced under increased ROS condition, or aerobic respiration which promotes an increased production of H_2O_2 (Lu, 2009a). ROS are neutralised by glutathione peroxidase (GPx) through the conversion of double GSH molecules to GSSG, the oxidized form, this reaction occurs in an expense of Nicotinamide adenine dinucleotide phosphate (NADPH) (Lu, 2009).

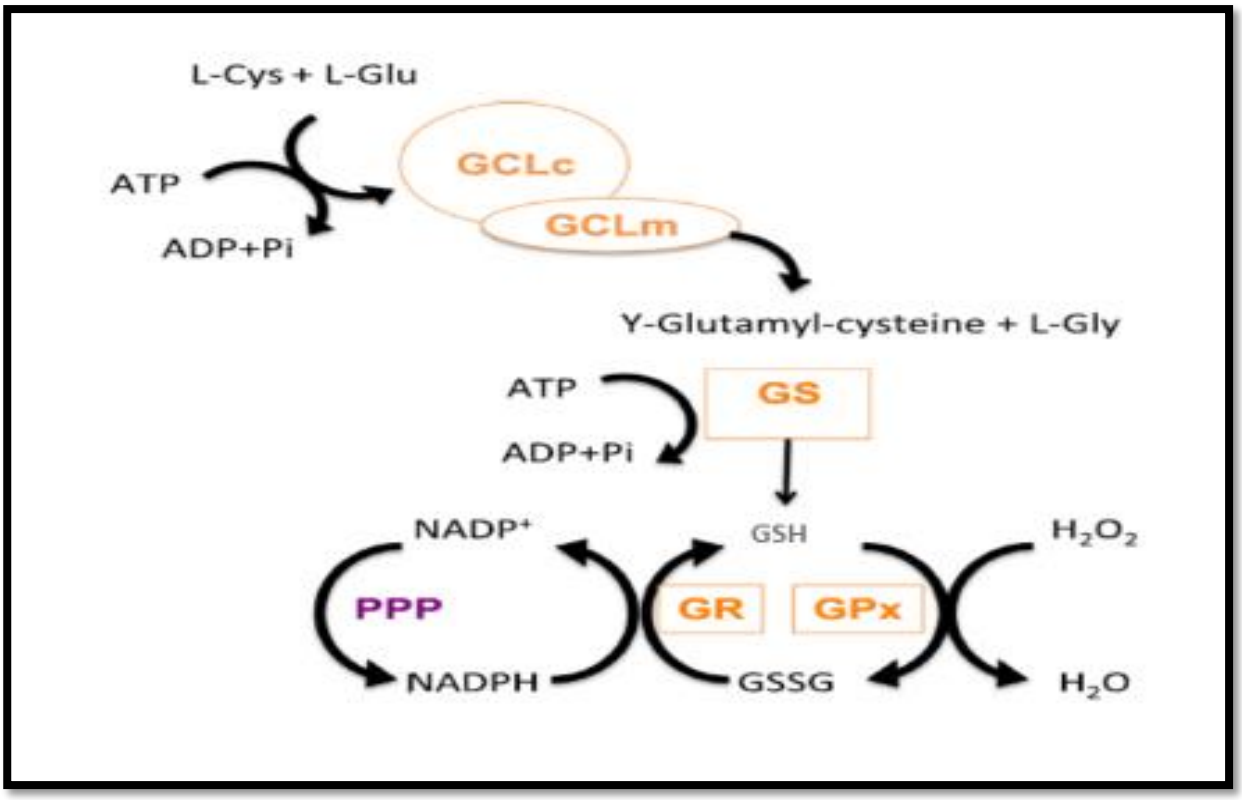

Figure 2.12: Diagrammatic presentation of GSH synthesis and GSH cycle Prepared by Author.

2.5.2 Keap1-NRF2

The nuclear factor erythroid 2-related-factor 2 (NRF2) is a member of the cap 'n' collar (CNC) subfamily of basic region leucine zipper (bZip) transcription factors. NRF2 is a strong transcriptional activator and an essential regulator of the expression of cytoprotective genes for molecules that express antioxidant functions in response to oxidative and electrophilic stresses within the cell (Nguyen et al., 2009). The Keap1 is a cysteine-rich, cytoplasmic, actin cytoskeleton-associated adapter protein of the Cullin3- (Cul3) based E3-ligase complex (McMahon et al., 2003). It has a huge responsibility in the regulation of the activity of NRF2. The Keap1-NRF2 pathway is the major antioxidant signalling mechanism in a cell, induced to respond to ROS and electrophiles activated endogenous and exogenous stresses (Nguyen et al., 2009).

Under homeostatic cell conditions, the NRF2 transcription factor is covalently bound to cysteine residues on its native repressor Keap1 in the cytoplasm. This results in the constitutive ubiquitination and proteosomal degradation of NRF2 causing an inhibition of the anti-oxidant response (McMahon et al., 2003). Under oxidative stress condition, cysteine residues on Keap1 are modified, resulting in the release, phosphorylation, stabilization and

shift of NRF2 into the nucleus, where it binds to the promoter region of the Antioxidant Response Elements (ARE) that possess structural and biological features that characterize its unique responsiveness to oxidative stress at the DNA and initiate the transcription of various cytoprotective enzymes (figure 2.13) (Nguyen et al., 2009, Nguyen et al., 2005).

These enzymes function to promote cellular survival through a variety of mechanisms, including the upregulation of antioxidant function, inflammatory inhibition, and the transport of toxic metabolites. Binding of phosphorylated NRF2 to the ARE results in the recruitment of elements required for the transcriptional activation of a variety of genes such as glutathione S-transferase A2 (GSTA2), NADPH quinone oxidoreductase (NQO-1), superoxide dismutase (SOD) (figure 2.13), and heme oxygenase-1 (Ho-1) and catalase (CAT) (Kensler et al., 2007).

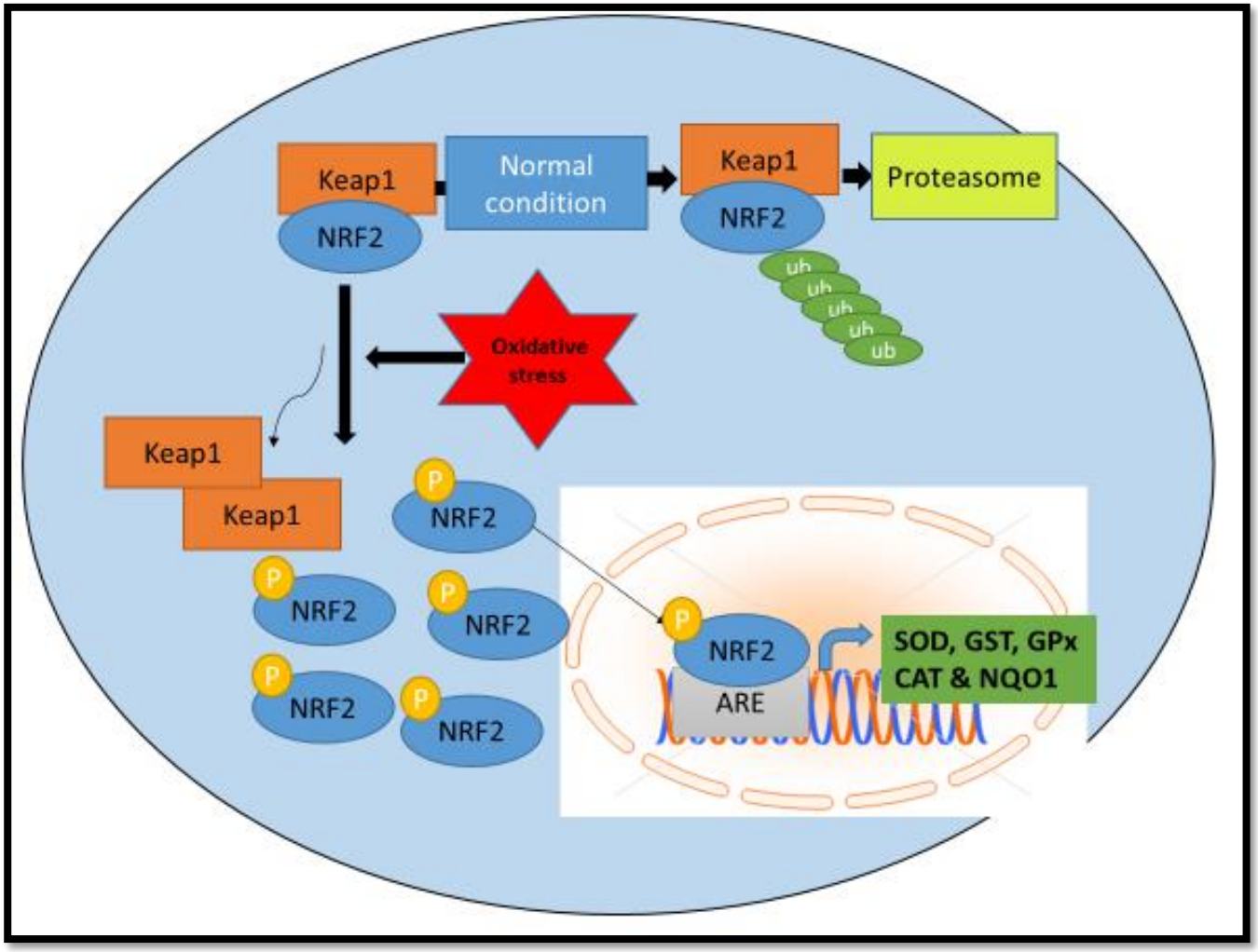

Figure 2.13: The Keap1-NRF2 antioxidant signalling Mechanism. Prepared by Author

The closer biochemical and structural analyses of the Keap1–NRF2 complex shows that under the normal condition, two molecules of Keap1 form a homodimer through the N-terminal BTB domain, and the C-terminal globular domains, called the DC domains, positioned apart from each other (Ogura et al., 2010) (figure 2.14A). Two DC domains of the Keap1 homodimer link with one molecule of NRF2. The N-terminal region of NRF2, called the Neh2 domain, bridges the two DC domains at two separate binding sites, namely the ETGE and DLG motifs (figure 2.14B) (Nioi and Nguyen, 2007, Mitsuishi et al., 2012). The lysine residues clustered in the Neh2 domain between the ETGE and DLG motifs serve as ubiquitination target sites. The two-site binding between Keap1 and NRF2 appears to be favourable for the efficient ubiquitination of NRF2. The ubiquitination thus triggers the proteasomal degradation of NRF2 molecule.

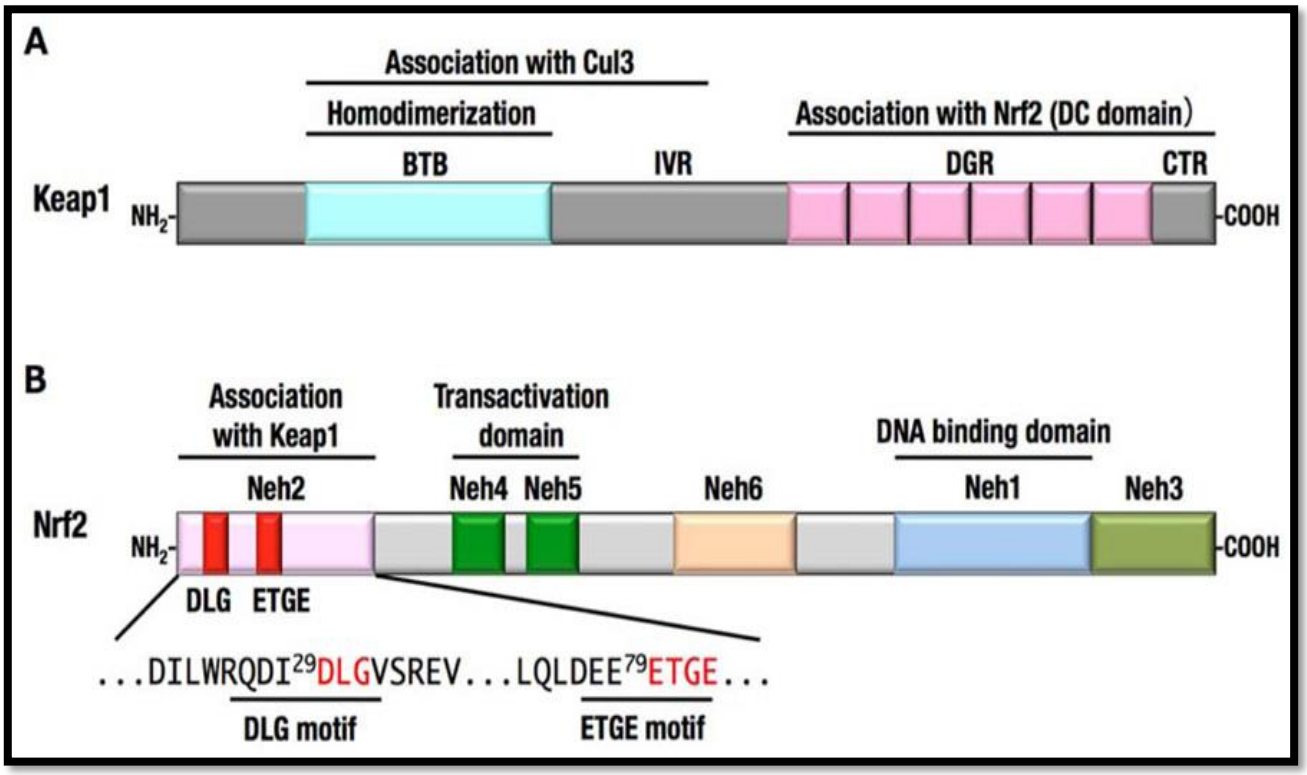

Figure 2.14: the closer illustration of Keap1-NRF2 complex (Mitsuishi et al., 2012)

2.5.3 NRF2 antioxidant response.

The protective response by NRF2 starts with the activation of ARE by phosphorylated NRF2. The consequence of the interaction of NRF2 and ARE is the synthesis of various enzymes that function to defend the cell against oxidative stress. There are three primary antioxidant proteins produced in mammalian cells that are considered necessary in all oxygen metabolizing cells (Weydert and Cullen, 2010). These include SOD, CAT, and a substrate

specific peroxidase and GPx which are produced upon the induction of the NRF2 antioxidant mechanism.

SODs function to protect the cell by catalysing the dismutation of two superoxide anion radicals (plus two protons), converting them into O_2 and H_2O_2, which can be eliminated by CAT and GPxs. Manganese superoxide dismutase (Mn-SOD)/(SOD2) is situated in mitochondria and its gene is located on chromosome 6q25. This enzyme plays a major role in defending the mitochondria from oxidative stress (Mikhak et al., 2008). A recent work reported that SOD2 activity is strongly regulated by its acetylation at several conserved lysine residues such as K68 and K122. Experiments identified a specific interaction between Sirt3 and SOD2 in cell lines and liver (Qiu et al., 2010). Deacetylation of SOD2 lysine residues by Sirt3 triggers and increases SOD2 activity, decreasing cellular ROS (Qiu et al., 2010, Bause and Haigis, 2013). Amazingly, overexpression of SOD2 by itself does not reduce cellular ROS to the same extent as co-expression with Sirt3 or overexpression of the specific lysine to arginine (KR) mutant that mimics the deacetylated state (Tao et al., 2010). This implies that deacetylation by Sirt3 is a highly important regulator of SOD2 activity and O_2^- detoxification (Chen et al., 2011).

CAT mainly functions in the subcellular organelles known as peroxisomes. It functions to reduce H_2O_2 to water and O_2. Such antioxidant enzymes co-operate for optimum detoxification of oxidants, especially with respect to oxidative stress (Dao et al., 2011). The lack of or/and failure to produce antioxidant enzyme could end in mutations, carcinogenesis or cell death as a consequence of ROS interaction with cellular molecules.

In summary, previous studies have showed that DON exerts adverse cytotoxic, genotoxic, and immunotoxic effects on experimental animal cell line. (Königs et al., 2008, Nielsen et al., 2009, Pestka, 2010, Sobrova et al., 2010). Studies on DON cytotoxicity, metabolism and cellular uptake tested in various human cell lines, including human hepatocellular carcinoma cells (HepG2) demonstrated that DON had a distinct cytotoxic effect on human cells, such as decreasing cell viability, inhibiting cell proliferation, inducing oxidative stress, causing mitochondria damage, and both necrotic and apoptotic cell death (Bensassi et al., 2009, Zhang et al., 2009, Warth et al., 2013)

At cellular level, DON has high affinity to ribosomes and can activate mitogen-activated protein kinases (Pestka et al., 2004). By interacting with the peptidyltransferase at the 28S ribosomal subunit, DON inhibits protein synthesis and triggers a ribotoxic stress, which results in cell death (Bonnet et al., 2012a, Hassan et al., 2015). In addition, DON induces the production of free radical and reactive ROS, which causes oxidative damage of target tissues (Sobrova et al., 2010, He and Pestka, 2010, Mishra et al., 2014).

The studies have shown that DON causes oxidative stress and causes both molecular and cellular modification in HepG2 cells (Zhang et al., 2009, Liu et al., 2016, Zhou et al., 2017). There is very limited study on how HepG2 cells respond to DON for survival at both acute and chronic exposure. This study focused on investigating NRF2 response triggered by DON in HepG2 cells, focusing on NRF2 cytoprotective response.

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

The HepG2 cell line was purchased from Highveld Biologicals (Johannesburg, SA). Cell culture equipment and reagents (cell culture flasks etc.) and reagents (Eagle's Minimum Essentials Medium (EMEM), trypsin, trypan blue etc.) were purchased from Lonza Biotechnology (Basel, Switzerland). Western Blot equipment and reagents were purchased from Bio-Rad (Hercules, CA). Deoxynivalenol (DON) was purchase from Sigma-Aldrich (St Louis, MO). The other reagents were purchased from Merck (Darmstadt, Germany), unless otherwise stated.

3.2 Methods

3.2.1 Cell culture and exposure assessment

HepG2 is a cell line comprising of human liver carcinoma cells, derived from the liver tissue of a 15-year-old *Caucasian* male with a well-differentiated hepatocellular carcinoma. These cells are adherent, non-tumorigenic and are epithelial in nature. This cell line is considered a good *in-vitro* model for the toxicity investigation since it has the ability to undergo detoxification responses as well as retaining many functions often lost by primary hepatocyte cultures (Knasmüller et al., 2004). The HepG2 cells were cultured in a monolayer, in a sterile 25 cm^3 culture flask. The cells were maintained in complete culture medium (CCM) consisting of EMEM supplemented with 10 % foetal calf serum, 1 % penicillin-streptomycin-fungizone, and 1 % L-glutamine). The cells were incubated at 37 ˚C.

3.2.2 Assessment

For the determination of a half maximal inhibitory concentration (IC_{50}), the Methyl Thiazol Tetrazolium (MTT) assay was performed (Denizot and Lang, 1986). Firstly, a stock solution of 50mM DON was prepared in 100% Dimethyl Sulphoxide (DMSO) and serially diluted to produce a range of 0-100 µM DON concentration. HepG2 cells were seeded in a 96-well microtiter plate and allowed adhere to the well surface overnight. The cells were then treated with DON (0-100 µM) for 24, 48 and 72 hr, respectively. The chosen time periods allowed the study to either be for acute period or long-term exposure, based on the cell viability response to DON in respect to half maximal inhibitory concentration (IC_{50}). In all other subsequent assays, cells were cultured to 70 % confluency in 25 cm^3 tissue sterile flasks after treatment with DON at the IC_{50} as determined by the MTT assay. The time period of 72 hr was used

throughout subsequent experiments. This is a quantitative measure that determines how much of a drug or another substance (inhibitor) is needed to inhibit a given biological process (or component of a process, i.e. an enzyme, cell, cell receptor or microorganism) by 50 %.

The negative control was used in all experimental assays and consisted of CCM only. The vehicle control (100% DMSO) was also used in all experiments. No significant difference was observed between vehicle control and untreated control; therefore, the vehicle was excluded from further analysis.

3.3 Metabolic activity

3.3.1 MTT assay

3.3.1.1 Introduction

The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay is based on the conversion of MTT into formazan crystals by living cells, which determines mitochondrial activity in living cells (figure 3.1) (Denizot and Lang, 1986). Methylthiazol tetrazolium is a yellow water-soluble dye that is reduced in the mitochondria of metabolically active cells through the activity of a mitochondrial dehydrogenase enzymes. The principle of the MTT assay is that for most viable cells, mitochondrial activity is continuous and thereby an increase or decrease in the number of viable cells is directly proportional to mitochondrial activity (Denizot and Lang, 1986). The mitochondrial activity of the cells is shown by the conversion of the tetrazolium salt MTT into formazan crystals, which can be solubilised for homogenous measurement by spectrophotometry.

The mitochondrial activity in the active cells is dependent on the production of the reducing equivalents produced from flavin adenine dinucleotide ($FADH_2$) and nicotinamide adenine dinucleotide (NADH) in the Krebs's cycle (figure 3.2).

