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An investigation into the mitochondrial toxicity of Fusaric acid associated with aberrant energy metabolism and inflammatory responses

By

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PREFACE AND DECLARATION

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Naeem Sheik Abdul

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PUBLICATIONS

1. Naeem Sheik Abdul, Savania Nagiah, Anil Chuturgoon (2016) Fusaric acid induces mitochondrial stress in human hepatocellular carcinoma (HepG2) cells. Toxicon. 119: 336-344 DOI: 10.1016/j.toxicon.2016.07.002.

2. Naeem Sheik Abdul, Savania Nagiah, Anil Chuturgoon (2019) Fusaric acid Induces NRF2 as a Cytoprotective Response to Prevent NLRP3 activation in an in vitro liver model. Toxicology *in vitro*. 55: Pages 151-159. DOI: 10.1016/j.tiv.2018.12.008.

3. Naeem Sheik Abdul, Savania Nagiah, Anil Chuturgoon (2018) Fusaric acid causes metabolic switch from Oxidative phosphorylation to aerobic glycolysis in hepatocytes. Scientific reports (Accepted, scientific reports, Ms. Ref. No.: SREP-18-28513).

4. Naeem Sheik Abdul, Savania Nagiah, Anil Chuturgoon (2018) Interactomic and molecular mechanisms of Fusaric acid induced mitochondrial sirtuin aberrations in glycolytically and oxidatively poised HepG2 liver cells. (In review Chemico-Biological Interactions - CHEMBIOINT_2018_1572).

PRESENTATIONS

1. Fusaric acid induces oxidative stress in human hepatocellular carcinoma (HepG2) cells N. Sheik Abdul, S. Nagiah, and A.A Chuturgoon Medical, Medicine and Health Sciences, Academic Fora, Cape Town, South Africa (3-4 November 2016) Oral Presentation

2. Fusaric acid dampens innate immune response through up-regulation of mitophagy N. Sheik Abdul, S. Nagiah, and A.A Chuturgoon, College of Health Sciences Research Symposium, University of KwaZulu-Natal, Durban, South Africa (5-6 October 2017). Oral presentation

3. Fusaric acid induces metabolic switch and phenotype reminiscent of the Warburg effect, N. Sheik Abdul, S. Nagiah, and A.A Chuturgoon. JSS Medical College, Mysore, India, (21-23 February 2018). Oral Presentation

4. Fusaric acid attenuates SIRT3 activity and expression in glycolytic and oxidative phosphorylation poised HepG2 cells, N. Sheik Abdul, S. Nagiah, and A.A Chuturgoon. School of laboratory medicine and medical science research day, University of KwaZulu-Natal, Durban, South Africa (5-6 October 2017). Oral presentation (29 August 2018)

5. Fusaric acid induces bioenergetic adaptations by switching energy metabolism from mitochondrial processes to glycolysis in a human liver (HepG2) cells. N. Sheik Abdul, S. Nagiah, and A.A Chuturgoon College of Health Sciences Research Symposium, University of KwaZulu-Natal, Durban, South Africa (11-12 October 2018). 1st place Oral Presentation – PhD student category.

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LIST OF ABBREVIATIONS

$\Delta\psi_m$	Mitochondrial membrane potential
ACSS2	Acetyl-coA synthetase 2
ADP	Adenosine diphosphate
AIF	Apoptosis-inducing factor
Apaf-1	Apoptotic protease activating factor-1
ASC	Apoptosis-associated speck-like protein
ATGs	Autophagy related genes
ATP	Adenosine triphosphate
Bcl-2	B-cell lymphoma 2
BAX	BCL2-Associated X Protein
CyP-D	Cyclophilin-D
DAMPS	Damage-associated molecular patterns
DBH	Dopamine-β-hydroxylase
DON	Deoxynivalenol
$FADH_2$	Flavin adenine dinucleotide
FA	Fusaric acid
FB_1	Fumonisin B1
FOXO3A	Forkhead box O3A
G6PD	Glucose-6-phosphate dehydrogenase
GSH	Reduced glutathione
H_2O_2	Hydrogen peroxide

HIFs	Hypoxia-inducible factors
IDH2	Isocitrate dehydrogenase 2
IL-1β	Interleukin 1β
Keap1	Kelch-like ECH-associated protein 1
kDa	Kilo Daltons
KO	Knockout
LC3	Microtubule-associated protein 1A/1B-light chain 3
LC–MS/MSL	Liquid chromatography tandem mass spectrometry
LD_{50}	Lethal dose
LON-P	LON protease
LPO	Lipid peroxidation
miRNA	Micro RNAs
MPP	Matrix peptidase
MPTP	Mitochondrial permeability transition pores
mtDNA	Mitochondrial DNA
mtROS	Mitochondrial ROS
NAD	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate hydrogen
NAM	nicotinamide
NFκB	Nuclear factor κB
NLRP3	Nucleotide-binding oligomerization domain like receptor protein 3
NRF1	Nuclear respiratory factor 1