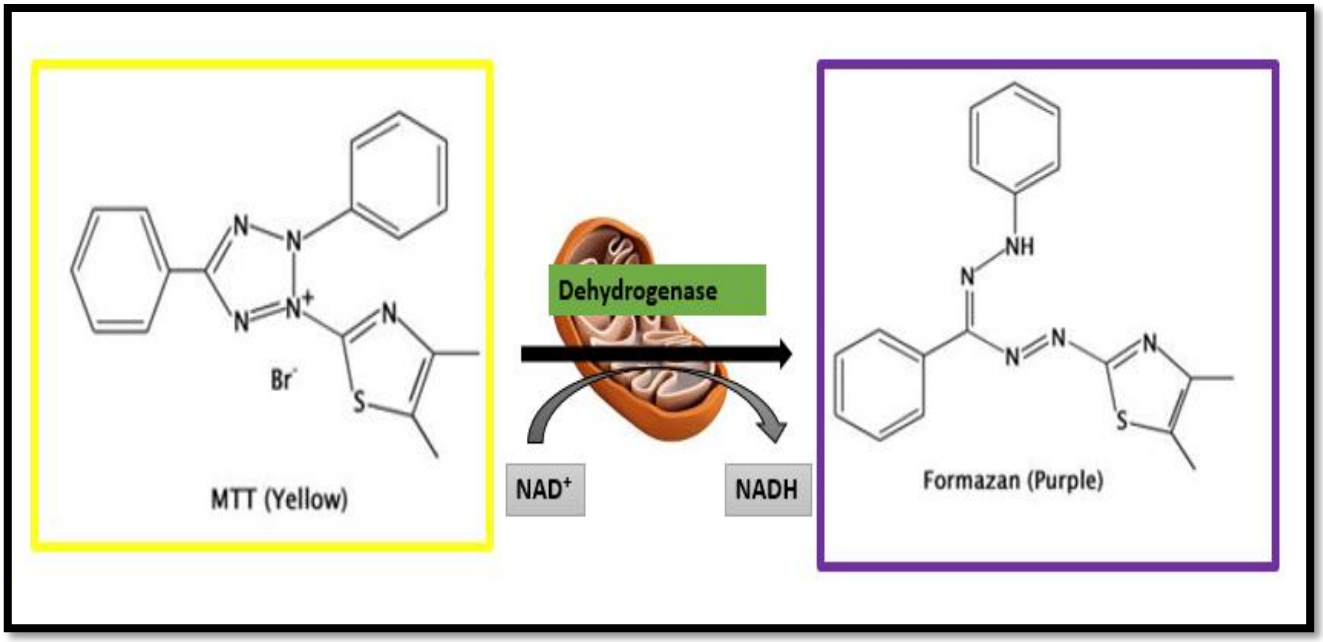

Figure 3.1: The schematic representation of the mitochondrial conversion of MTT salt to formazan crystals in the metabolically active cells during MTT assay. Prepared by Author.

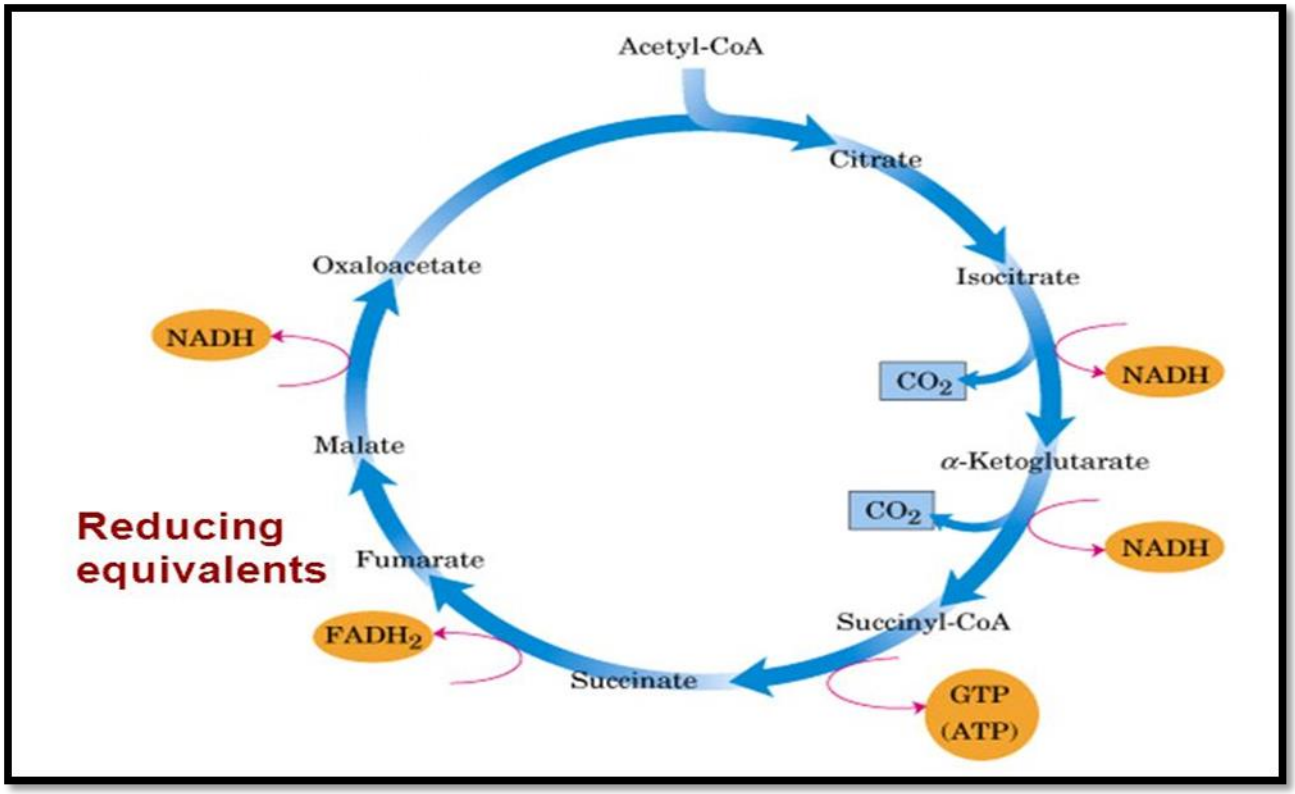

Figure 3.2: The Reducing equivalents produced by the Krebs cycle to accelerate the electron transport chain and maintain the ATP production in HepG2 cells (Fernie et al., 2004)

3.3.1.2 Protocol

HepG2 cells (15 000 cells) were seeded into a 96-well microtiter plate in 6 replicates. At 70% confluency, the cells were treated with DON concentrations (0, 2.5, 5, 10, 25, 50, and 100 µM) DON for 24, 48 and 72 hr. Following treatment, cells were washed with 0.1M Phosphate Buffer Saline (PBS) 3 times and incubated with MTT salt solution (5 mg/ml) in 0.1M PBS in CCM 4 h, 37˚ C. After the 4 hr incubation, the MTT salt solution was discarded, DMSO (100 µl/well) added to the well, and incubated further for 1 hr. The formazan product's optical density was measured using a spectrophotometer (Bio-Tek µQuant) at 570/690 nm. The IC_{50} was determined from the percentage cell viability vs the log concentration. The time period of 72 hr was used throughout the subsequent experiments since it showed a better dose dependent response compared to a 24 and 48 hr period, was 26.17 µM at 72 hr. The IC_{50} value obtained was used in all subsequent experiments.

3.3.2 ATP assay

3.3.2.1 Introduction

ATP is a nucleotide consisting of an adenine attached to a ribose sugar, which is bound to three phosphate groups linked to each another by high-energy bonds called phosphoanhydride bonds. It is the primary source energy in the cell because of these high-energy bonds. ATP acts as an allosteric effector of numerous biochemical cell processes. Most intracellular ATPs are obtained from cytosolic glycolysis and mitochondrial oxidative phosphorylation in the electron transport chain (St-Pierre et al., 2000). The final process in the ETC couples the oxidation of reduced cofactors via the respiratory chain to ATP synthesis by mitochondrial ATP synthase (figure 3.3). The levels of intracellular ATP are indicative of respiratory capacity and mitochondrial function.

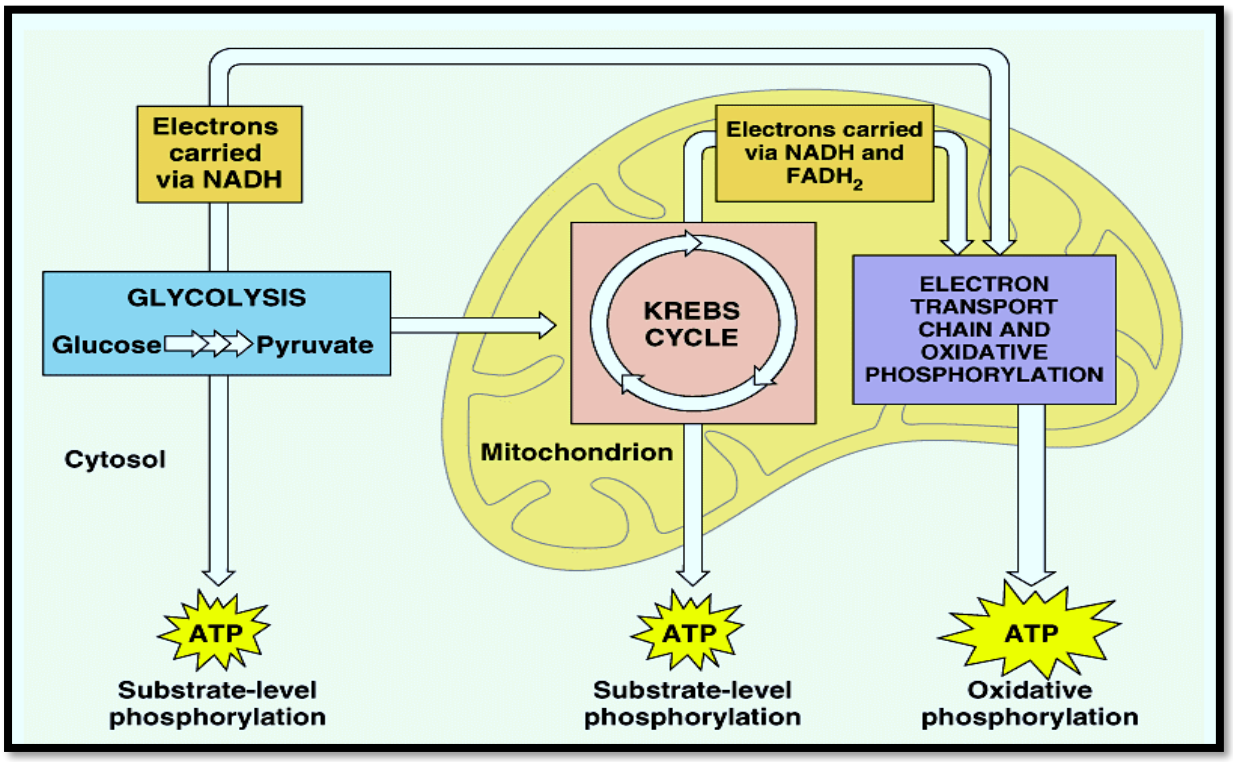

Figure 3.3: The intracellular ATP production, namely glycolysis, the TCA cycle and the ETC. ATP synthesis in the ETC is propelled by an electron transfer via reducing equivalents. Prepared by Author.

3.3.2.2 Protocol

CellTitre-Glo™ (Promega) assay was used for the assessment of intracellular levels of ATP. In this assay, the quantification of intracellular ATP in cells is based on bioluminescence emitted through a luciferase dependent reaction. Luciferin is mono-oxygenated to oxy-luciferin in the presence of Mg^{2+}, molecular oxygen and ATP, resulting in the release of energy in the form of luminescence (figure 3.4). This luminescent signal is directly proportional to the intracellular levels of cellular ATP.

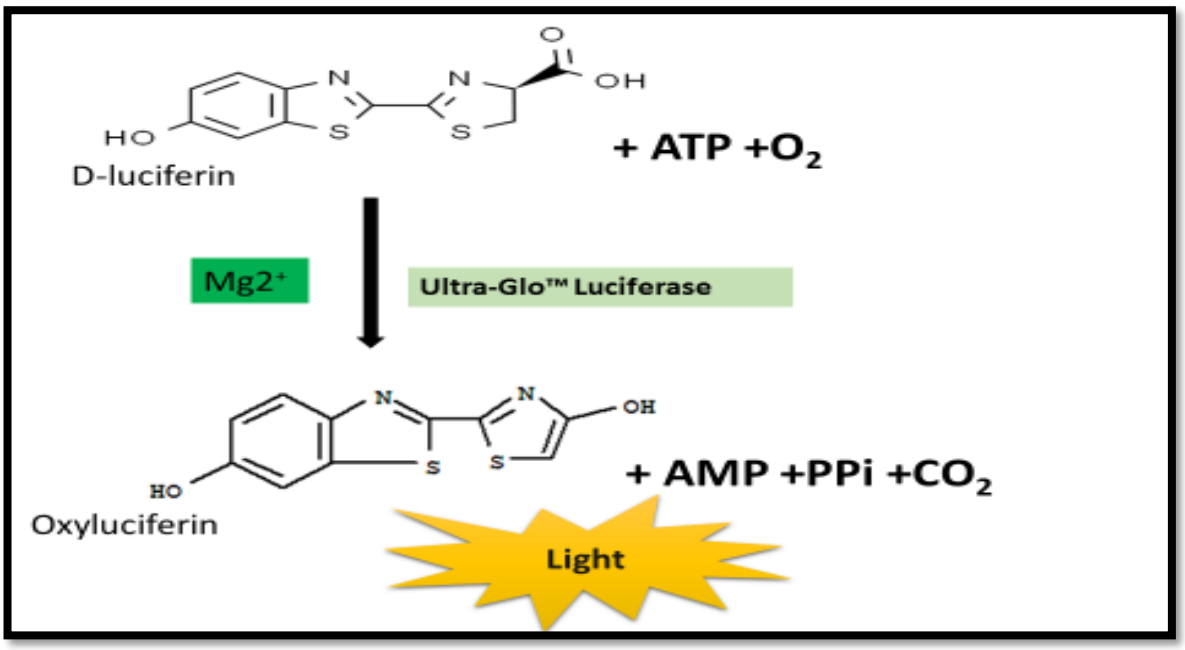

Figure 3.4: Diagrammatic presentation of the CellTitre Glo™ used to quantify intracellular ATP concentration. Prepared by Author.

For the CellTitre-Glo™ assay, 50 µl of HepG2 cell suspension (20,000 cells/well in 0.1M PBS) was seeded into a white, opaque 96-well luminometer plate in triplicate. Following that, 20 µl CellTitre Glo™ reagent was added into each well followed by incubation of the plate in the dark for 30 minutes (min) at room temperature (RT) to let the luciferin-luciferase reaction occur. Luminescence, which is linearly related to the levels of intracellular ATP, was therefore detected using a Modulus™ microplate luminometer (Turner Biosystems, Sunnyvale, USA). The levels of ATP were presented as Relative Light Units (RLU) which is the measurement of ATP; in light, following the luminescence reaction.

3.4 Oxidative stress

3.4.1 Glutathione assay

3.4.1.1 Introduction

Glutathione is a tripeptide consisting of amino acids- cysteine, glutamic acid and glycine. It is an essential antioxidant produced by the body. It prevents a cell from damage caused by free radicals, electrophiles and peroxides (Iwaoka and Tomoda, 1994). Its roles include antioxidant defence, detoxification of electrophilic xenobiotics, redox modulation regulated signal transduction, storage and transport of cysteine, synthesis of deoxyribonucleotide synthesis, regulation of cell proliferation, regulation of immune responses, and regulation of leukotriene and prostaglandin metabolism (Sen, 1999).

Glutathione exists predominantly in its biologically active form as reduced glutathione (GSH). When the cellular environment experiences the presence of ROS, GSH acts as an electron donor and is oxidised to form GSSG. Oxidation of GSH results in a decreased ratio of GSH: GSSG (Iwaoka and Tomoda, 1994). The recycling of GSH and GSSG are carried out by glutathione-S-transferase (GST) and GSH peroxidase (GPx) (Figure 3.5).

The GSH luminometric assay is also based on the reaction of luciferin-luciferase, in this case, Luciferin-NT is converted to Luciferin by GST. This reaction consumes GSH and gives off ATP and oxygen as by-products. Therefore, the conversion of Luciferin by Luciferase to emit light is detected using a luminometer.

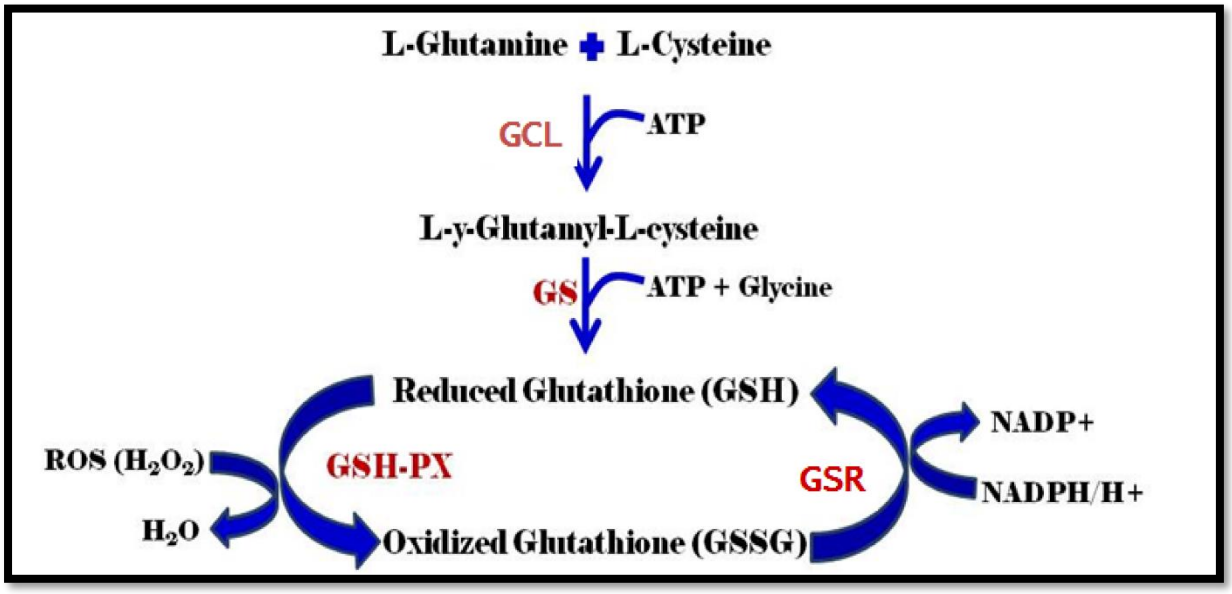

Figure 3.5: The antioxidant activity of GPx in ROS (H_2O_2) cause GSH to generate water and GSSG, while regeneration of GSH from GSSG by GST requires NADPH as a cofactor Prepared by Author.

3.4.2.1 Protocol.

To assess the intracellular concentration of GSH in HepG2 cells, the GSH assay (Promega, Madison, USA) was used. The cells were treated with DON (26.17 µM, 72 hr). Cells were then transferred to a luminometry microtiter plate (50 µl of 20 000 cells in 0.1 M PBS per well, 6 replicates. GSH standards (0, 3.125, 6.25, 12.5, 25, 50 µM) were made from a 5mM stock solution and used to generate the standard curve of a known GSH concentrations. The 2x GSH-Glo™ reagents were prepared according to manufacturer's instructions and added to the plated cells (50 µl/well). The luminometry plate was incubated at RT, 30 min, in darkness. Following incubation, Reconstituted Luciferin detection reagent (50 µl) was added to each well and incubated then further for RT, 15 min, in darkness. Luminescence emitted was then measured on a Modulus™ microplate luminometer (Turner Biosystems, Sunnyvale, CA). The concentration of GSH in HepG2 cells was determined using the standard curve generated using GSH standard concentration. The results are represented in a concentration (µM).

3.4.2 Thiobarbituric acid reactive substances (TBARS) assay

3.4.2.1 Introduction

Lipid peroxidation is a process whereby oxidants such as free radicals, radically attack lipids having carbon-carbon double bond(s), particularly PUFAs in the lipid membrane. This results in the formation and production of a variety of aldehydes (Ayala et al., 2014a). These products are very reactive with other cellular components and the extracellular matrix; they are represented as biomarkers of lipid peroxidation. Among reactive aldehydes, malondialdehyde (MDA) is toxic and an end-product of lipid peroxidation.

In the first step of lipid peroxidation (initiation), oxidizing agents extract a hydrogen atom forming a lipid radical thal radical can be stabilized by a rearrangement of a molecule to form a conjugated diene (Step 1) (figure 3.6). In step 2, the propagation phase, there is a reaction of oxygen with the fatty acid radical producing a fatty acid peroxyl radical, and because of its instability, it removes a hydrogen from another lipid molecule generating a new lipid radical. In the termination reaction (Step 3) the formation of the end-product of lipid peroxidation, malondialdehyde (MDA) is obtained (Fernández et al., 1997, Ayala et al., 2014a).

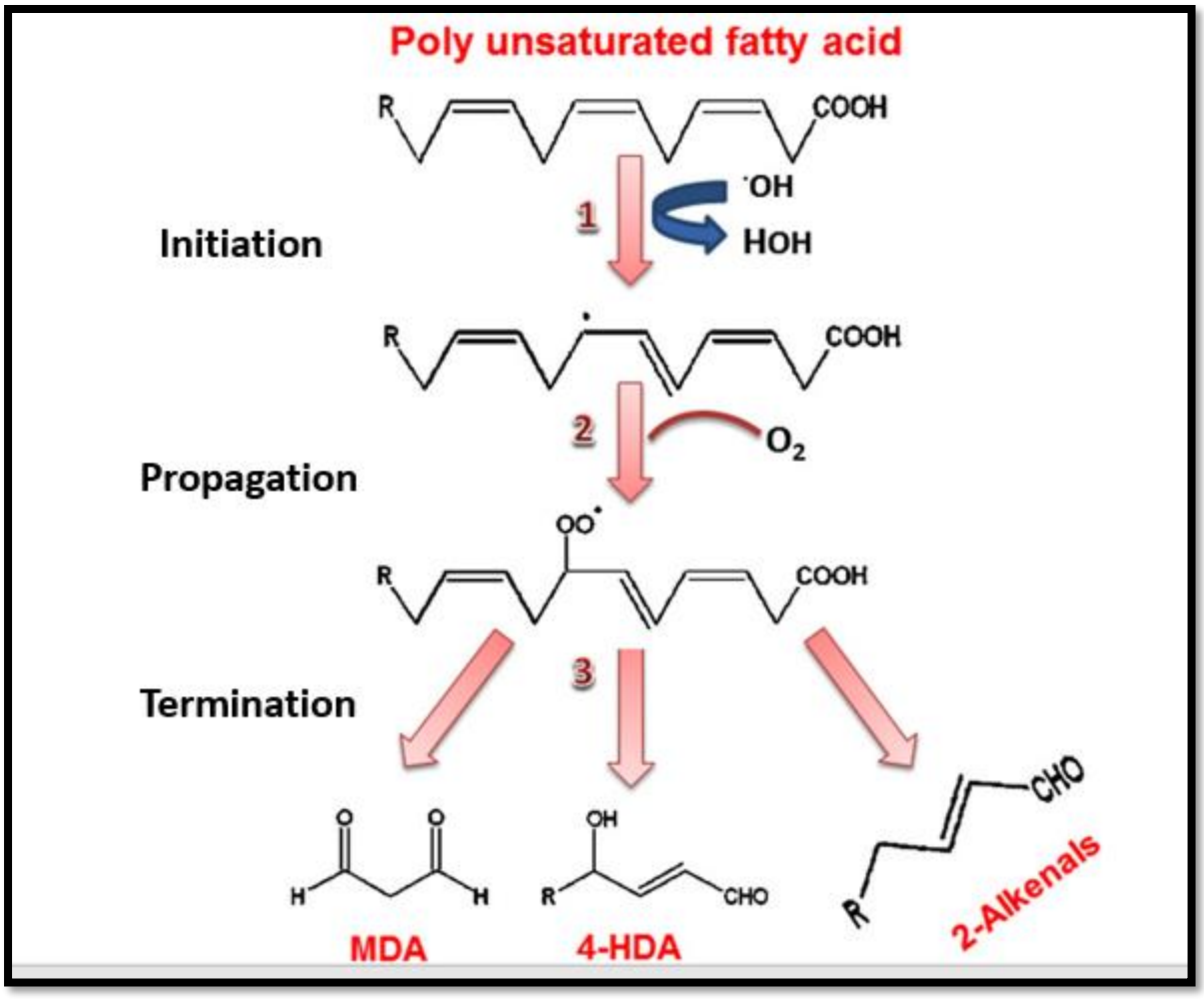

Figure 3.6: The diagrammatic presentation of the lipid peroxidation process. Step 1- Initiation, step 2 - propagation and finally termination on step 3. Prepared by Author.

TBARS assay is carried out to establish the concentration of the end-product of lipid peroxidation, MDA (Asakawa and Matsushita, 1979). The principle behind this assay is based on the ability of two molecules of thiobarbituric acid (TBA) to condense with one molecule of MDA at a high temperature and low pH to form a pink pigment that absorbs light at 532 nm (figure 3.7). MDA reacts with the methylene group of TBA to form adducts. The colour intensity is directly proportional to the concentration of MDA, which is measured using a spectrophotometer (Fernández et al., 1997).

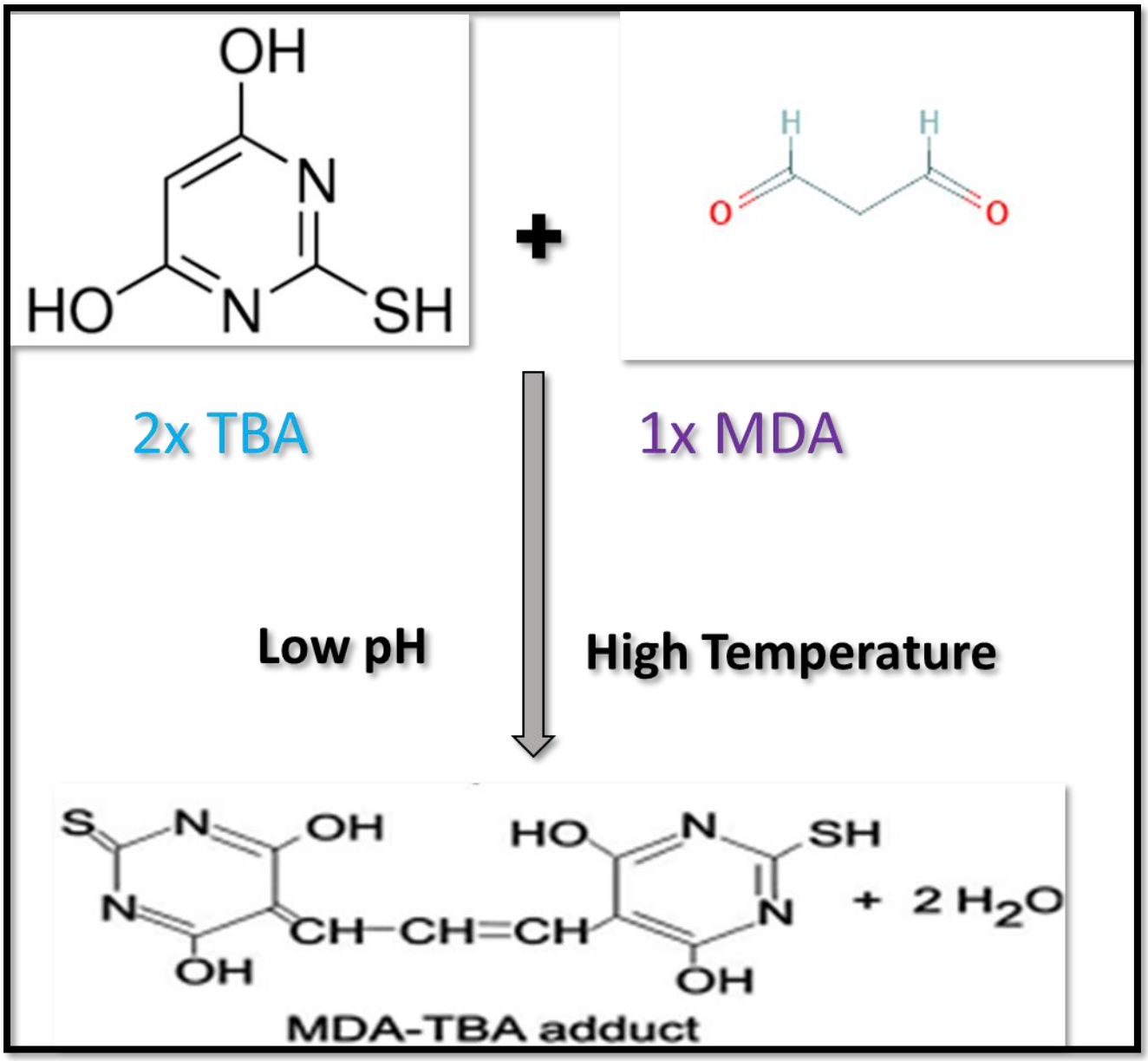

Figure 3.7: The schematic presentation of the TBARS assay principle. Prepared by Author.

3.4.2.2 Protocol

Thiobarbituric acid assay was perfomed to determine oxidative stress (lipid peroxidation). Cell culture supernatant (200 µl) from all treatments were added into the test tubes containing 2% H_3PO_4 (200 µl), 7% H_3PO_4 (200 µl) and TBA/butylated hydroxytoluene solution (400 µl) (Asakawa and Matsushita, 1979, Fernández et al., 1997)**.** Subsequently, MDA (1 µl) was added to the positive control test tube and hydrochloric acid (HCl 400 µl) to the blank test tube. Individually, samples were vortexed and adjusted to pH 1.5, and were boiled for 15 min, then allowed to cool to RT prior to the addition of butanol (1.5 ml). Each tube was vortexed and allowed to separate into two separate phases. The upper phase (butanol) was transferred

from each sample to a sterile test tube and further centrifuged (2 500 x g, 24˚C, 6 min). Following centrifugation, the butanol phase of each sample was transferred to the microtiter plate in 4 replicates. The absorbance was measured on a spectrophotometer at 532 nm with a reference wavelength of 600 nm. The mean optical density of 4 replicates per treatment was calculated and divided by absorption coefficient (156 nm^{-1}) and results expressed in concentration of MDA (µM).

3.5 Cytotoxicity – Lactate Dehydrogenase (LDH) assay

3.5.1 Introduction

Lactate dehydrogenase (LDH) is a soluble cytoplasmic enzyme that is present in the cytoplasm of intact, healthy cells (Korzeniewski and Callewaert, 1983). An increased in LDH's extracellular activity, is the consequence of disrupted cell membrane integrity. This disruption of the cell membrane integrity occurs during lipid peroxidation, under the influence of free radicals and in an oxidative stress environment.

To investigate the leakage of LDH into cell culture medium, the activity of LDH is assessed via an enzymatic test where tetrazolium salt is used (Burd and Usategui-Gomez, 1973). The primary reaction, is production of reduced (NADH) by LDH when it catalyses the oxidation of lactate to pyruvate (figure 3.8). The secondary reaction, is a conversion of tetrazolium salt to a coloured formazan product using newly synthesized NADH in the presence of an electron acceptor.

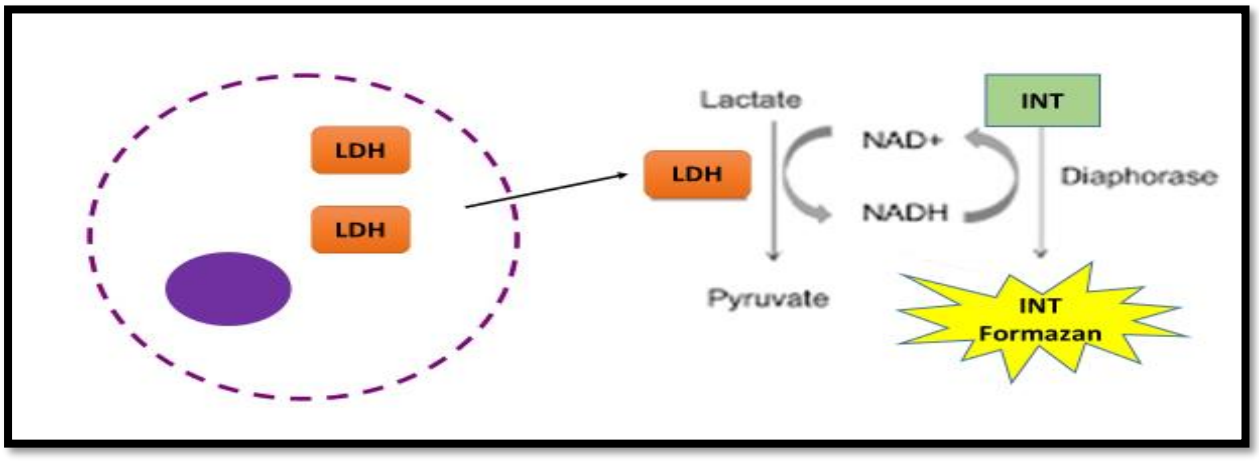

Figure 3.8: The principle of an LDH assay. Intracellular LDH is released into the extracellular matrix when cell membrane integrity is disrupted. Extracellular LDH is detected and measured by means of the oxidation of lactate which is associated to reduction of NAD to NADH which is then used to produce a coloured substrate iodonitroteformazan (INT formazan) quantifiable via colorimetric analysis. (Prepared by Author).

3.5.2 Protocol

Extracellular levels of LDH were quantified using a Cytotoxicity Detection Kit (11644793001) (Roche, Mannheim, Germany). The supernatant (100 µl) from control or treated sample was added into a microtiter plate in 4 replicates. The substrate mixture (100 µl) consisting of a catalyst (diaphorase/NAD^+) and a dye solution (INT/sodium lactate) was added to the supernatant (cell culture medium) and the mixture was allowed to react at RT for 25 min. Optical density was measured at 500 nm wavelength (Microplate reader -Bio-Teck µQuant) and results were presented as mean optical density.

3.6 Protein expression - Western Blotting.

3.6.1. Introduction

Western Blot is a very important laboratory technique used in cellular and molecular biology. It is a technique followed to identify the expression of specific proteins from a complex mixture extracted from the cells. In this technique, proteins are separated through a gel based on their molecular weight (Burnette, 1981). Separated proteins are electro-transferred to a firm membrane which is probed by antibodies (Abs) that bind to the targeted specific proteins. The detection of the protein of interest is magnified in a membrane where the chemiluminescence is developed using a substrate that binds to an enzyme attached to the Ab. Proteins are represented in a membrane as bands and the band width is linearly related to the concentration of the respective protein.

3.6.1.1 Protein quantification - Bicinchoninic Acid (BCA) Assay

The Bicinchoninic Acid (BCA) assay was used to quantify the crude protein in samples. The BCA assay allows for the measurement of Cu^+ production which result when peptide bond in a sample react with Cu^{2+} under alkaline conditions (figure 3.9) (Hill and Straka, 1988). Two BCA molecules react with the Cu^+ chromophore to produce a purple chromophore, which can be measured at 562 nm. The interaction between BCA, Cu^{2+} with the amino acid residues (Tryptophan, Tyrosine and Cysteine) in the protein sample result in the production of the purple colour and can be measured in a spectrophotometer (Bainor et al., 2011). The intensity of the colour is directly proportional to the concentration of the proteins.

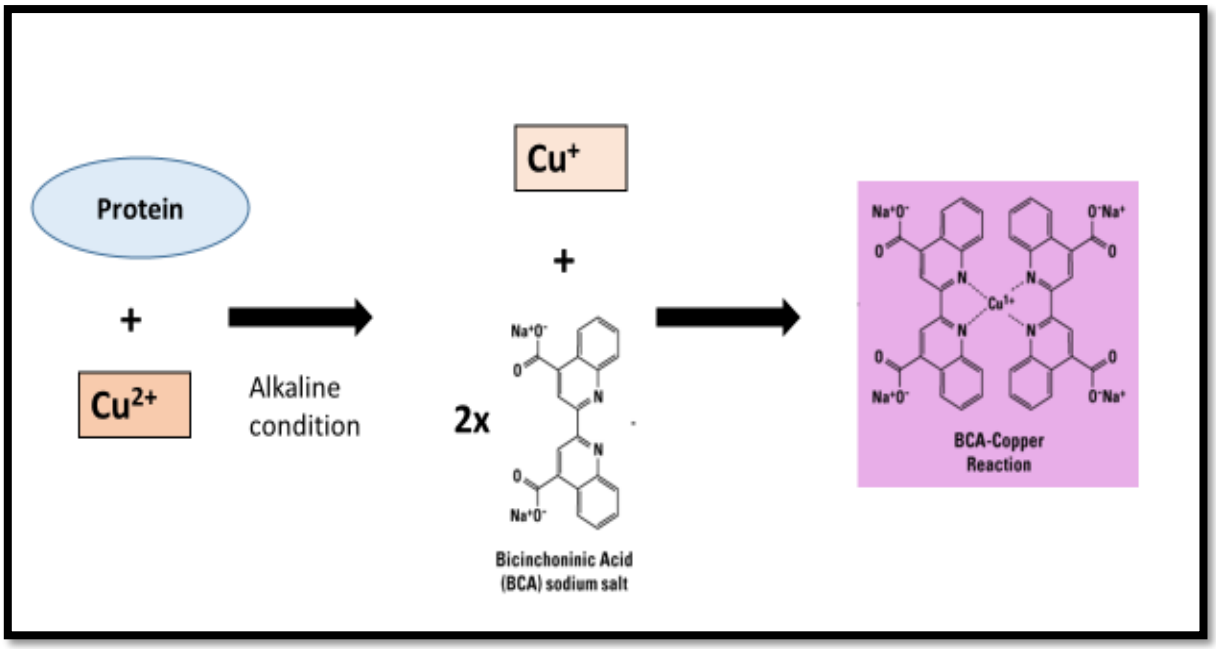

Figure 3.9: The principle of the BCA assay used to quantify protein concentration. Prepared by Author.

3.6.1.2 SDS – Protein Denaturing

This technique allows for the separation of macromolecules in an electric field via electrophoresis. The separation of proteins by electrophoresis uses an immobile polyacrylamide gel as a medium and sodium dodecyl sulphate (SDS) which binds to the polypeptide to denature the proteins by disturbing the non-covalent bonds (figure 3.10) (Mahmood and Yang, 2012, Burnette, 1981). The negative charge of SDS causes the proteins to be strongly attracted towards the anode (positively charged) in an electric field.

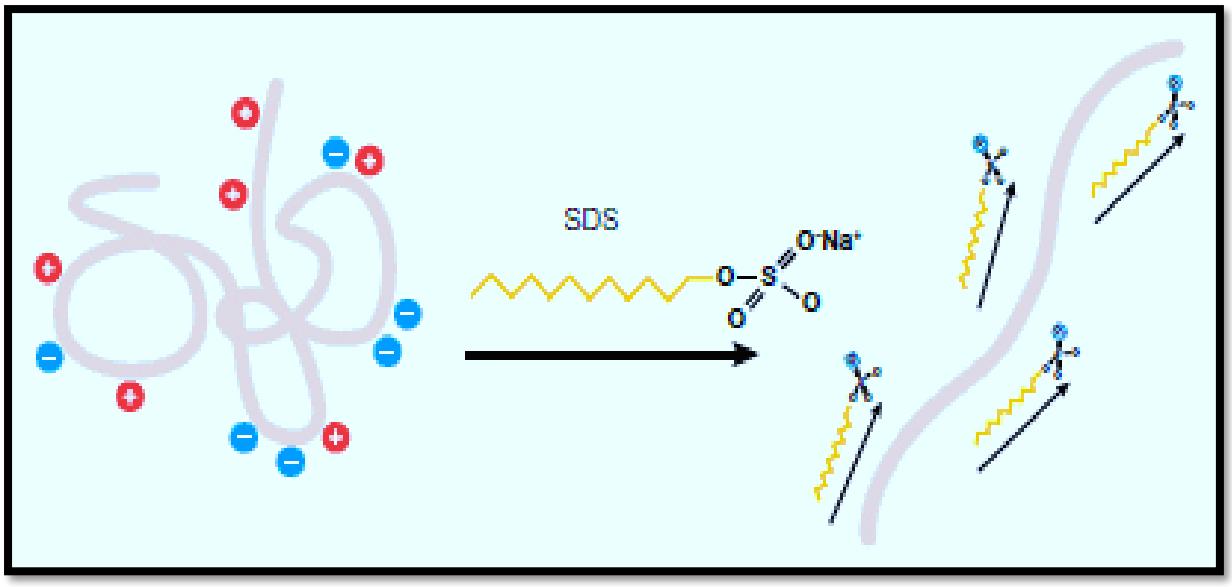

Figure 3.10: The denaturing of proteins by SDS, producing the negatively charged unfolded protein version (Laemmli, 1970).

3.6.1.3 Protein Separation

PAGE (Polyacrylamide Gel Electrophoresis), is an analytical technique used to separate components of a protein mixture according to their sizes in a gel. For the formation of the gel, there is a polymerization of reaction, based on bis-acrylamide, which forms cross-links between two acrylamide molecules. The concentration of bis-acrylamide is inversely proportional to the pore sizes in the gel. PAGE is made of 2 gels: stacking and separating gel (Mahmood and Yang, 2012). Above, is a slightly acidic (pH 6.8) stacking gel which contains a lower concentration of acrylamide making a porous gel, that separates protein poorly yet allows them to form sharply, thin clear bands (Kurien and Scofield, 2006). The lower gel is called the resolving gel. This one is basic (pH 8.8) and has a higher concentration of polyacrylamide, making the gel's pores thinner (figure 3.11). Proteins are therefore separated by their sizes this in gel with the smaller proteins allowed to travel more easily, and rapidly than larger proteins.

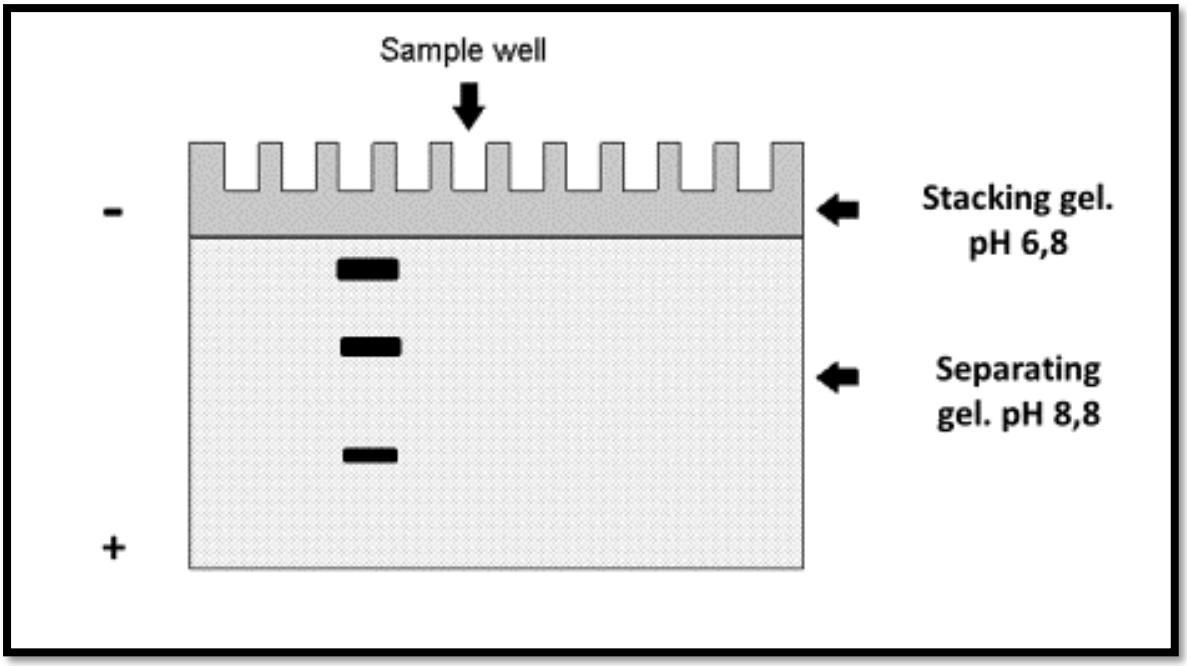

Figure 3.11: The migration of proteins in the polyacrylamide gels. Smaller proteins (bands) migrate faster towards the anode than larger proterns. Prepared by Author.

3.6.1.4 Protein transfer

Following the separating the protein mixture, the transfer of proteins to a membrane protein takes place. The proteins are electrically transferred from the resolving gel to the nitrocellulose membrane (figure 3.12). Negatively charged proteins to migrate out of the gel and onto the nitrocellulose membrane. The proteins that are transferred from the gels become stationary at their corresponding migratory positions at a time point when the electric current on the gel run is stopped.