NRF2	Nuclear factor erythroid 2-related factor 2
OxPhos	Oxidative phosphorylation
O_2^-	Superoxide
OMM	Outer mitochondrial membrane
PA	Picolinic acid
PARL	PINK1/PGAM5- associated rhomboid-like protease
PDH	Pyruvate dehydrogenase
PDK1	PDH kinase 1
PHH	Primary human hepatocytes
Pi	Inorganic phosphate
PK	Pyruvate kinase
PTMs	Post translational modifications
PINK1	PTEN-induced putative kinase 1
ROS	Reactive oxygen species
SQSTM1/ p62	Sequestosome 1
SIRT	Sirtuin
SOD2	Superoxide dismutase 2
TCA cycle	Tricarboxylic acid cycle
TIM	Translocase of the inner membrane
TOM	Translocase of the outer membrane
TSH	Thyroid stimulating hormone
UPR^{mt}	Mitochondrial unfolded protein response

VDAC	Voltage dependent anion channel
WT	Wild type

ABSTRACT

Mycotoxins pose a serious threat to global food security due to their ubiquitous presence in food and feed crops. Mycotoxins became the subject of intense research when aflatoxin was implicated in "turkey X disease", and subsequently many other mycotoxins have been deemed hazardous. Mycotoxins are of particular concern in Africa where environmental factors contribute to accelerated fungal growth and production of toxins; this coupled with poor understanding of the population on mycotoxin contamination and lack of regulatory measures highlight the need for active rather than reactive research into the molecular mechanisms of toxicity.

Fusarium spp, are common contaminants of maize and produce a plethora of mycotoxins, including the neglected fusariotoxin fusaric acid (FA). Fusaric acid is described as a putative mitochondrial toxin and the canonical mechanism of FA toxicity is associated with the induction of oxidative stress; however its molecular mechanisms of toxicity are not fully elucidated. This study aimed to investigate the molecular mechanisms underlying FA-induced mitochondrial dysfunction and its effects on inflammation, cell stress responses and cellular metabolic processes in human hepatocellular carcinoma (HepG2) cells.

It is well-established that mitochondria are the major sources of reactive oxygen species (ROS) and damage associated molecular patterns (DAMPs) that activate inflammatory responses via nucleotide-binding domain (NOD)-like receptor protein 3 (NLRP3). Chronic inflammation or aberrantly regulated activation will result in pathological outcomes. However, the selective elimination of dysfunctional mitochondria via autophagy can maintain mitochondrial homeostasis and protect against the hyperinflammation induced by ROS and DAMPs. The effects of FA (25, 50, 104 and 150 µg/mL, 24 hours) on interleukin-1β (IL-1β) processing, NLRP3 inflammasome priming and activation along with the potential of FA to initiate anti-inflammatory and cytoprotective responses through mitophagy were investigated. Fusaric acid disrupted synthesis and maturation of IL-1β by inhibiting NRLP3 priming and activation. Activation of NF-κB, a master regulator of inflammatory responses, was inhibited by FA through downregulation of upstream activator regulators - IKKβ, TRAF6, IRAK1 and IRAK4. Further experimentation revealed an up-regulation of NRF2 with concomitant elevation in the antioxidant enzyme SOD2 and autophagy/mitophagy markers suggesting that FA induces NRF2 cytoprotective programs and removal of damaged mitochondria in these cells. These results provide insight into a novel mechanism of immune dysregulation by FA.

Metabolic flexibility defines the capacity of cells to respond to changes in nutrient status. Mitochondria are important mediators of metabolic flexibility and dysfunction is associated with metabolic inflexibility and pathology. Foodborne toxins are often overlooked as potential factors contributing to metabolic toxicity. Thus, the effects of FA on energy homeostasis in cultured human liver (HepG2) cells were evaluated. HepG2 cells poised to undergo oxidative and glycolytic metabolism were exposed to a range of FA concentrations (4, 63 and 250 µg/mL) for 6 hours. The data connects a constitutive expression of HIF-1α in response to FA to the inhibition of pyruvate decarboxylation through up-regulation of PDK-1 and phosphorylation of pyruvate dehydrogenase E1α subunit. There was a loss of ATP and metabolite shunt to lactate production in oxidatively poised HepG2 cells. This was confirmed by elevated expression of PKM2 and LDHa Moreover, the potential of FA to induce a glucose "addiction" and phenotype reminiscent of the Warburg effect is highlighted. The findings provide novel insights into the impact of this neglected foodborne mycotoxin in the dysregulation of energy metabolism.