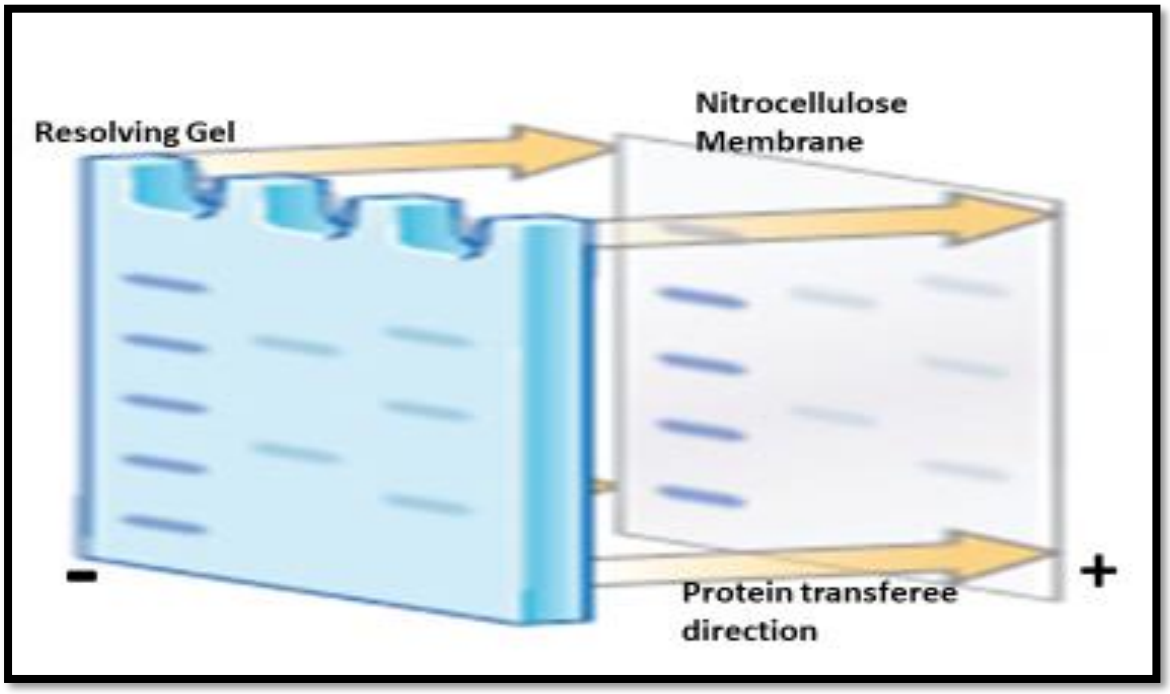

Figure 3.12: Transfer of proteins from resolving gel to nitrocellulose membrane. Proteins are transferred from the gel to the membrane in contact with the gel. Prepared by Author.

3.1.1.5 Immuno-Blotting

Upon completion of protein transfer to a membrane, the target protein is immunoprobed using specific Abs. Prior to immune-probing, it is required to block the membranes where the protein did not bind to prevent non-specific binding of antibodies. An unlabelled primary antibody is applied to the membrane for the protein of interest and a species specific, and labelled secondary antibody is directed against the primary anti-body (Mahmood and Yang, 2012, Kurien and Scofield, 2006). The secondary antibody is conjugated with horseradish peroxidase (HRP) (figure 3.13), which oxidises luminol to generate light and serve as a carrier of the label and is involved in signal amplification, since in theory, many secondary antibodies can simultaneously bind to a single primary antibody.

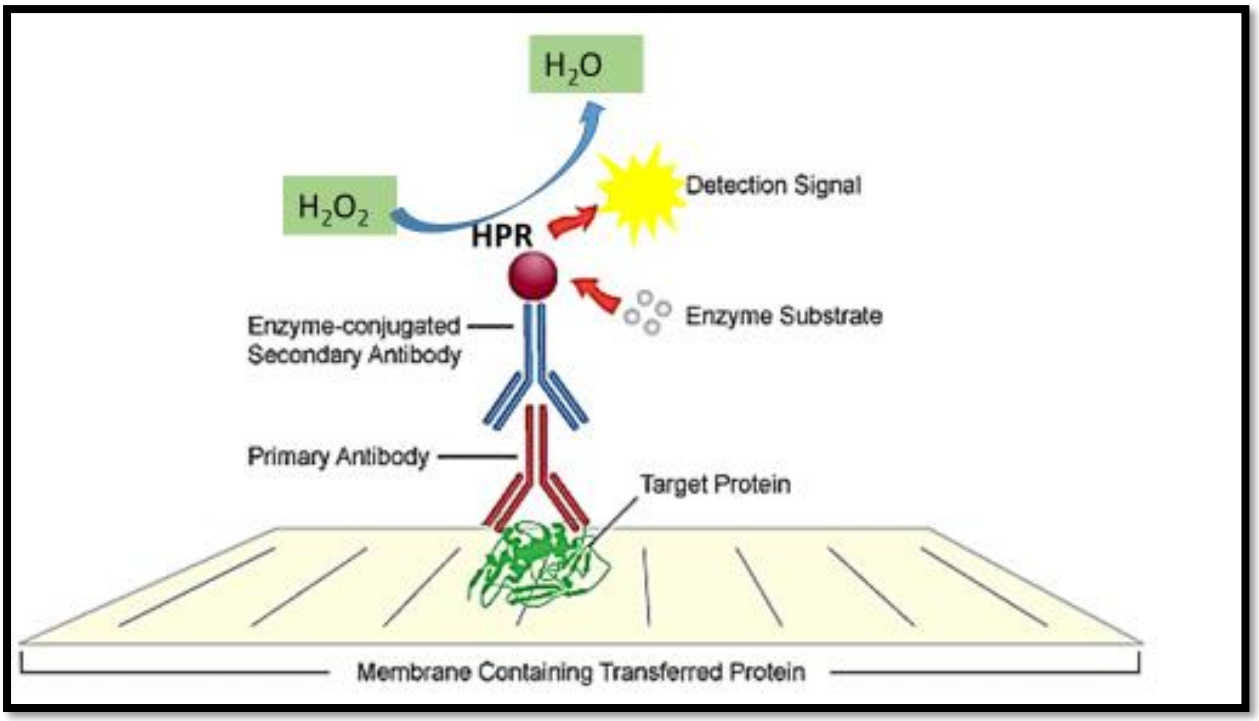

Figure 3.13: The chemiluminescence detection of the target specific protein through the primary and secondary antibodies. Prepared by Author.

3.6.2 Protocol

3.6.2.1 Protein Isolation

Firstly, total protein was isolated from the previously cultured HepG2 cell lysates, Cytobuster™ reagent (catalogue no. 710093, Novagen, San Diego, CA, USA), supplemented with protease (0589279100) and phosphatase inhibitor (04906837001) Roche, Germany, as per manufacturer's instructions. Cytobuster reagent (200 µl) was added to cells in a 25 cm^3 flask and left on ice for 30 min. The cells were lysed mechanically, scrapped out of the flask, and centrifuged (4°C, 10 min, 10,000 x g) to isolate the crude protein extract from the HepG2 cells.

The supernatant containing the crude protein was aspirated into a fresh 1.5 ml microcentrifuge tubes and kept at -80˚C overnight. Samples were left at RT and allowed thaw and used for protein quantification using a Bicinchoninic Acid (BCA) assay (Sigma, Germany) (Bainor et al., 2011). Bovine serum albumin (BSA) was used to prepare protein standards (0, 0.2, 0.4, 0.6, 0.8, 1 mg/ml) to measure the generate the calibration curve. Both the standards and samples were incubated with a working solution (BCA and $CuSO_4$) at 37°C for 30 min and the OD was measured using a µQuant Biotek ELISA plate reader set at a wavlenght of 562 nm. The OD values obtained for the standard were used to generate the standard curve used to

determine the concentration of the total protein in samples. The samples were subsequently standardised to a concentration of 1.5 mg/ml.

Samples were prepared in Laemmli buffer (dH_2O, 0.5M Tris-HCl (pH 6.8), glycerol, 10% SDS, β-mercaptoethanol, 1% bromophenol blue]) and electrophoresed (150V, 1 hr) in sodium-dodecyl-sulphate polyacrylamide gels (4% stacking, 10% resolving) using BioRad compact power supply. Denatured protein samples were then separated via SDS-PAGE; firstly, 7.5% resolving gel was prepared [dH_2O, 1.5 M Tris, 10% SDS, Bis/acrylamide, 10% ammonium persulphate (APS), tetramethylene diamine (TEMED)] and allowed to polymerise (1 hr). Thereafter, 4% stacking gel was prepared (dH_2O, 0.5 M Tris, 10% SDS, Bis/acrylamide, 10% APS, TEMED) and added on top of the resolving gel (1 hr). Protein samples were then subjected to an electric field (150 V, 1 hr) using a Bio-Rad compact power supply. To facilitate the electric field by providing conducting ions, 1 x running (electrode) buffer (dH_2O, Tris, glycine, SDS, 4°C) was used during electrophoresis.

Electrophoresed protein bands were then electro-transferred from the resolving gel to a nitrocellulose membrane. Prior to that, there was an equilibration of the membranes, fibre pads and gels in transfer buffer (dH_2O, Tris, glycine, methanol, pH 8.3, 4°C) for 10 min. Following transfer, of the protein to the nitrocellulose membrane, the membranes were blocked in blocking buffer consisting of 5 % BSA (phosphorylated proteins) or 5% non-fat dry milk (NFDM) in TTBS [TTBS; 150 mM NaCl, 3 mM KCl, 25 mM Tris, 0.05% Tween 20, dH2O, pH 7.5]; for 1 hr at RT with gentle shaking probed with primary antibody against p-NRF2 and total NRF2 [(ab76026, Abcam and ab31163, Abcam respectively); 1:5000 dilution in 1% BSA] and against SOD2, CAT and Sirt3 [(HPA001814, Sigma-Aldrich; C0979, Sigma-Aldrich and ab86671, Abcam1)] :1000 in 5% NFDM then overnight at 4^{o}C. Membranes were washed with TTBS (5 x, 10 minutes) and then incubated with secondary antibody conjugated to HRP [goat anti-mouse (31800): 1:10 000 dillution in 5% NFDM, goat anti-rabbit (ab6112) 1:10 000 dillution in 1% BSA and 5% NFDM] for 1 h RT on shaker.

The membranes were then washed with 10 ml TTBS, (5 x, 10 minutes). The Clarity Western Enhanced Chemiluminescence (ECL) substrate (Catalogue no. 1705061, Bio-Rad) was used to detect the reaction in the Biorad ChemiDoc. Protein expression (bands) was analysed in a BioRad ChemiDoc. Membranes were stripped with 5% H_2O_2 for 30 min, 37 ˚C, incubated in blocking solution (5% NFDM; 1 h; RT), rinsed thrice in TTBS and probed with HP-conjugated antibody for the house-keeping protein, β-actin (Sigma). The relative band intensity was normalised against β-Actin. Results were expressed as Relative band density (RBD) which is he ratio of the band density of a protein sample to that of a standard protein.

3.7 Gene expression – Quantitative polymerase chain reaction

3.7.1 Introduction

Quantitative polymerase chain reaction (qPCR) is used to simultaneously detect a specific target DNA sequence in a sample and determine the absolute copy number of this sequence relative to that of a standard. qPCR use the linearity of DNA amplification to determine actual or quantities of a known sequence in a sample. In qPCR, DNA strand is generated as the DNA polymerase stretches primers of DNA and initiate the PCR reaction (Ramakers et al., 2003).

PCR consists of a process of heating and cooling named thermal cycling which is done by machine. Here are the three main stages of qPCR (figure 3.14).

1. **Denaturing** – The two-stranded DNA template is heated (90 ˚C) to divide it into two single strands.
2. **Annealing** – The temperature is optimized to the specificity of the primer, allowing the complementary primers to bind to the target sequence of the template DNA.
3. **Extension** – The temperature is elevated to 72 ˚C and the new strand of DNA is synthesised from the annealed primers by the enzyme DNA polymerase. The DNA copy of interest is amplified in each cycle, resulting in an exponential amplification of the original DNA fragment.

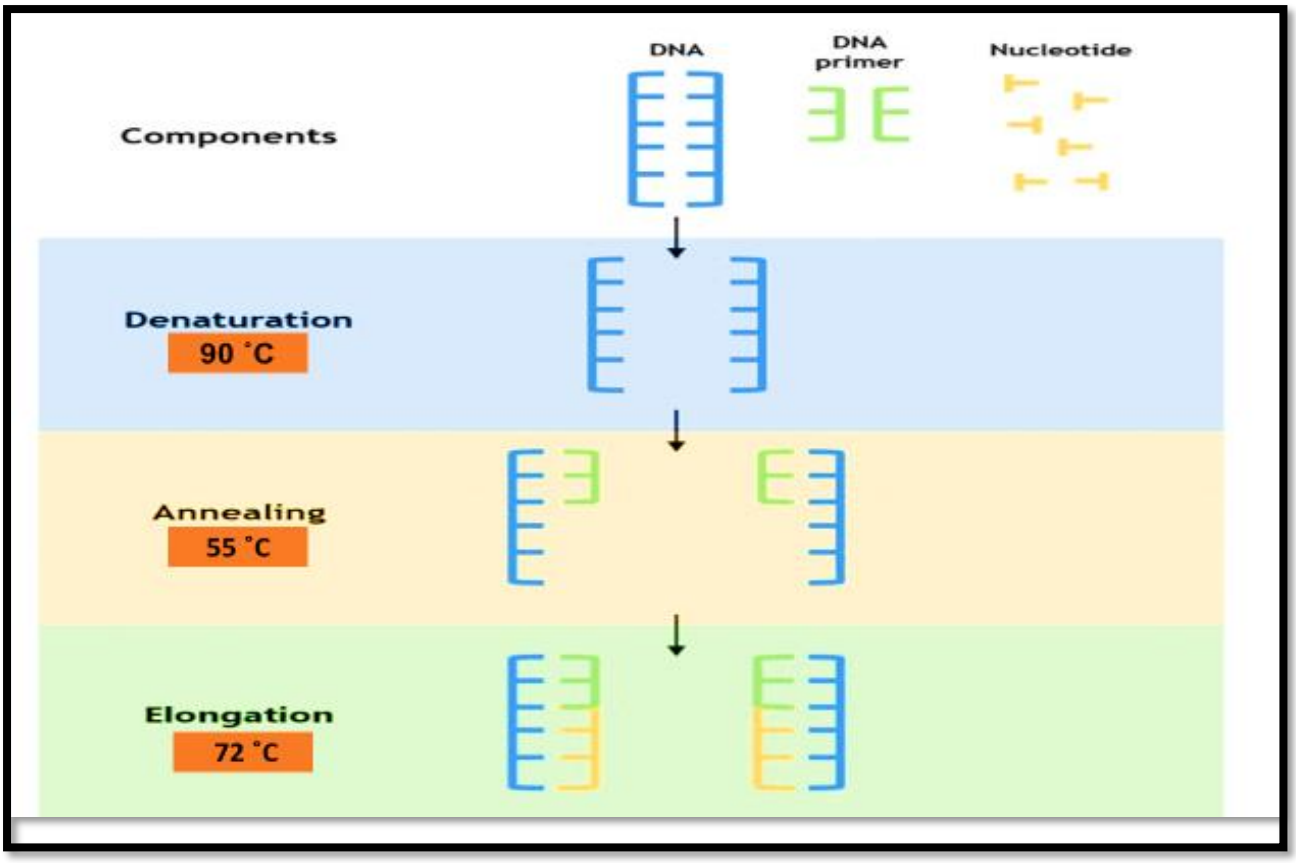

Figure 3.14: The Illustration showing the main steps in the qPCR. Prepared by Author.

Firstly, the RNA is extracted from the cells and used to generate single stranded (ss) complementary DNA strand (cDNA). This strand is used in the amplification of DNA (figure 3.15). Following the amplification of DNA, SYBR Green, dsDNA-binding dye is used for the quantification of the DNS. SYBR green is believed to bind to the minor groove of dsDNA and upon binding, it increases its fluorescence by over a hundred folds (Maeda et al., 2003). This allows the measurement of the targeted gene expression. qPCR differs from conventional PCR in that by using this fluorescent probe (SYBR green) it allows one to not only amplify the PCR product, but quantify it as well.

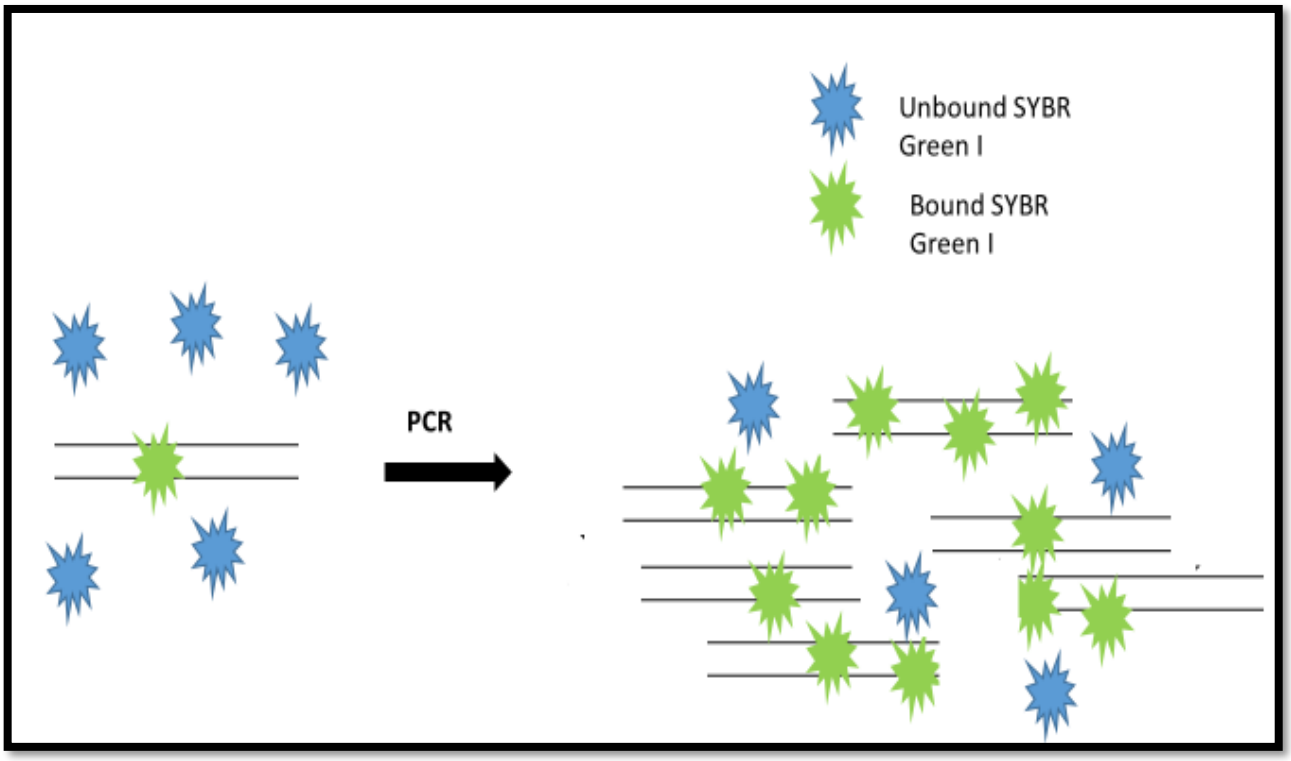

Figure 3.15: Florescence detection of dsDNA. Prepared by Auther

3.7.2 Protocol

3.7.2.1 RNA extraction

RNA was isolated from treated HepG2 cells using an in-house protocol. Qiaizol (Qiagen, Hilden, Germany) reagent (500 µl) was added to each flask and incubated for 15 min, RT. Cells were scrapped out of the flask and transferred to a 1.5 ml microcentrifuge tubes, and stored in stored in Qiazol at -80˚C overnight. In the phase separation step, samples were thawed down at RT prior to addition of chloroform (100 µl) to each sample. Samples were then shaken vigorously for 15 sec, incubated at RT (3 min) and centrifuged (15 min, 12 000 x g, 4˚C).

The aqueous phase containing RNA was carefully transferred to a new 1.5 ml microcentrifuge tubes. RNA precipitation step: Isopropanol (250 µl) was added to each sample and incubated of samples at -80˚C overnight. This was followed by the RNA washing step, wherein samples were thawed at TR samples and centrifuged 12 000 x g, 4˚C for 20min. The supernatant was discarded, pellet washed with 75% ethanol (500 µl) and centrifuged 7 400 x g, 15 min, 4˚C. Ethanol was removed and the samples were allowed to air dry under the hood. The pellets were then resuspended in nuclease free water (15 µl) and incubated at -80˚C overnight. The

RNA was quantified using the Nanodrop2000 spectrophotometer (Thermo-Scientific) and the A260/A280 nm ratio was used to standardize the RNA concentration to 1000 ng/ml.

Standardized RNA samples were used to synthesize complementary DNA (cDNA) using a commercially available kit (iScript™ cDNA Synthesis kit, BioRad; catalogue no 107-8890). A 20 µl reaction volume containing 1 µl RNA template, 4 ml 5X iScript™ reaction mix, 1 µl iScript reverse transcriptase and nuclease-free water was made up. Thermocycler conditions were 25˚C for 5 min, 42˚C for 45 min, 85˚C for 5 min and a final hold at 4˚C.

3.7.2.2 cDNA Synthesis

Gene expression of *NRF2* [sense 5'-AGTGGATCTGCCAACTACTC-3'; antisense 5'-CATCTACAAACGGGAATGTCTG-3') (58 ˚C)], *SOD2* [Sense 5'GAGATGTTACACGCCCAGATAGC-3'; Antisense 5'AATCCCCAGCAGTGGAATAAGG-3'(57 ˚C)], *CAT* [Sense5'-TAAGACTGACCAGGGCATC-3'; Antisense 5'CAACCTTGGTGAGATCGAA-3' (58 ˚C)], and GPx [Sense 5'GACTACACCCAGATGAACGAGC- 3'; Antisense 5'CCCACCAGGAA CTTCTCAAAG-3'(58 ˚C)] were evaluated using the iQ™ SYBR® Green PCR kit (BioRad; 170-880).

3.7.2.3 Quantitative PCR

The following thermocycler profile was used to initiate PCR: An initial denaturation for 8 min at 95 ˚C followed by 39 cycles of 95 ˚C denaturations for 15 sec and annealing for 40 sec at 57˚C for SOD2 and 58˚C for both CAT and GPx. Denaturing was followed by the extension at 72 ˚C for 30 sec. At 70ºC, the final extension was performed for 30 sec. Each measurement was performed in triplicates and then normalized against β-actin, which was assessed under similar conditions and used as a housekeeping gene. Data was analysed and presented as fold change relative to the housekeeping gene, *β-actin* (Sense5"-TGACGGGTCACCCACACTGTGCCCAT-3", Anti sense5 "CTAGAAGCATTTGCGGTGGACGATGGAGGG-3").

3.8. Statistical analysis

For data analysis,GraphPad prism V5.0 software (GraphPad Software Inc., La Jolla, USA.) was used. Data was considered to be statistically significant with a p value < 0.05. Unpaired t-test with Welch correction (data reported as mean ± standard deviation) or the one-way analysis of variance (ANOVA) followed by a Bonferroni test for multiple group comparison (data is presented as 95% CI) was used to determine statistical significance.

CHAPTER FOUR

RESULTS

4.1 Mitochondrial output

Cell viability and intracellular ATP levels were assessed to examine the effect of DON on the mitochondrial productivity.

4.1.1: Cell Viability Assay

A serial dilution of DON (0, 2.5, 5, 10, 25, 50, and 100 µM) was used to determine a dose-response (MTT assay) in HepG2 cells. The analysis of the dose-response curve revealed that DON caused a 50% metabolic activity inhibition (IC_{50}) at 26.17 µM, over 72 hr (figure 4.1). This IC_{50} value was selected for the subsequent experiments because it showed a better response of the cell viability compared to the 24 hr and 48 hr.

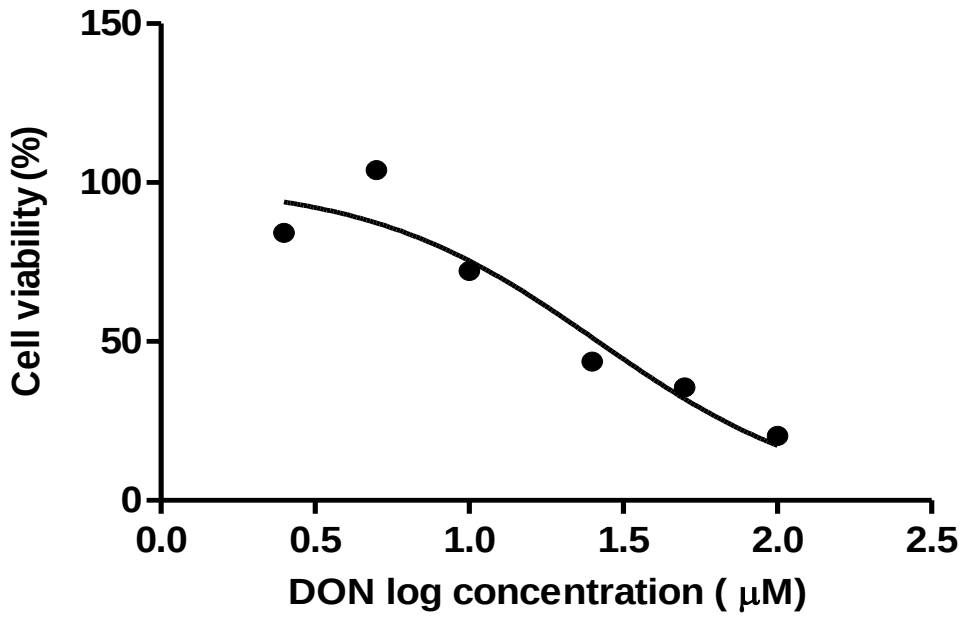

Figure 4.1: The induction of a dose-dependent decline in HepG2 cell viability was observed. HepG2 cells were treated with various concentrations of DON (0-100µM) over 72 hr.

4.1.2 ATP assay

The intracellular concentration of ATP in HepG2 cells exposed to DON (26.17µM, 72 hr) was assessed by using luminometry. DON significantly reduced the ATP levels in HepG2 cells with a 1.92-fold decrease, (4890130 ± 4449970 RLU) compared to the control ($1.00x10^7$ ± 9586910 RLU) (figure 4.2)

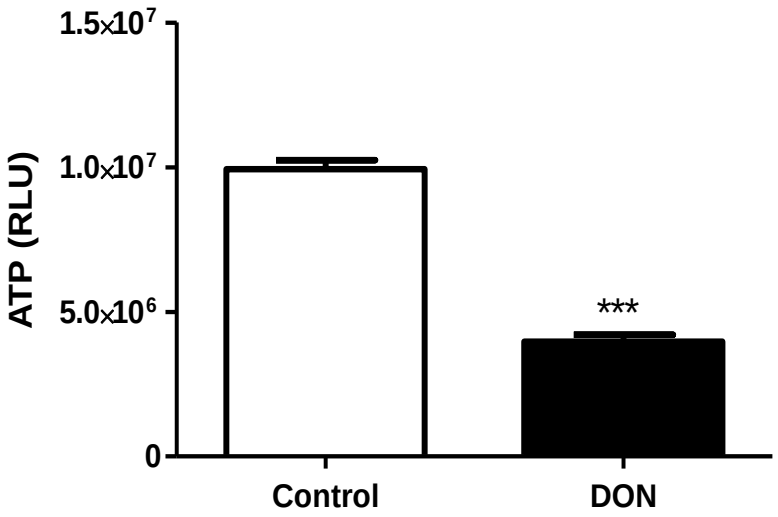

Figure 4.2: The exposure of HepG2 cells to DON (26.17 µM, 72 hr) caused a significant decrease in the intracellular concentration of ATP, ***p =0.0002.

4.2 Cellular redox status

The effect of DON in HepG2 cells on oxidative stress was assessed by measuring a by-product of lipid peroxidation (MDA) and the main endogenous antioxidant, GSH.

4.2.1 Lipid peroxidation

Extracellular levels of MDA were measured using the TBARS assay. There was membrane damage following the exposure of HepG2 cells to DON (26.17 µM; 72 hr). DON significantly increased the extracellular levels of MDA in DON-treated HepG2 cells compared to the control by 1.89-fold, (0.6538 ± 0.1678 mM vs 0.1423 ± 0.0588 mM) (figure 4.3).

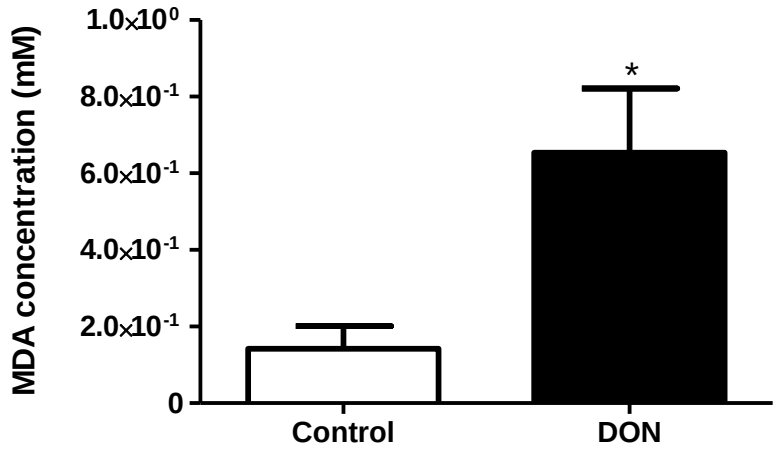

Figure 4.3: DON significantly increased the extracellular levels of MDA in HepG2 cells after a 72-hr period, *p= 0.0104

4.2.2 Intracellular GSH

Intracellular concentration of GSH was measured using luminometry. DON significantly decreased the intracellular concentration of GSH in HepG2 cells as compared to the controls over a period of 72 hr (figure 4.4; 27.724 ± 26.372 μM vs 14.023 ± 12.234 μM) with a 1.77-fold decrease.

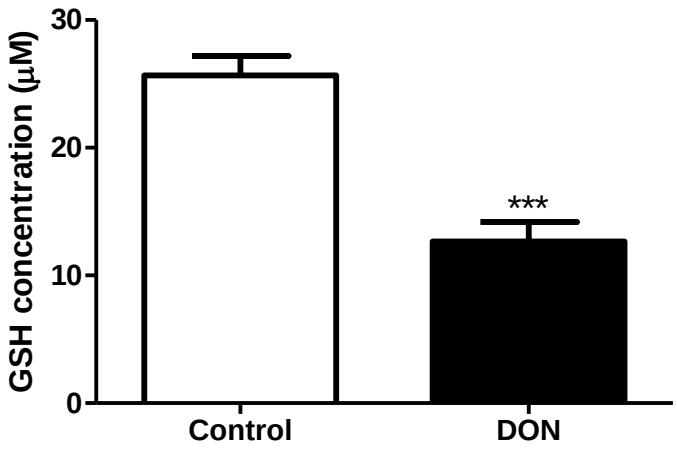

Figure 4.4: DON significantly reduced the intracellular concentration of GSH in HepG2 cells over a 72-hr period, ***p =0.0005.

4.3 Cytotoxicity

4.3.1: Cell membrane integrity and Cell death

Exposure of HepG2 cells to DON led to the induction of membrane damage and the leakage of LDH (and an indicator of cell membrane damage). DON significantly increased extracellular levels of LDH compared to the control by 1.35-fold, treatment (3.567 ± 3.990 OD), control (2.715 ± 2.825 OD); (figure 4.5). The increased extracellular levels of MDA assessed also reveal that DON causes the disruption of the membrane integrity.

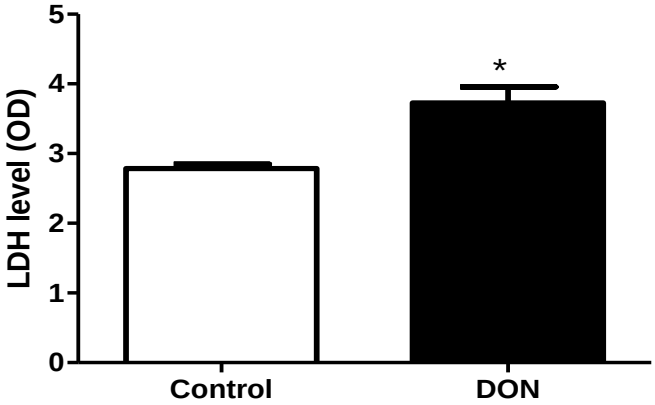

Figure 4.5: DON significantly increased the extracellular levels of LDH in HepG2 cells after 72 hr exposure, *p =0.0207.

4.4 Mitochondrial stress response – Sirtuin 3

Sirt3 is a primarily mitochondrial NAD^+-dependent acetyl-lysine deacetylase that modulates various proteins involved in mitochondrial function and antioxidant defence (Kim et al., 2010, Verdin et al., 2010a). Western blotting analysis showed a significant decrease in Sirt3 protein expression with a 0.14-fold treatment (0.1541 ± 0.1308 RBD) compared to control (0.2664 ± RBD) (figure 4.6; p =0.0058).

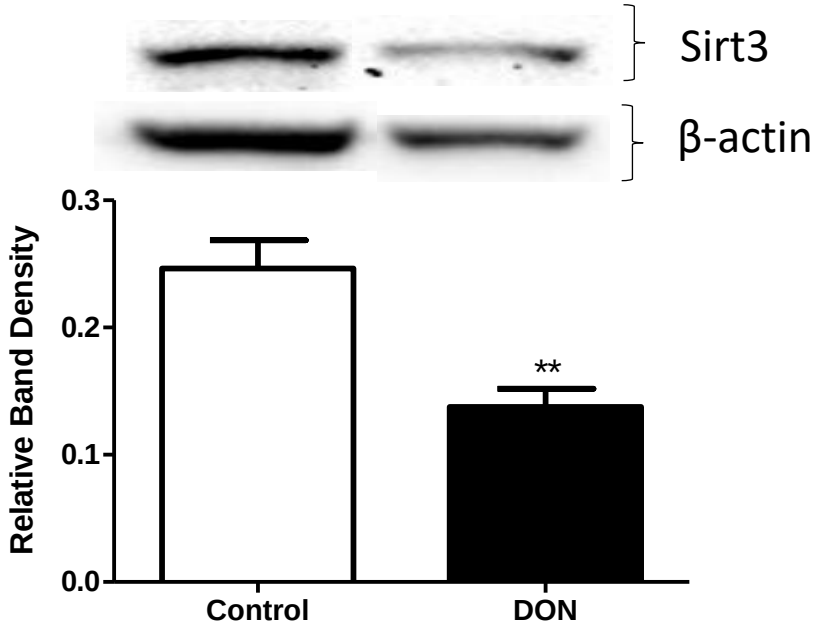

Figure 4.6: A significant decrease of Sirt3 expression in HepG2 cells after exposure to DON over 72-hour period was observed (**p= 0.0058).

4.5 Endogenous antioxidant response

4.5.1 NRF2

NRF2 is considered the master regulator of the antioxidant response. Phosphorylated NRF2 (p-NRF2) is marked for dissociation from its cytosolic inhibitor Keap1, allowing NRF2 to translocate to the nucleus and bind to the ARE; thus, allowing the transcription of several antioxidant genes. We assessed protein expression of p-NRF2 and NRF2, as well as downstream antioxidants regulated by this pathway.

Phosphorylated-NRF2 (active) was significantly decreased by 0.74-fold, (DON 0,7403 ± 0,05104 vs control 1,509 ± 0,1807 RBD, p =0.0193,) (figure 4.7A), and total NRF2 protein levels were significantly decreased by 0.30-fold (0.3132 ± 0.2858 RBD; p =0.0017) in DON treated HepG2 cells compared to control cells (0.9358 ± 0.8673 RBD; figure 4.7B). The p-NRF2 normalised against the total NRF2, was significantly increased by a 3.54-fold (DON: 3.9228 ± 3.1953 vs Control: .2625 ± 0.9991 RBD; p =0.0085; figure 4.7C).

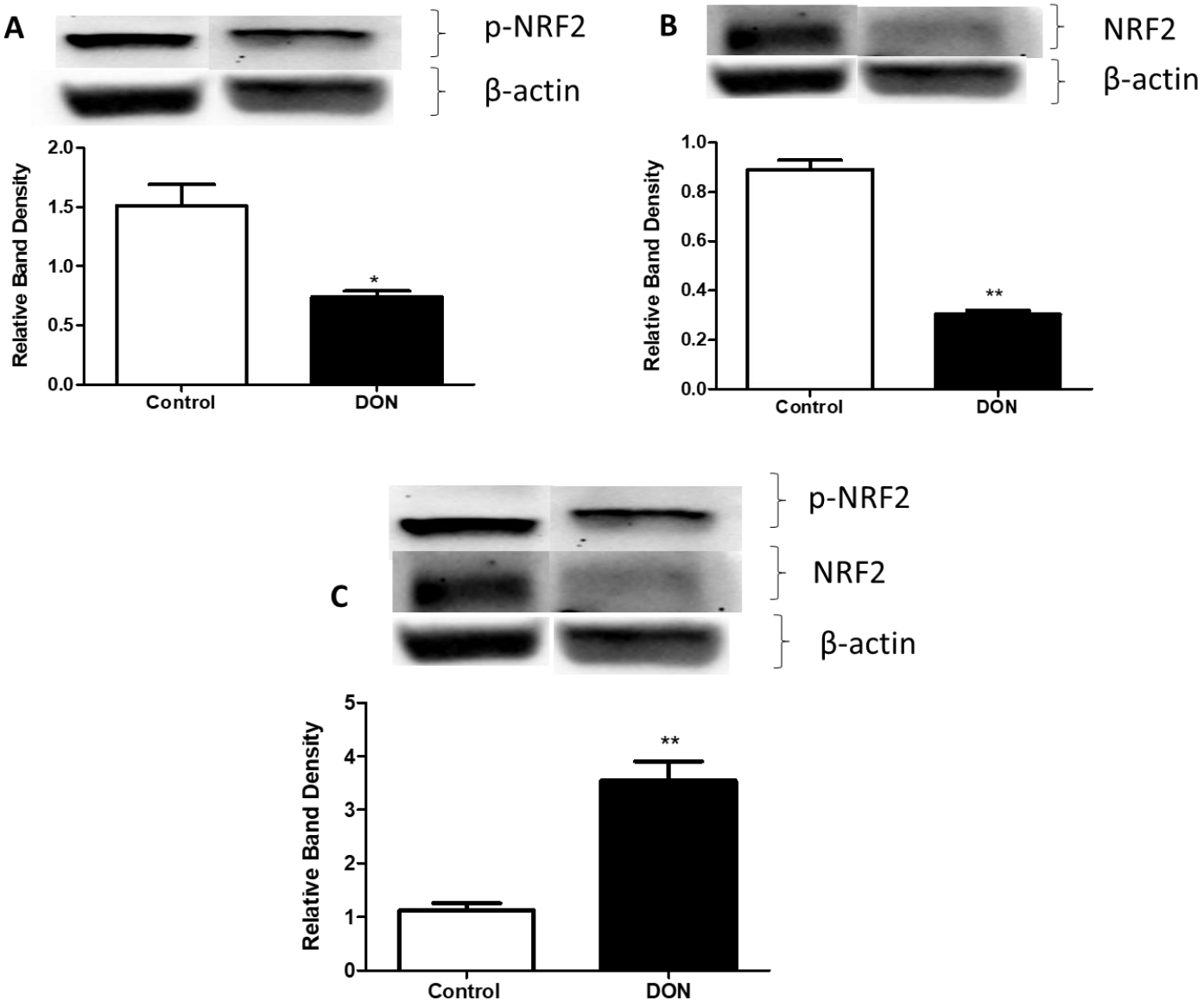

Figure 4.7: DON significantly reduced p-NRF2 (A) expression in HepG2 cells over a 72-hr period of exposure (*p= 0.0193). DON significantly reduced the total NRF2 (B) expression in HepG2 cells over a 72-hr period of exposure (**p =0.0017). The Comparison of pNRF2 to total NRF2 (C) showed that DON significantly increased the expression of p-NRF2 in HepG2 cells (**p= 0.0085).

4.5.2. NRF2 target genes

The first enzymatic response to detoxifying superoxide in the mitochondrion is SOD2. Quantitative PCR results showed DON significantly decreased *SOD2* mRNA levels by ($2^{-\Delta\Delta Ct}$ = 0.063-fold; p =0.0020; figure 4.8 B); while Western blot data showed a similar trend in SOD2 protein levels (DON: 1.2886 ± 1.1942 vs Control = 0.0937 ± 0.0843; 0.02-fold, p= 0.0137 figure 4.80A)

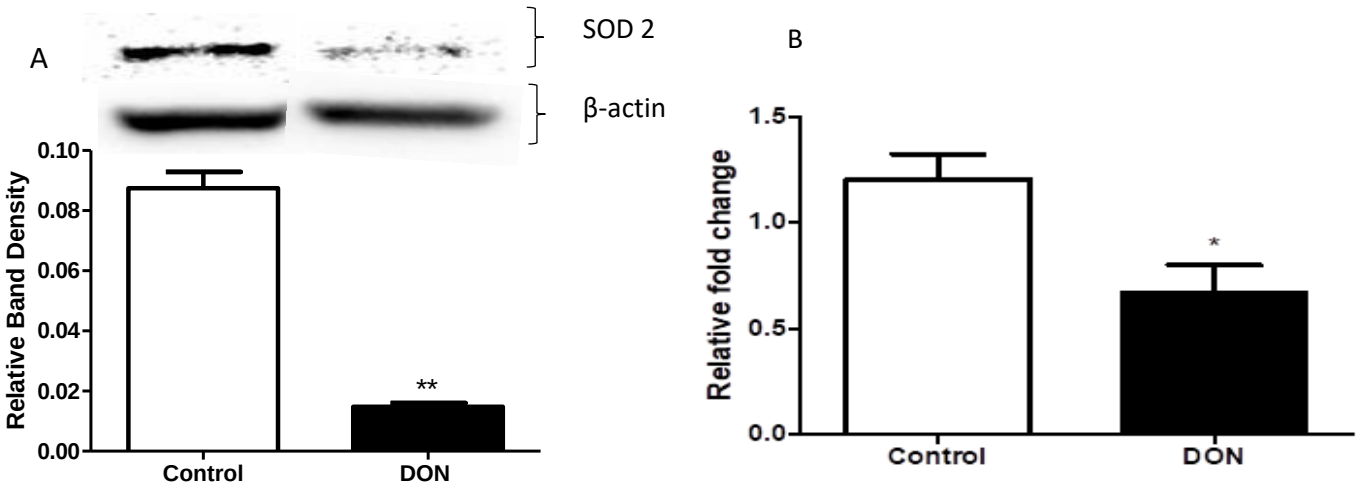

Figure 4.8: A significant decrease in SOD2 protein expression (A) and *SOD2* mRNA levels (B) in HepG2 cells after exposure to DON over 72 hr period (*p=0.0137; **p= 0.0020)

Detoxification of H_2O_2 is mediated by CAT. Protein levels of CAT were reduced significantly by DON (DON 0,3304 ± 0,06479 vs control 1,249 ± 0,04895, 0.33-fold, p =0.0003; figure 4.9A) with a concomitant reduction in *CAT* mRNA levels ($2^{-\Delta\Delta Ct}$ = 0.021-fold; p =0.0050; figure 4.9B).

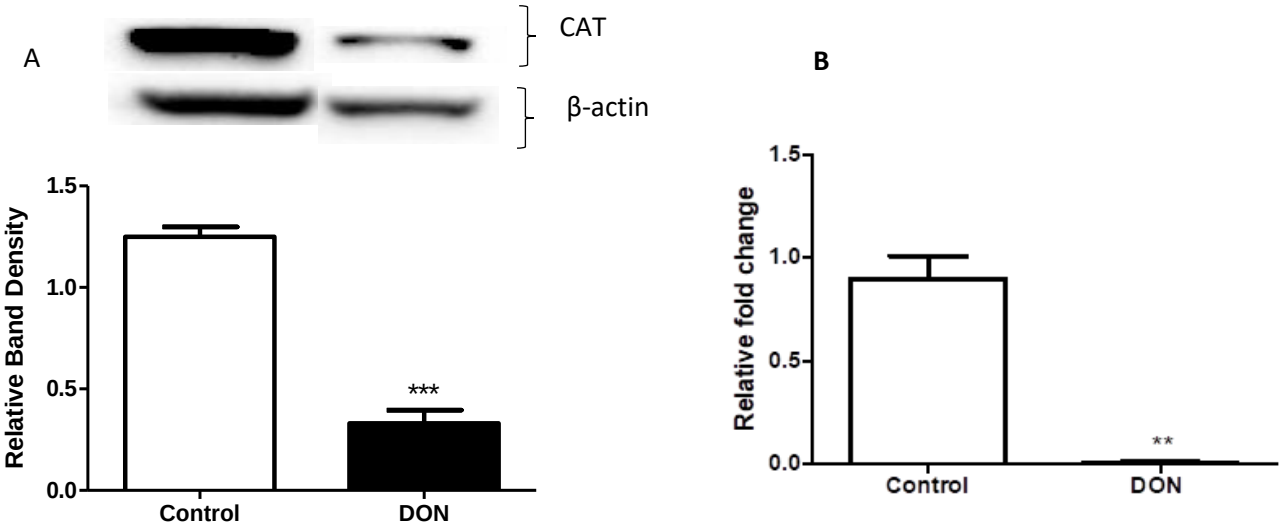

Figure 4.9: DON significantly reduced the protein expression (A) and mRNA levels (B) of catalase in HepG2 cells over a 72-hr period of exposure (**p=0.005; ***p = 0.0003).

The mRNA expression of *GPx*, which regulates GSH functioning and detoxification of H_2O_2 was assessed using qPCR. There was a significant decreased in *GPx* mRNA after the treatment of HepG2 cells ($2^{-\Delta\Delta Ct}$ = 0.036-fold; p =0.0006 (figure 4.10).

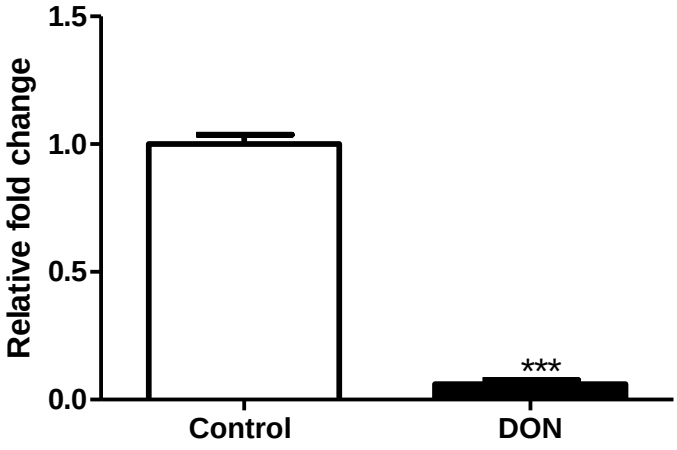

Figure 4.10: DON significantly reduced the expression of *GPx* in HepG2 cells, (***p =0.0006).

CHAPTER 5

DISCUSSION

DON is a common contaminant of food and feed and has severe consequences in both human and animal health. DON is produced by *Fusarium* species and it affects agricultural foods (Bennett and Klich, 2003). The majority of studies evaluating DON toxicity has focussed on the immune system (Pestka et al., 2004, Bracarense et al., 2017), kidney (Liang et al., 2015) and brain cells (Bonnet et al., 2012a, Pinton and Oswald, 2014) as well as intestinal cells (Flannery et al., 2012). Known molecular outcomes of DON exposure include oxidative stress and the inhibition of protein translation synthesis (Mishra et al., 2014, Zhang et al., 2009).

The MTT assay measures cell viability based on the cell's ability to reduce yellow MTT salt to purple formazan using mitochondrial NADPH-dependent oxidoreductase (Mosmann, 1983). Our studies show that DON decreases cell viability in a dose-dependent manner, with a strong decrease between 8 to 30 µM (figure 4.1). The decrease in metabolic activity in DON exposed HepG2 cells may be caused by the impaired mitochondria and the reduced functioning of the Kreb's cycle. This leads to decreased products of reducing equivalents required for MTT salt reduction to produce the purple formazan products.

DON, like T-2 toxin, is a trichothecene. Both toxins share similar basic structures and DON might mimic the binding of T-2 toxin and inhibit the succinate dehydrogenase in the Krebs cycle by influencing the mitochondrial proton gradient. T-2 toxin inhibits mitochondrial electron transport system by inhibiting the functioning of mitochondrial succinate dehydrogenase activity and complex (II) leading to an increase mitochondrial NADH dehydrogenase activity (Pace, 1983, Koshinsky et al., 1988). The chemical structure of DON contains 3 hydroxyl groups (-OH), which are linked to its toxicity (Sobrova et al., 2010). These hydroxyl groups may also be responsible for the impairment of the mitochondrial function.

DON is a weak acid under physiological conditions, causing change in pH leading to increase in ion pump into the electron transport chain, therefore increasing mitochondrial ROS production. The results in this study show a significant increase of extracellular MDA, a by-product of lipid peroxidation and a biomarker of oxidative stress (figure 4.3). The common target of *Fusarium*-derived toxins is the mitochondria (Kouadio et al., 2005), which is one of the most important cellular sources of ROS production and particularly susceptible to oxidative stress (Cadenas and Davies, 2000). A strong ROS production leads to oxidative modification of lipids in cellular membranes. This type of interaction yields lipid peroxides. DON increased

extracellular MDA levels in HepG2 cells, indicating oxidative damage to lipid membranes. This study is in agreement with the study done by (Zhang et al., 2009) who showed a significant increase of extracellular MDA in HepG2 cells treated with DON.

The current study showed a significant decrease in intracellular ATP concentrations in DON exposed HepG2 cells (figure 4.2). DON, through its methanol group, may decrease the availability of all oxidative phosphorylation complexes, contributing to the decline in the functioning of the oxidative phosphorylation system and suppressed rates of ATP synthesis. This study is in agreement with the study that displays that the reduced activity of the electron transport chain is linked to the decrease in ATP-production in an electron transport chain. The similar study was done in IPEC-J2 cell line, where ATP was reduced after a 48 hr exposure to DON (Awad et al., 2012).

In the present study, HepG2 cells responded to DON with an increase in the release of LDH into the culture medium (figure 4.5). LDH is an enzyme found in the cytoplasm of healthy cells with intact cell membranes, catalysing the conversion of lactate to pyruvic acid and vice versa (Bagchi et al., 1995). An increase in extracellular LDH levels indicates cell membrane damage caused by lipid peroxidation. DON causes lipid peroxidation through increasing the levels of ROS. The study on HepG2 and IPEC-J2 cell lines is in agreement with these results (Königs et al., 2008, Awad et al., 2012).
GSH is the main non-enzymatic antioxidant defence in HepG2 cells and plays a crucial role in protecting cells against oxidative stress (Scharf et al., 2003). It is a substrate in GPx-catalysed detoxification of organic peroxides (Scharf et al., 2003, Alía et al., 2005), reacting with free radicals and repairing free radicals-induced damage through electron transfer reaction. GSH reduction or depletion reflects intracellular oxidative stress (Alía et al., 2006). In this experimental study, treatment of HepG2 cells with DON led to the generation of ROS with a significant decrease in intracellular GSH concentrations observed (figure 4.4), strongly supporting that DON causes oxidative stress.

Glutathione peroxidase (GPx) converts GSH into oxidized glutathione (also called glutathione disulphide, GSSG) and during this process, reduces H_2O_2 to H_2O and lipid hydroperoxides (ROOH) to corresponding stable alcohols (Bhattacharyya et al., 2014), leading to a reduction in oxidative stress (Miyamoto et al., 2003). Our study showed a significant decrease in the gene expression of GPx (figure 4.10). This GPx reduction is concomitant with the GSH reduction, which implies that cells experienced an increase in intracellular ROS hence oxidative stress. The reduction of GPx is accompanied by an increase of oxidative stress

(Yildirim et al., 2011). These data display that DON reduced the antioxidant defence in HepG2 cells.

The ability of HepG2 cells to induce NRF2 mediated antioxidant response against oxidative stress was downregulated. DON significantly decreased the expression of NRF2 (figure 4.7B). It exerts its toxicity by binding to the A site of the 28S ribosomal subunit and inhibits peptidyl transferase, leading to an inhibition of protein elongation [(Pestka, 2010, Bonnet et al., 2012b, Zhang et al., 2016)]. The observed reduction may be due to the inhibition of protein translation. Although NRF2 protein levels were significantly downregulated; it was found that active p-NRF2 expression was significantly higher in DON-treated HepG2 cells (figure 4.7A). This implies that a higher level of NRF2 is present in the activated form due to oxidative stress. NRF2 is phosphorylated when free radicals interact with cysteine residues, binding NRF2 to Keap1 in the cytoplasm. This NRF2 translocates to the nucleus and binds to ARE in the promoter region in DNA, and transcribes the synthesis of the cytoprotective enzymes, including SOD and CAT (Tang et al., 2014). There was a significant decrease in the expression of NRF2 targets: CAT and SOD2 at both protein and mRNA levels (figure 4.9 and 4.8) respectively. The reduction of these cytoprotective enzymes supports downregulation of an NRF2 response mechanism.

SOD2 plays an important role in protecting against cellular damage by ionising radiation. The decrease of SOD2 also supports that DON does affect the cell via the mitochondrial pathway. This is in agreement with the decrease of Sirt3 expression noted in this study. Sirt3 controls NAD^+ dependent mitochondrial substrate deacetylation and attenuates ROS by deacetylating and activating SOD2 and CAT ultimately supplementing the synthesis of these enzymes in response to oxidative stress (Verdin et al., 2010b, Zhong and Mostoslavsky, 2011). The decrease in the expression of Sirt3 might also contribute to the downregulated production of SOD2 and CAT.

Sirt3 is a NAD+-dependent acetyl-lysine deacetylase primarily located in the mitochondrion. Sirt3 modulates mitochondrial biogenesis, antioxidant and unfolded protein response via its deacetylase activity (Kim et al., 2010, Verdin et al., 2010a). Our study shows a significant decrease in the protein expression of Sirt3 in HepG2 cells exposed to DON (figure 4.6). This offers further support that DON has mitochondrial-linked effects in HepG2 cells. Also, it supports that DON does downregulate the stress response protein expression, through the inhibition of protein translation.

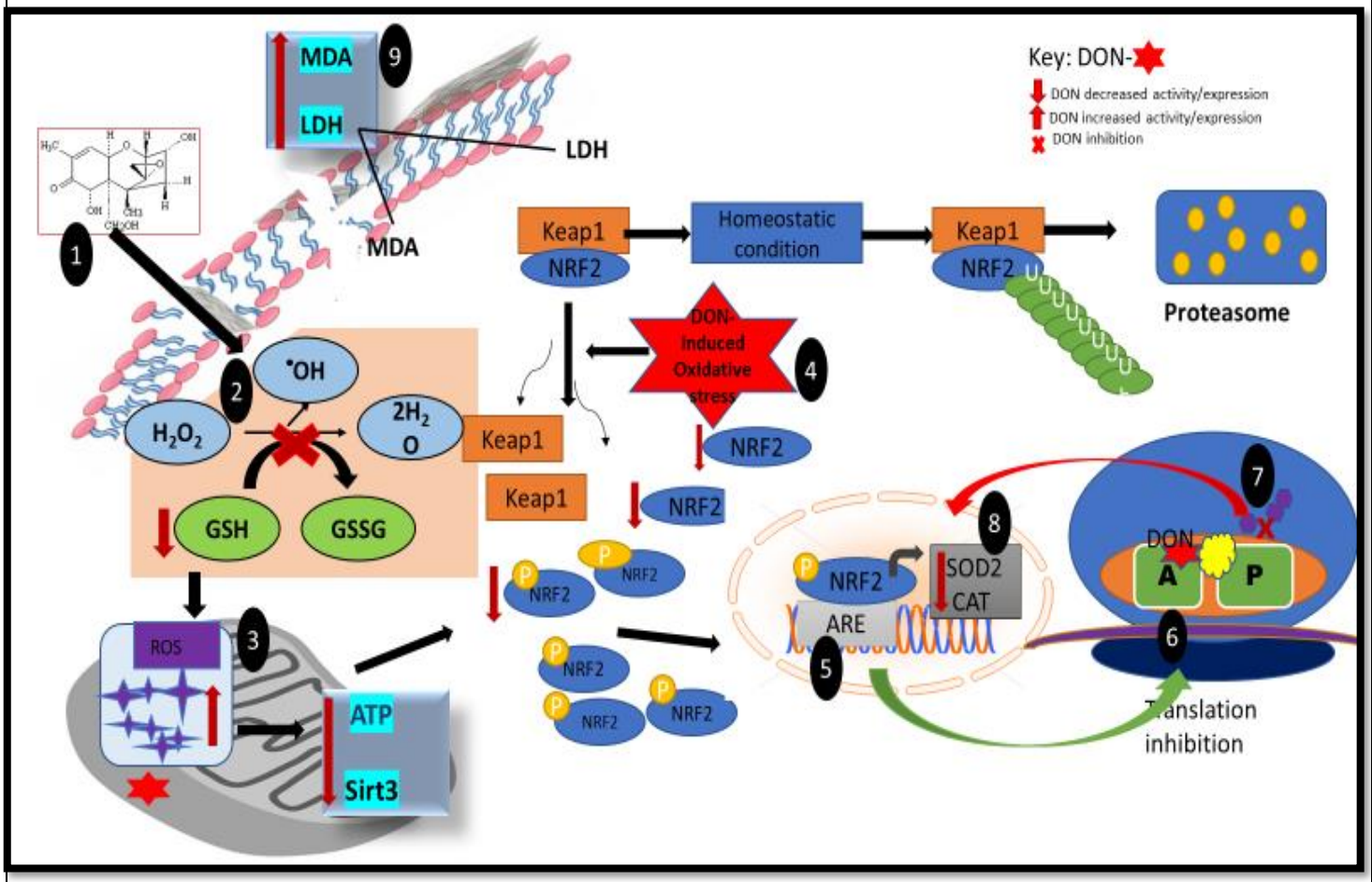

Figure 5.1: The schematic summary of the biochemical effect of DON on HepG2 cells. An induction of oxidative stress, protein translation synthesis inhibition, mitochondrial dysfunction, downregulation of the NRF2-induced cytoprotective response and membrane integrity disruption. Prepared by Author

CHAPTER 6

CONCLUSION

Mycotoxin contamination is a serious problem for agricultural commodities globally, due to their adverse health effects on humans and animals which ultimately compromise food safety and security. The eradication of mycotoxins during food processing is not entirely achieved due to factors such as the lack of proper equipment and nature of mycotoxin. Developing countries like South Africa are prone to mycotoxin contamination, as a result of inadequate facilities to reduce the contamination of mycotoxins. The population in rural areas is highly dependent on maize (which is prone to DON contamination), making the population highly susceptible to mycotoxin exposure.

DON is documented to have negative effects on human health. DON exerts toxic effects on micromolecular synthesis, cell signalling, and silencing of genes promoting programmed cell death. DON acts on cellular cytoplasm, it targets the A site of 28S ribosomal subunit and inhibits the functioning of the peptidyl transferase, hence the peptide elongation. DON is reported to exert its toxicity mostly through the induction of oxidative stress (Pestka et al., 2004, Bracarense et al., 2017).

For the first time, the NRF2 induced cytoprotective response to DON in HepG2 cells is revealed. DON reduced the primary antioxidant GSH and affected mitochondrial functioning, and as a result, triggered the elevation of ROS and concomitantly the impairment of intracellular ATP production. This study also revealed that DON induced lipid peroxidation and membrane damage which is primarily a consequence of oxidative stress.

The study also revealed that DON affects phase (ll) endogenous antioxidant response to oxidative stress. The results indicated that DON suppresses the NRF2 expression, however, the increased presence of p-NRF2 indicates a response to oxidative stress. The downregulation of cytoprotective enzymes expression CAT and SOD2 in HepG2 cells was observed, and this downregulation is associated with the mechanism of DON in inhibiting protein synthesis. This indicates that DON-induced NRF2 protein expression depletion resulted in reduced expression of NRF2 target genes.

Collectively, this study demonstrated that DON induced oxidative stress, promoting cytotoxicity, but most importantly, the study revealed that DON downregulates the NRF2-induced cytoprotective response in HepG2 cells. The study might be limited by the period of

exposure (72 hr). Further studies on *in vivo* studies are required using laboratory animal model to further investigate the effect of DON in brain, kidney liver and lungs.

REFERENCES

ALÍA, M., MATEOS, R., RAMOS, S., LECUMBERRI, E., BRAVO, L. & GOYA, L. 2006. Influence of quercetin and rutinon growth and antioxidant defense systemof a human hepatoma cell line (HepG2). *European Journal of Nutrition,* 45**,** 19-28.

ALÍA, M., RAMOS, S., MATEOS, R., BRAVO, L. & GOYA, L. 2005. Response of the antioxidant defense system to tert-butyl hydroperoxide and hydrogen peroxide in a human hepatoma cell line (HepG2). *Journal of Biochemical and Molecular Toxicology,* 19**,** 119-128.

AMUZIE, C. J. & PESTKA, J. J. 2010. Suppression of Insulin-Like Growth Factor Acid-Labile Subunit Expression—A Novel Mechanism for Deoxynivalenol-Induced Growth Retardation. *Toxicological Sciences,* 113**,** 412-421.

ANITA, S., TEJ, K., BHAT, & OM, P. S. 2011. Clinical Biochemistry of Hepatotoxicity. *Journal of Clinical Toxicology* s4**,** 2514-2542.

ANZENBACHER, P. & ANZENBACHEROVÁ, E. 2001. Cytochromes P450 and metabolism of xenobiotics. *Cellular and Molecular Life Sciences CMLS,* 58**,** 737-747.

ASAKAWA, T. & MATSUSHITA, S. 1979. Thiobarbituric acid test for detecting lipid peroxides. *Lipids,* 14**,** 401-406.

AWAD, W., GHAREEB, K., BÖHM, J. & ZENTEK, J. 2013. The Toxicological Impacts of the Fusarium Mycotoxin, Deoxynivalenol, in Poultry Flocks with Special Reference to Immunotoxicity. *Toxins,* 5**,** 912.

AWAD, W. A., ASCHENBACH, J. R. & ZENTEK, J. 2012. Cytotoxicity and metabolic stress induced by deoxynivalenol in the porcine intestinal IPEC-J2 cell line. *Journal of Animal Physiology and Animal Nutrition,* 96**,** 709-716.

AYALA, A., MU, #XF1, OZ, M. F., ARG, #XFC & ELLES, S. 2014a. Lipid Peroxidation: Production, Metabolism, and Signaling Mechanisms of Malondialdehyde and 4-Hydroxy-2-Nonenal. *Oxidative Medicine and Cellular Longevity,* 2014**,** 31.

AYALA, A., MUÑOZ, M. F. & ARGÜELLES, S. 2014b. Lipid Peroxidation: Production, Metabolism, and Signaling Mechanisms of Malondialdehyde and 4-Hydroxy-2-Nonenal. *Oxidative Medicine and Cellular Longevity,* 2014**,** 360438.

BAGCHI, D., BAGCHI, M., HASSOUN, E. A. & STOHS, S. J. 1995. In vitro and in vivo generation of reactive oxygen species, DNA damage and lactate dehydrogenase leakage by selected pesticides. *Toxicology,* 104**,** 129-40.

BAINOR, A., CHANG, L., MCQUADE, T. J., WEBB, B. & GESTWICKI, J. E. 2011. Bicinchoninic acid (BCA) assay in low volume. *Analytical Biochemistry,* 410**,** 310-312.

BAUSE, A. S. & HAIGIS, M. C. 2013. SIRT3 regulation of mitochondrial oxidative stress. *Experimental Gerontology,* 48**,** 634-639.

BENNETT, J. W. & KLICH, M. 2003. Mycotoxins. *Clinical Microbiology Reviews,* 16**,** 497-516.

BENSASSI, F., EL GOLLI-BENNOUR, E., ABID-ESSEFI, S., BOUAZIZ, C., HAJLAOUI, M. R. & BACHA, H. 2009. Pathway of deoxynivalenol-induced apoptosis in human colon carcinoma cells. *Toxicology,* 264**,** 104-109.

BHATTACHARYYA, A., CHATTOPADHYAY, R., MITRA, S. & CROWE, S. E. 2014. Oxidative Stress: An Essential Factor in the Pathogenesis of Gastrointestinal Mucosal Diseases. *Physiological Reviews,* 94**,** 329-354.

BIRBEN, E., SAHINER, U. M., SACKESEN, C., ERZURUM, S. & KALAYCI, O. 2012. Oxidative Stress and Antioxidant Defense. *The World Allergy Organization journal,* 5**,** 9-19.

BOHR, V. A. 2002. Repair of oxidative DNA damage in nuclear and mitochondrial DNA, and some changes with aging in mammalian cells1,2 1Guest Editor: Miral Dizdaroglu 2This article is part of a series of reviews on "Oxidative DNA Damage and Repair." The full list of papers may be found on the homepage of the journal. *Free Radical Biology and Medicine,* 32**,** 804-812.

BONNET, M., ROUX, J., MOUNIEN, L., DALLAPORTA, M. & TROADEC, J.-D. 2012a. Advances in Deoxynivalenol Toxicity Mechanisms: The Brain as a Target. *Toxins,* 4**,** 1120.

BONNET, M. S., ROUX, J., MOUNIEN, L., DALLAPORTA, M. & TROADEC, J. D. 2012b. Advances in Deoxynivalenol Toxicity Mechanisms: The Brain as a Target. *Toxins,* 4**,** 1120-1138.

BRACARENSE, A., BASSO, K. M., DA SILVA, E. O., PAYROS, D. & OSWALD, I. P. 2017. Deoxynivalenol in the liver and lymphoid organs of rats: effects of dose and duration on immunohistological changes. *World Mycotoxin Journal,* 10**,** 89-96.

BULLERMAN, L. B. & BIANCHINI, A. 2007. Stability of mycotoxins during food processing. *International Journal of Food Microbiology,* 119**,** 140-146.

BURD, J. F. & USATEGUI-GOMEZ, M. 1973. A colorimetric assay for serum lactate dehydrogenase. *Clinica Chimica Acta,* 46**,** 223-227.

BURNETTE, W. N. 1981. "Western Blotting": Electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. *Analytical Biochemistry,* 112**,** 195-203.

CADENAS, E. & DAVIES, K. J. A. 2000. Mitochondrial free radical generation, oxidative stress, and aging11This article is dedicated to the memory of our dear friend, colleague, and mentor Lars Ernster (1920–1998), in gratitude for all he gave to us. *Free Radical Biology and Medicine,* 29**,** 222-230.

CASTEEL, M., NIELSEN, C., KOTHLOW, S., DIETRICH, R. & MÄRTLBAUER, E. 2010. Impact of DUSP1 on the apoptotic potential of deoxynivalenol in the epithelial cell line HepG2. *Toxicology Letters,* 199**,** 43-50.

CASTELL, J. V., GÓMEZ-LECHÓN, M. J., PONSODA, X. & BORT, R. 1997. In vitro Investigation of the Molecular Mechanisms of Hepatotoxicity. *In:* SEILER, J. P. & VILANOVA, E. (eds.) *Applied Toxicology: Approaches Through Basic Science: Proceedings of the 1996 EUROTOX Congress Meeting Held in Alicante, Spain, September 22-25, 1996.* Berlin, Heidelberg: Springer Berlin Heidelberg.

CHEN, Y., ZHANG, J., LIN, Y., LEI, Q., GUAN, K.-L., ZHAO, S. & XIONG, Y. 2011. Tumour suppressor SIRT3 deacetylates and activates manganese superoxide dismutase to scavenge ROS. *EMBO Reports,* 12**,** 534-541.

CUI, H., KONG, Y. & ZHANG, H. 2012. Oxidative Stress, Mitochondrial Dysfunction, and Aging. *Journal of Signal Transduction,* 2012**,** 13.

DAO, V. T.-V., FLOEREN, M., KUMPF, S., BOTH, C., PETER, B., BALZ, V., SUVORAVA, T. & KOJDA, G. 2011. Catalase activity prevents exercise-induced up-regulation of vasoprotective proteins in venous tissue. *Journal of Cellular and Molecular Medicine,* 15**,** 2326-2334.

DARWISH, W. S., IKENAKA, Y., NAKAYAMA, S. M. M. & ISHIZUKA, M. 2014. An Overview on Mycotoxin Contamination of Foods in Africa. *The Journal of Veterinary Medical Science,* 76**,** 789-797.

DAVIES, MICHAEL J. 2016. Protein oxidation and peroxidation. *Biochemical Journal,* 473**,** 805-825.

DE RUYCK, K., DE BOEVRE, M., HUYBRECHTS, I. & DE SAEGER, S. 2015. Dietary mycotoxins, co-exposure, and carcinogenesis in humans: Short review. *Mutation Research/Reviews in Mutation Research,* 766**,** 32-41.

DEGLI ESPOSTI, D., HAMELIN, J., BOSSELUT, N., SAFFROY, R., #XEB, SEBAGH, M., #XE8, NE, POMMIER, A., MARTEL, C., #XE9, CILE & LEMOINE, A. 2012. Mitochondrial Roles and Cytoprotection in Chronic Liver Injury. *Biochemistry Research International,* 2012**,** 16.

DEL RÍO, L. A., SANDALIO, L. M., CORPAS, F. J., PALMA, J. M. & BARROSO, J. B. 2006. Reactive Oxygen Species and Reactive Nitrogen Species in Peroxisomes. Production, Scavenging, and Role in Cell Signaling. *Plant Physiology,* 141**,** 330-335.

DENIZOT, F. & LANG, R. 1986. Rapid colorimetric assay for cell growth and survival: Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. *Journal of Immunological Methods,* 89**,** 271-277.

DINU D., BODEA G.O., CEAPA C.D., MUNTEANU MC, R. F. I., SERBAN A.I., HERMENEAN A., COSTACHE M., ZARNESCU O. & A, D. 2011. Adapted response of the antioxidant defense system to oxidative stress induced by deoxynivalenol in Hek-293 cells. *Toxicon,* 7**,** 1023-1032.

EDITE BEZERRA DA ROCHA, M., FREIRE, F. D. C. O., ERLAN FEITOSA MAIA, F., IZABEL FLORINDO GUEDES, M. & RONDINA, D. 2014. Mycotoxins and their effects on human and animal health. *Food Control,* 36**,** 159-165.

ENGLAND, K., O'DRISCOLL, C. & COTTER, T. G. 2003. Carbonylation of glycolytic proteins is a key response to drug-induced oxidative stress and apoptosis. *Cell Death And Differentiation,* 11**,** 252.

FERNÁNDEZ, J., PÉREZ-ÁLVAREZ, J. A. & FERNÁNDEZ-LÓPEZ, J. A. 1997. Thiobarbituric acid test for monitoring lipid oxidation in meat. *Food Chemistry,* 59**,** 345-353.

FERNIE, A. R., CARRARI, F. & SWEETLOVE, L. J. 2004. Respiratory metabolism: glycolysis, the TCA cycle and mitochondrial electron transport. *Current Opinion in Plant Biology,* 7**,** 254-261.

FLANNERY, B. M., CLARK, E. S. & PESTKA, J. J. 2012. Anorexia Induction by the Trichothecene Deoxynivalenol (Vomitoxin) Is Mediated by the Release of the Gut Satiety Hormone Peptide YY. *Toxicological Sciences,* 130**,** 289-297.

FUKAI, T. & USHIO-FUKAI, M. 2011. Superoxide Dismutases: Role in Redox Signaling, Vascular Function, and Diseases. *Antioxidants & Redox Signaling,* 15**,** 1583-1606.

FUKAO, T., HOSONO, T., MISAWA, S., SEKI, T. & ARIGA, T. 2004. The effects of allyl sulfides on the induction of phase II detoxification enzymes and liver injury by carbon tetrachloride. *Food and Chemical Toxicology,* 42**,** 743-749.

GASCHLER, M. M. & STOCKWELL, B. R. 2017. Lipid peroxidation in cell death. *Biochemical and Biophysical Research Communications,* 482**,** 419-425.

GRANT, D. M. 1991. Detoxification Pathways in the Liver. *In:* HARKNESS, R. A., POLLITT, R. J. & ADDISON, G. M. (eds.) *Journal of Inherited Metabolic Disease.* Dordrecht: Springer Netherlands.

GUGUEN-GUILLOUZO, C. & GUILLOUZO, A. 2010. General Review on In Vitro Hepatocyte Models and Their Applications. *In:* MAUREL, P. (ed.) *Hepatocytes: Methods and Protocols.* Totowa, NJ: Humana Press.

HANSEN, J. L., MOORE, P. B. & STEITZ, T. A. 2003. Structures of Five Antibiotics Bound at the Peptidyl Transferase Center of the Large Ribosomal Subunit. *Journal of Molecular Biology,* 330**,** 1061-1075.

HASSAN, Y., WATTS, C., LI, X.-Z. & ZHOU, T. 2015. A Novel Peptide-Binding Motifs Inference Approach to Understand Deoxynivalenol Molecular Toxicity. *Toxins,* 7**,** 1989.

HAZEL, C. M. & PATEL, S. 2004. Influence of processing on trichothecene levels. *Toxicology Letters,* 153**,** 51-59.

HE, K. & PESTKA, J. 2010. Deoxynivalenol-induced modulation of microRNA expression in RAW 264.7 macrophages-A potential novel mechanism for translational inhibition. *Toxicologist (Toxicol. Sci. Suppl.),* 114**,** 310.

HE, K., ZHOU, H.-R. & PESTKA, J. J. 2012a. Mechanisms for ribotoxin-induced ribosomal RNA cleavage. *Toxicology and Applied Pharmacology,* 265**,** 10-18.

HE, K., ZHOU, H.-R. & PESTKA, J. J. 2012b. Targets and Intracellular Signaling Mechanisms for Deoxynivalenol-Induced Ribosomal RNA Cleavage. *Toxicological Sciences,* 127**,** 382-390.

HILL, H. D. & STRAKA, J. G. 1988. Protein determination using bicinchoninic acid in the presence of sulfhydryl reagents. *Analytical Biochemistry,* 170**,** 203-208.

HOLMSTROM, K. M. & FINKEL, T. 2014. Cellular mechanisms and physiological consequences of redox-dependent signalling. *Nat Rev Mol Cell Biol,* 15**,** 411-421.

HWANG, C., SINSKEY, A. J. & LODISH, H. F. 1992. Oxidized redox state of glutathione in the endoplasmic reticulum. *Science,* 257**,** 1496-1502.

IWAOKA, M. & TOMODA, S. 1994. A Model Study on the Effect of an Amino Group on the Antioxidant Activity of Glutathione Peroxidase. *Journal of the American Chemical Society,* 116**,** 2557-2561.

JAESCHKE, H., GORES, G. J., CEDERBAUM, A. I., HINSON, J. A., PESSAYRE, D. & LEMASTERS, J. J. 2002. Mechanisms of Hepatotoxicity. *Toxicological Sciences,* 65**,** 166-176.

KATERERE, D. R., SHEPHARD, G. S. & FABER, M. 2008. Infant malnutrition and chronic aflatoxicosis in Southern Africa: is there a link? *International Journal of Food Safety, Nutrition and Public Health,* 1**,** 127-136.

KELLY, F. J. & MUDWAY, I. S. 2003. Protein oxidation at the air-lung interface. *Amino Acids,* 25**,** 375-396.

KENSLER, T. W., WAKABAYASHI, N. & BISWAL, S. 2007. Cell Survival Responses to Environmental Stresses Via the Keap1-Nrf2-ARE Pathway. *Annual Review of Pharmacology and Toxicology,* 47**,** 89-116.

KIM, H.-S., PATEL, K., MULDOON-JACOBS, K., BISHT, K. S., AYKIN-BURNS, N., PENNINGTON, J. D., VAN DER MEER, R., NGUYEN, P., SAVAGE, J., OWENS, K. M., VASSILOPOULOS, A., OZDEN, O., PARK, S.-H., SINGH, K. K., ABDULKADIR, S. A., SPITZ, D. R., DENG, C.-X. & GIUS, D. 2010. SIRT3 Is a Mitochondria-Localized Tumor Suppressor Required for Maintenance of Mitochondrial Integrity and Metabolism during Stress. *Cancer Cell,* 17**,** 41-52.

KIMANYA, M. E. 2015. The health impacts of mycotoxins in the eastern Africa region. *Current Opinion in Food Science,* 6**,** 7-11.

KNASMÜLLER, S., MERSCH-SUNDERMANN, V., KEVEKORDES, S., DARROUDI, F., HUBER, W. W., HOELZL, C., BICHLER, J. & MAJER, B. J. 2004. Use of human-derived liver cell lines for the detection of environmental and dietary genotoxicants; current state of knowledge. *Toxicology,* 198**,** 315-328.

KÖNIGS, M., SCHWERDT, G., GEKLE, M. & HUMPF, H.-U. 2008. Effects of the mycotoxin deoxynivalenol on human primary hepatocytes. *Molecular Nutrition & Food Research,* 52**,** 830-839.

KORZENIEWSKI, C. & CALLEWAERT, D. M. 1983. An enzyme-release assay for natural cytotoxicity. *Journal of Immunological Methods,* 64**,** 313-320.

KOSHINSKY, H., HONOUR, S. & KHACHATOURIANS, G. 1988. T-2 toxin inhibits mitochondrial function in yeast. *Biochemical and Biophysical Research Communications,* 151**,** 809-814.

KOUADIO, J. H., MOBIO, T. A., BAUDRIMONT, I., MOUKHA, S., DANO, S. D. & CREPPY, E. E. 2005. Comparative study of cytotoxicity and oxidative stress induced by deoxynivalenol, zearalenone or fumonisin B1 in human intestinal cell line Caco-2. *Toxicology,* 213**,** 56-65.

KURIEN, B. T. & SCOFIELD, R. H. 2006. Western blotting. *Methods,* 38**,** 283-293.

LAEMMLI, U. K. 1970. Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. *Nature,* 227**,** 680.

LIANG, Z., REN, Z., GAO, S., CHEN, Y., YANG, Y., YANG, D., DENG, J., ZUO, Z., WANG, Y. & SHEN, L. 2015. Individual and combined effects of deoxynivalenol and zearalenone on mouse kidney. *Environmental Toxicology and Pharmacology,* 40**,** 686-691.

LIU, Y., FISKUM, G. & SCHUBERT, D. 2002. Generation of reactive oxygen species by the mitochondrial electron transport chain. *Journal of Neurochemistry,* 80**,** 780-787.

LIU, Y., RAN, R., HU, C., CUI, B., XU, Y., LIU, H., QUAN, S., LI, D., LI, X., WU, Y., ZHANG, D. & SHI, J. 2016. The metabolic responses of HepG2 cells to the exposure of mycotoxin deoxynivalenol. *World Mycotoxin Journal,* 9**,** 577-586.

LU, S. C. 2009. REGULATION OF GLUTATHIONE SYNTHESIS. *Molecular aspects of medicine,* 30**,** 42-59.

LU, S. C. 2013. Glutathione synthesis. *Biochimica et Biophysica Acta (BBA) - General Subjects,* 1830**,** 3143-3153.

LUONGO, D., SEVERINO, L., BERGAMO, P., D'ARIENZO, R. & ROSSI, M. 2010. Trichothecenes NIV and DON modulate the maturation of murine dendritic cells. *Toxicon,* 55**,** 73-80.

MAHMOOD, T. & YANG, P.-C. 2012. Western Blot: Technique, Theory, and Trouble Shooting. *North American Journal of Medical Sciences,* 4**,** 429-434.

MARESCA, M. 2013. From the Gut to the Brain: Journey and Pathophysiological Effects of the Food-Associated Trichothecene Mycotoxin Deoxynivalenol. *Toxins,* 5**,** 784-820.

MARIN, S., RAMOS, A. J., CANO-SANCHO, G. & SANCHIS, V. 2013a. Mycotoxins: Occurrence, toxicology, and exposure assessment. *Food and Chemical Toxicology,* 60**,** 218-237.

MARIN, S., RAMOS, A. J., CANO-SANCHO, G. & SANCHIS, V. 2013b. Mycotoxins: Occurrence, toxicology, and exposure assessment. *Food and Chemical Toxicology,* 60**,** 218-237.

MARNETT, L. J. 1999a. Lipid peroxidation—DNA damage by malondialdehyde. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis,* 424**,** 83-95.

MARNETT, L. J. 1999b. Lipid peroxidation—DNA damage by malondialdehyde. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis,* 424**,** 83-95.

MCCORMICK, S. P., STANLEY, A. M., STOVER, N. A. & ALEXANDER, N. J. 2011. Trichothecenes: From Simple to Complex Mycotoxins. *Toxins,* 3**,** 802-814.

MCMAHON, M., ITOH, K., YAMAMOTO, M. & HAYES, J. D. 2003. Keap1-dependent Proteasomal Degradation of Transcription Factor Nrf2 Contributes to the Negative Regulation of Antioxidant Response Element-driven Gene Expression. *Journal of Biological Chemistry,* 278**,** 21592-21600.

MERSCH-SUNDERMANN, V., KNASMÜLLER, S., WU, X.-J., DARROUDI, F. & KASSIE, F. 2004. Use of a human-derived liver cell line for the detection of cytoprotective, antigenotoxic and cogenotoxic agents. *Toxicology,* 198**,** 329-340.

MIKHAK, B., HUNTER, D. J., SPIEGELMAN, D., PLATZ, E. A., WU, K., ERDMAN, J. W. & GIOVANNUCCI, E. 2008. Manganese superoxide dismutase (MnSOD) gene polymorphism, interactions with carotenoid levels and prostate cancer risk. *Carcinogenesis,* 29**,** 2335-2340.

MISHRA, S., DWIVEDI, P. D., PANDEY, H. P. & DAS, M. 2014. Role of oxidative stress in Deoxynivalenol induced toxicity. *Food and Chemical Toxicology,* 72**,** 20-29.

MISIHAIRABGWI, J. M., EZEKIEL, C. N., SULYOK, M., SHEPHARD, G. S. & KRSKA, R. 2017. Mycotoxin contamination of foods in Southern Africa: A 10-year review (2007–2016). *Critical Reviews in Food Science and Nutrition***,** 1-16.

MITSUISHI, Y., MOTOHASHI, H. & YAMAMOTO, M. 2012. The Keap1–Nrf2 system in cancers: stress response and anabolic metabolism. *Frontiers in Oncology,* 2.

MITTAL, M., SIDDIQUI, M. R., TRAN, K., REDDY, S. P. & MALIK, A. B. 2014. Reactive Oxygen Species in Inflammation and Tissue Injury. *Antioxidants & Redox Signaling,* 20**,** 1126-1167.

MITTLER, R. 2002. Oxidative stress, antioxidants and stress tolerance. *Trends in Plant Science,* 7**,** 405-410.

MIYAMOTO, Y., KOH, Y. H., PARK, Y. S., FUJIWARA, N., SAKIYAMA, H., MISONOU, Y., OOKAWARA, T., SUZUKI, K., HONKE, K. & TANIGUCHI, N. 2003. Oxidative Stress Caused by Inactivation of Glutathione Peroxidase and Adaptive Responses. *Biological Chemistry.*

MOSMANN, T. 1983. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *Journal of Immunological Methods,* 65**,** 55-63.

NARUSE, K., TANG, W. & MAKUUCHI, M. 2007. Artificial and bioartificial liver support: A review of perfusion treatment for hepatic failure patients. *World Journal of Gastroenterology : WJG,* 13**,** 1516-1521.

NGUYEN, T., NIOI, P. & PICKETT, C. B. 2009. The Nrf2-Antioxidant Response Element Signaling Pathway and Its Activation by Oxidative Stress. *The Journal of Biological Chemistry,* 284**,** 13291-13295.

NGUYEN, T., SHERRATT, P. J., NIOI, P., YANG, C. S. & PICKETT, C. B. 2005. Nrf2 Controls Constitutive and Inducible Expression of ARE-driven Genes through a Dynamic Pathway Involving Nucleocytoplasmic Shuttling by Keap1. *Journal of Biological Chemistry,* 280**,** 32485-32492.

NIELSEN, C., CASTEEL, M., DIDIER, A., DIETRICH, R. & MÄRTLBAUER, E. 2009. Trichothecene-induced cytotoxicity on human cell lines. *Mycotoxin Research,* 25**,** 77-84.

NIOI, P. & NGUYEN, T. 2007. A mutation of Keap1 found in breast cancer impairs its ability to repress Nrf2 activity. *Biochemical and Biophysical Research Communications,* 362**,** 816-821.

OGURA, T., TONG, K. I., MIO, K., MARUYAMA, Y., KUROKAWA, H., SATO, C. & YAMAMOTO, M. 2010. Keap1 is a forked-stem dimer structure with two large spheres enclosing the intervening,

double glycine repeat, and C-terminal domains. *Proceedings of the National Academy of Sciences,* 107**,** 2842-2847.
OTT, M., GOGVADZE, V., ORRENIUS, S. & ZHIVOTOVSKY, B. 2007. Mitochondria, oxidative stress and cell death. *Apoptosis,* 12**,** 913-922.
PACE, J. G. 1983. Effect of T-2 mycotoxin on rat liver mitochondria electron transport system. *Toxicon,* 21**,** 675-680.
PACE, J. G., WATTS, M. R. & CANTERBURY, W. J. 1988. T-2 mycotoxin inhibits mitochondrial protein synthesis. *Toxicon,* 26**,** 77-85.
PASTORE, A. & PIEMONTE, F. 2012. S-Glutathionylation signaling in cell biology: Progress and prospects. *European Journal of Pharmaceutical Sciences,* 46**,** 279-292.
PENG, Z., CHEN, L., NÜSSLER, A. K., LIU, L. & YANG, W. 2017. Current sights for mechanisms of deoxynivalenol-induced hepatotoxicity and prospective views for future scientific research: A mini review. *Journal of Applied Toxicology,* 37**,** 518-529.
PERAICA, M., RADIC, B., LUCIC, A. & PAVLOVIC, M. 1999. Toxic effects of mycotoxins in humans. *World Health Organisation,* 9**,** 762-791.
PÉREZ-CARRERAS, M., DEL HOYO, P., MARTÍN, M. A., RUBIO, J. C., MARTÍN, A., CASTELLANO, G., COLINA, F., ARENAS, J. & SOLIS-HERRUZO, J. A. 2003. Defective hepatic mitochondrial respiratory chain in patients with nonalcoholic steatohepatitis. *Hepatology,* 38**,** 999-1007.
PESTKA J.J., S. A. T. 2005. Deoxynivalenol : Toxicity and potential effect on humans. . *Toxicology and Enviromental helth part B:,* 8:1**,** 39-69.
PESTKA, J. J. 2007. Deoxynivalenol: Toxicity, mechanisms and animal health risks. *Animal Feed Science and Technology,* 137**,** 283-298.
PESTKA, J. J. 2010. Deoxynivalenol: mechanisms of action, human exposure, and toxicological relevance. *Archives of Toxicology,* 84**,** 663-679.
PESTKA, J. J., ISLAM, Z. & AMUZIE, C. J. 2008. Immunochemical assessment of deoxynivalenol tissue distribution following oral exposure in the mouse. *Toxicology Letters,* 178**,** 83-87.
PESTKA, J. J., ZHOU, H.-R., MOON, Y. & CHUNG, Y. J. 2004. Cellular and molecular mechanisms for immune modulation by deoxynivalenol and other trichothecenes: unraveling a paradox. *Toxicology Letters,* 153**,** 61-73.
PIERRON, A., ALASSANE-KPEMBI, I. & OSWALD, I. P. 2016. Impact of two mycotoxins deoxynivalenol and fumonisin on pig intestinal health. *Porcine Health Management,* 2**,** 21.
PINTON, P. & OSWALD, I. 2014. Effect of Deoxynivalenol and Other Type B Trichothecenes on the Intestine: A Review. *Toxins,* 6**,** 1615.
POHLAND, A. E. 1993. Mycotoxins in review. *Food Additives & Contaminants,* 10**,** 17-28.
QINGHUA, W., VLASTIMIL, D., KAMI, K. & ZONGHUI, Y. 2013. Trichothecenes: Structure-Toxic Activity Relationships. *Current Drug Metabolism,* 14**,** 641-660.
QIU, X., BROWN, K., HIRSCHEY, M. D., VERDIN, E. & CHEN, D. 2010. Calorie Restriction Reduces Oxidative Stress by SIRT3-Mediated SOD2 Activation. *Cell Metabolism,* 12**,** 662-667.
RÅDMARK, O., WERZ, O., STEINHILBER, D. & SAMUELSSON, B. 2015. 5-Lipoxygenase, a key enzyme for leukotriene biosynthesis in health and disease. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids,* 1851**,** 331-339.
RAMAKERS, C., RUIJTER, J. M., DEPREZ, R. H. L. & MOORMAN, A. F. M. 2003. Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. *Neuroscience Letters,* 339**,** 62-66.
ROTTER, B. A. 1996. Invited Review: TOXICOLOGY OF DEOXYNIVALENOL (VOMITOXIN). *Journal of Toxicology and Environmental Health,* 48**,** 1-34.
SALGADO, J. D., WALLHEAD, M., MADDEN, L. V. & PAUL, P. A. 2011. Grain Harvesting Strategies to Minimize Grain Quality Losses Due to Fusarium Head Blight in Wheat. *Plant Disease,* 95**,** 1448-1457.
SAUKKONEN, J. J., COHN, D. L., JASMER, R. M., SCHENKER, S., JEREB, J. A., NOLAN, C. M., PELOQUIN, C. A., GORDIN, F. M., NUNES, D., STRADER, D. B., BERNARDO, J., VENKATARAMANAN, R. &

STERLING, T. R. 2006. An Official ATS Statement: Hepatotoxicity of Antituberculosis Therapy. *American Journal of Respiratory and Critical Care Medicine,* 174**,** 935-952.

SCHARF, G., PRUSTOMERSKY, S., KNASMÜLLER, S., SCHULTE-HERMANN, R. & HUBER, W. W. 2003. Enhancement of Glutathione and γ-Glutamylcysteine Synthetase, the Rate Limiting Enzyme of Glutathione Synthesis, by Chemoprotective Plant-Derived Food and Beverage Components in the Human Hepatoma Cell Line HepG2. *Nutrition and Cancer,* 45**,** 74-83.

SEN, C. K. 1999. Glutathione homeostasis in response to exercise training and nutritional supplements. *Molecular and Cellular Biochemistry,* 196**,** 31-42.

SHARMA, P., JHA, A. B., DUBEY, R. S. & PESSARAKLI, M. 2012. Reactive Oxygen Species, Oxidative Damage, and Antioxidative Defense Mechanism in Plants under Stressful Conditions. *Journal of Botany,* 2012**,** 26.

SHAW, L. B., ZIA, R. K. P. & LEE, K. H. 2003. Totally asymmetric exclusion process with extended objects: A model for protein synthesis. *Physical Review E,* 68**,** 021910.

SHEPHARD, G. S. 2008. Impact of mycotoxins on human health in developing countries. *Food Additives & Contaminants: Part A,* 25**,** 146-151.

SOBROVA, P., ADAM, V., VASATKOVA, A., BEKLOV, M., ZEMAN, L. & KIZEK, R. 2010. Deoxynivalenol and its toxicity *Interdisciplinary Toxicology* 3**,** 94-99.

SOBROVA, P., VASATKOVA, A., SKLADANKA, J., BEKLOVA, M., ZEMAN, L., KIZEK, R. & ADAM, V. 2012. Study of deoxynivalenol effect on metallothionein and glutathione levels, antioxidant capacity, and glutathione-S-transferase and liver enzymes activity in rats. *Chemical Papers,* 66**,** 1092-1102.

ST-PIERRE, J., BRAND, M. D. & BOUTILIER, R. G. 2000. Mitochondria as ATP consumers: Cellular treason in anoxia. *Proceedings of the National Academy of Sciences,* 97**,** 8670-8674.

TANG, W., JIANG, Y.-F., PONNUSAMY, M. & DIALLO, M. 2014. Role of Nrf2 in chronic liver disease. *World Journal of Gastroenterology : WJG,* 20**,** 13079-13087.

TAO, R., COLEMAN, M. C., PENNINGTON, D., OZDEN, O., PARK, S.-H., JIANG, H., KIM, H.-S., FLYNN, C. R., HILL, S., MCDONALD, W. H., OLIVIER, A. K., SPITZ, D. R. & GIUS, D. 2010. Sirt3-Mediated Deacetylation of Evolutionarily Conserved Lysine 122 Regulates MnSOD Activity in Response to Stress. *Molecular Cell,* 40**,** 893-904.

TIMOTHY, P. D., HOWARD, G. S., AND & ALVARO, P. 1999. REGULATION OF GENE EXPRESSION BY REACTIVE OXYGEN. *Annual Review of Pharmacology and Toxicology,* 39**,** 67-101.

TSUBOI, KOUDA, TAKEUCHI, TAKIGAWA, MASAMOTO, TAKEUCHI & OCHI 1998. 8-Hydroxydeoxyguanosine in urine as an index of oxidative damage to DNA in the evaluation of atopic dermatitis. *British Journal of Dermatology,* 138**,** 1033-1035.

TURNER, P. C., ROTHWELL, J. A., WHITE, K. L. M., GONG, Y., CADE, J. E. & WILD, C. P. 2008. Urinary Deoxynivalenol Is Correlated with Cereal Intake in Individuals from the United Kingdom. *Environmental Health Perspectives,* 116**,** 21-25.

TURRENS, J. F. 2003. Mitochondrial formation of reactive oxygen species. *The Journal of Physiology,* 552**,** 335-344.

VALKO, M., RHODES, C. J., MONCOL, J., IZAKOVIC, M. & MAZUR, M. 2006. Free radicals, metals and antioxidants in oxidative stress-induced cancer. *Chemico-Biological Interactions,* 160**,** 1-40.

VERDIN, E., HIRSCHEY, M. D., FINLEY, L. W. S. & HAIGIS, M. C. 2010a. Sirtuin Regulation of Mitochondria - Energy Production, Apoptosis, and Signaling. *Trends in biochemical sciences,* 35**,** 669-675.

VERDIN, E., HIRSCHEY, M. D., FINLEY, L. W. S. & HAIGIS, M. C. 2010b. Sirtuin regulation of mitochondria: energy production, apoptosis, and signaling. *Trends in Biochemical Sciences,* 35**,** 669-675.

VOSS, K. A. 2010. A New Perspective on Deoxynivalenol and Growth Suppression. *Toxicological Sciences,* 113**,** 281-283.

WAGACHA, J. M. & MUTHOMI, J. W. 2008. Mycotoxin problem in Africa: Current status, implications to food safety and health and possible management strategies. *International Journal of Food Microbiology,* 124**,** 1-12.

WANDA, M. & HASCHEK, V. R. 2009. Trichothecene Mycotoxins. *Handbook of Toxicology of Chemical Warfare Agents,* **,** 655-676.

WANG, Z., WU, Q., KUČA, K., DOHNAL, V. & TIAN, Z. 2014. Deoxynivalenol: signaling pathways and human exposure risk assessment—an update. *Archives of Toxicology,* 88**,** 1915-1928.

WARTH, B., SULYOK, M., BERTHILLER, F., SCHUHMACHER, R. & KRSKA, R. 2013. New insights into the human metabolism of the Fusarium mycotoxins deoxynivalenol and zearalenone. *Toxicology Letters,* 220**,** 88-94.

WEYDERT, C. J. & CULLEN, J. J. 2010. MEASUREMENT OF SUPEROXIDE DISMUTASE, CATALASE, AND GLUTATHIONE PEROXIDASE IN CULTURED CELLS AND TISSUE. *Nature protocols,* 5**,** 51-66.

WILD, C. P. & GONG, Y. Y. 2010. Mycotoxins and human disease: a largely ignored global health issue. *Carcinogenesis,* 31**,** 71-82.

YILDIRIM, Z., UCGUN, N. I. & YILDIRIM, F. 2011. The role of oxidative stress and antioxidants in the pathogenesis of age-related macular degeneration. *Clinics,* 66**,** 743-746.

YIN, H., XU, L. & PORTER, N. A. 2011. Free Radical Lipid Peroxidation: Mechanisms and Analysis. *Chemical Reviews,* 111**,** 5944-5972.

ZAIN, M. E. 2011. Impact of mycotoxins on humans and animals. *Journal of Saudi Chemical Society,* 15**,** 129-144.

ZHANG, X., JIANG, L., GENG, C., CAO, J. & ZHONG, L. 2009. The role of oxidative stress in deoxynivalenol-induced DNA damage in HepG2 cells. *Toxicon,* 54**,** 513-518.

ZHANG, Z.-Q., WANG, S.-B., WANG, R.-G., ZHANG, W., WANG, P.-L. & SU, X.-O. 2016. Phosphoproteome Analysis Reveals the Molecular Mechanisms Underlying Deoxynivalenol-Induced Intestinal Toxicity in IPEC-J2 Cells. *Toxins,* 8**,** 270.

ZHONG, L. & MOSTOSLAVSKY, R. 2011. Fine Tuning our Cellular Factories: Sirtuins in Mitochondrial Biology. *Cell metabolism,* 13**,** 621-626.

ZHOU, H.-R., JIA, Q. & PESTKA, J. J. 2005. Ribotoxic Stress Response to the Trichothecene Deoxynivalenol in the Macrophage Involves the Src Family Kinase Hck. *Toxicological Sciences,* 85**,** 916-926.

ZHOU, H., GEORGE, S., HAY, C., LEE, J., QIAN, H. & SUN, X. 2017. Individual and combined effects of Aflatoxin B1, Deoxynivalenol and Zearalenone on HepG2 and RAW 264.7 cell lines. *Food and Chemical Toxicology,* 103**,** 18-27.

APPENDICES

Appendix A

Cell Viability Raw Data

HepG2 cells were treated with serial dilution of DON (0-100 µM) over 24, 48 and 72 hr for the determination of an IC_{50} value

Table 1: Effect of DON concentration on HepG2 cell viability after 24hr of exposure.

DON concentration (µM)	Log (DON)	Average Absorbance	% Cell Viability
0	0	0,6595	100
2.5	0,399	0,59975	90,94011
5	0,699	0,6475	98,18044
10	1	0,6055	91,81198
25	1,399	0,4025	61,03108
50	1,699	0,3845	58,30174
100	2	0,1355	20,54587

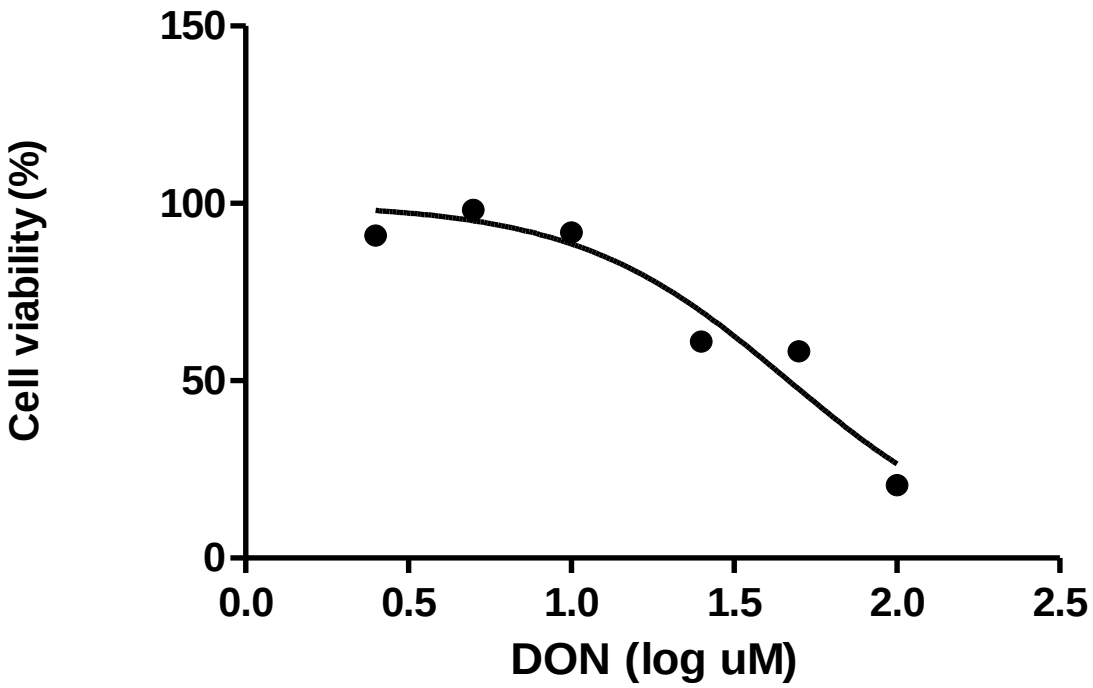

Figure 1: DON dose dependent effect on HepG2 cell viability by DON, over 24-hr exposure

Table 2: Effect of DON concentration on HepG2 cell viability after 48hr of exposure.

DON concentration (µM)	Log (DON)	Average Absorbance	% Cell Viability
0	0	0,8655	100
2.5	0,399	0,72825	84,14211
5	0,699	0.9	103,9861
10	1	0,62475	72,18371
25	1,399	0,27275	43,65746
50	1,699	0,308	35,58637
100	2	0,0176	20,33507

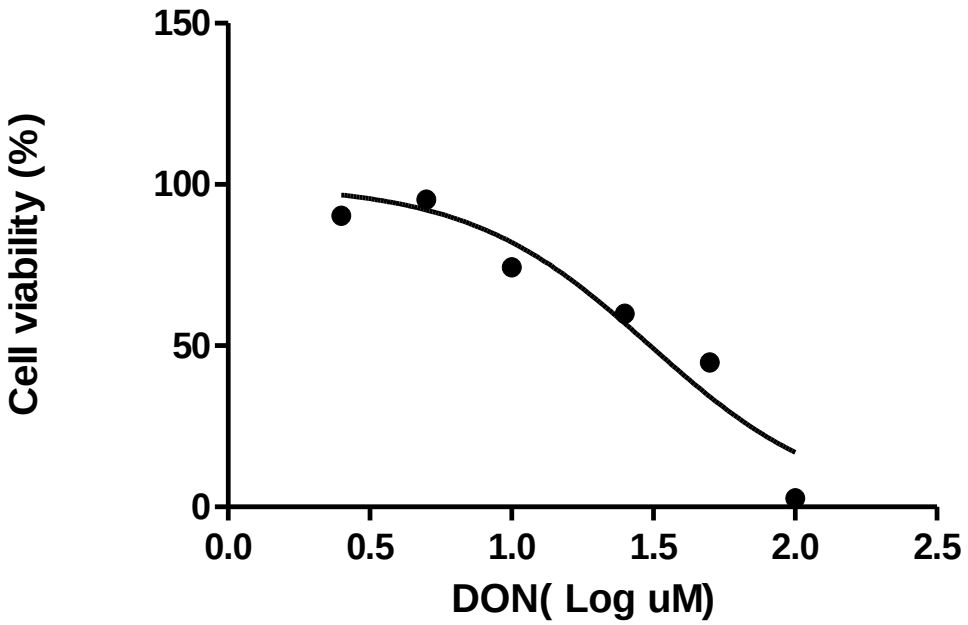

Figure 2: DON dose dependent effect on HepG2 cell viability by DON, over 48-hr exposure

Table 3: Effect of DON concentration on HepG2 cell viability after 72 hr of exposure.

DON concentration (µM)	Log (DON)	Average Absorbance	% Cell Viability
0	0	0,9844	100
2.5	0,399	0,8888	90,2885
5	0,699	0,9384	95,3271
10	1	0,732	74,36002
25	1,399	0,5904	59,97562
50	1,699	0,4412	44,81918
100	2	0,0264	2,681837

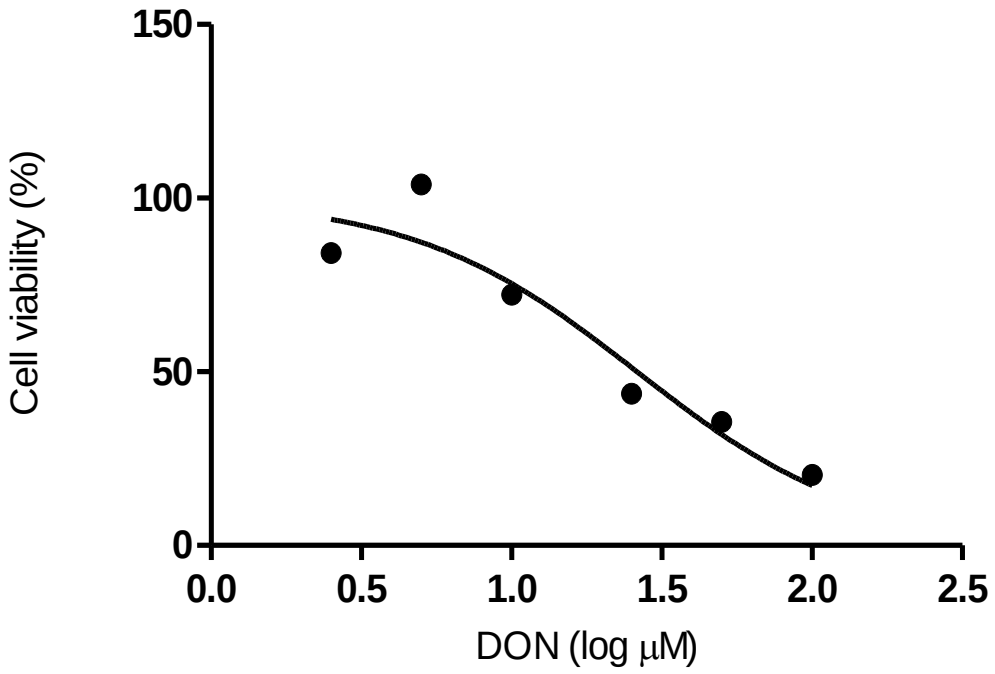

Figure 3: DON dose dependent effect on HepG2 cell viability by DON, over 72-hr exposure

Appendix B

GHS Assay Raw Data

Table 1: The serially diluted GSH used as standards absorbance values (RLU)

GSH STANDARDS (µM)	AVERAGE RLU
0	870700
3,125	1774005
6,25	2348415
12.5	3452800
25	4769320
50	6643900

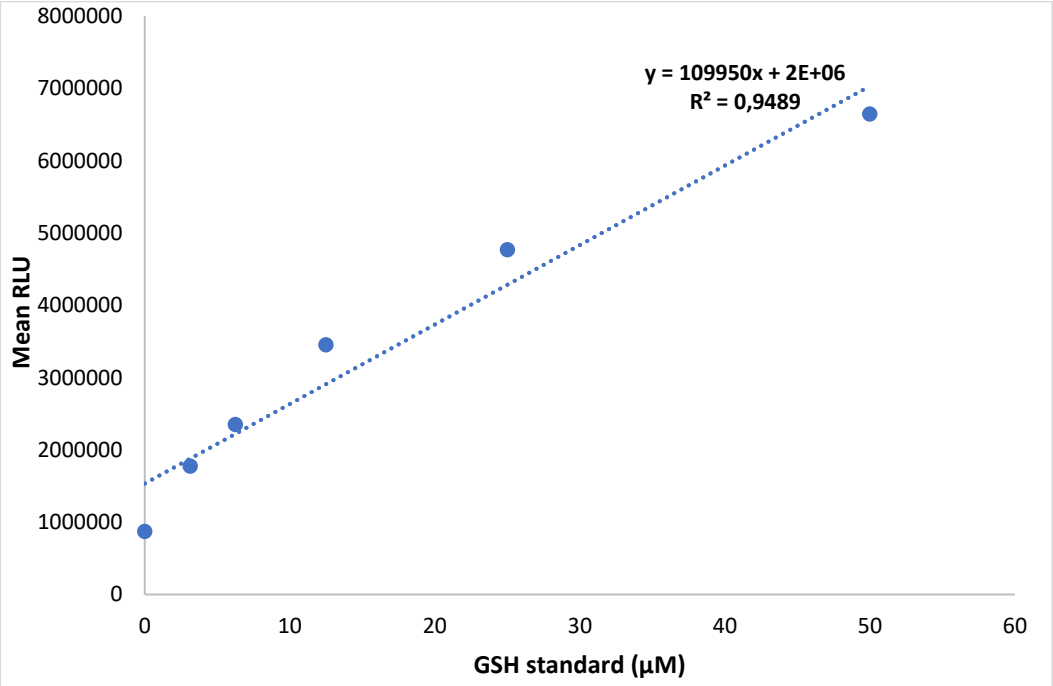

Figure 1**:** GSH concentrations standard curve representing average absorbance values (RLU).

Table 2: Absorbance values (RLU) of samples.

SAMPLE	AVERAGE RLU
CONTROL	4679123
DON	2936870

Appendix C

Standard curve for protein isolation

Known concentrations of Bovine Serum Albumin (BSA) were used to determine the concentration of the samples using the Bicinchoninic Acid (BCA).

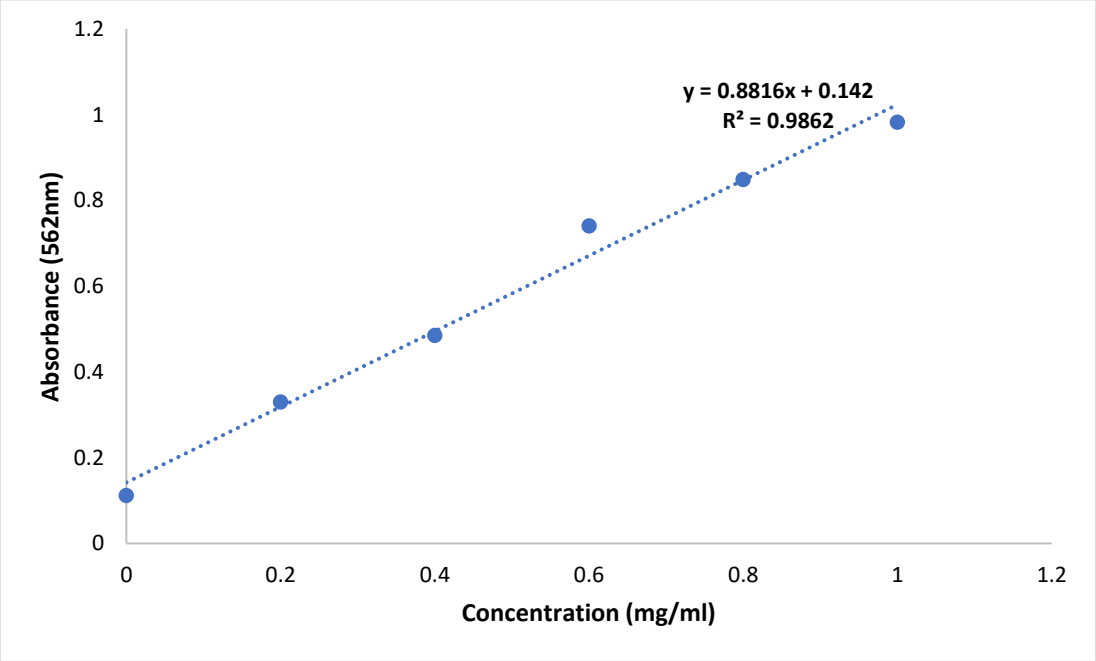

Figure 1: The standard curve of known concentration of BSA