Fusaric acid is a niacin related compound and established chelator of divalent cations. This mycotoxin is a putative mitochondrial toxin and inducer of oxidative stress. Mitochondrial sirtuins are NAD dependent deacetylases that play a critical role in cytoprotection against oxidative and mitochondrial stressors as well as maintaining metabolic flexibility. The role of FA in the dysregulation of the mitochondrial SIRTs (3and 5) was examined in this study. Molecular docking revealed strong ligand affinity of FA to all mitochondrial SIRTs, with SIRT3 showing the highest binding affinity. The results indicate that FA binds to the active site of SIRT3 preventing binding of NAD (co-factor). All SIRTs were down regulated at the protein level but showed elevated mRNA expression. The expression profile of the common transcription factor, PGC-1α showed strong induction at the protein level suggesting that FA affects SIRT translational processes. The phosphorylation status of eif2α (an indicator of translational inhibition) was elevated. Furthermore, the expression profile of miRNA 30c, which suppresses SIRT translation was significantly elevated. The study suggests that FA inhibits SIRT activity; moreover this mycotoxin hinders SIRT expression through interference with translational and transcriptional processes as well as removal of zinc ions needed for SIRT stability.

In conclusion, FA mediated mitochondrial stress induced cytoprotective mechanisms that perturb cellular homeostasis leading to aberrant inflammatory processes and energy metabolism. Foodborne toxins contribute to mitochondrial toxicity and may have implications in disease conditions such as cancer and diabetes which stem from oxidative stress, aberrant immune regulation and metabolic inflexibility.

INKULUMO YOPHANDO EFINGQIWE

Ama-Mycotoxins atshengisa ubungozi obusiriyasi ekudleni emhlabeni jikelele ngenxa yokuba a-ubiquitous ngobukhona bawo ekudleni nase makhobeni okudliwa yimfuyo. Ama- Mycotoxins aba undabuzekwayo kuphando ngenkathi u-aflatoxin wayesoleka kwi “turkey X disease”, kuphinde nje emvakwalokho asemaningi amanye ama-mycotoxins esevezwe njenganobungozi. Ama-mycotoxins ayinkinga kakhulu e-Africa lapho isimo sendawo sinomuthelela ekukhuleni ngokushesha kwe-fungal nokukhiqizeka kwama-toxins; lokhu kuhambisana nokungabi nolwazi oluphusile emuphakathini mayelana nezinhlobo zama- mycotoxin contamination kanti nokuxega kwemigomo nemibandela kuveza isidingo sokwenza umunyakazo ngophando lwe-molecular mechanisms ye-toxicity.

I-Fusarium spp, abangcolisi abadumile bombila phinde nje bakhiqize inqwaba yama- mycotoxins, Kanye Kanye nalena enganakiwe i-fusariotoxin fusaric acid (FA). I-Fusaric acid ichazwe njenge mbangela ye-mitochondrial toxin kuphinde nje i-mechanism evamile ye-FA toxicity ibandakanyeka nokukhiqizeka kwe-oxidative stress; kodwa ke ama-molecular mechanisms ayo ukuze ibe i-toxicity ebengakaze avezwe kahle ngokuphelele. Ucwaningo lolu luhlose ukuphanda kabanzi ama-molecular mechanisms ayimbangela ye-FA-induced mitochondrial dysfunction Kanye negalelo kwi-inflammation, ukuzivikela kweseli ekucindazelekeni Kanye nendlela ekwenzeka ngayo i-cellular metabolic ngaphakathi kuma-hepatocellular carcinoma (HepG2) seli omuntu.

Sekungqanyiswe ngokusobala ukuthi ama-mitochondria awumgodlagodla omukhulu ka-ROS Kanye nama-DAMPs lawa avusa i-inflamatory response ngosizo luka NLRP3. I- inflammation yafuthi noma imvuselelo e-aberrantly regulated iphelela kwimiphumela e- pathological. Kodwa ke, uhlungo olukhethekileyo lama-dysfunctional mitochondria nge- autophangy ingangcina i-homeostasis ye-mitochondria iphinde ivikele kwi- hyperinflammation eqalwa u-ROS Kanye nama-DAMPs. Igalelo lika-FA (25, 50, 104, and 150 μg/mL, amahora awu 24) kwi IL-1β processing, NRLP3 inflammasome priming and activation Kanye-Kanye ne-potential ka-FA yokuqalisa i-anti-inflammatory kanye nama-cytoprotective responses nge mitophagy liphenyiwe. U-Fusaric acid uphazamise i-synthesis nokukhula kuka- IL-1β ngokucindazela u-NRLP3 priming and activation. I-activation ka-NF-κB, umulawuli owu-master kuma-inflammatory responses, wacindezelwa ngu-FA nge- downregulation ye-upstream activator regulators - IKKβ, TRAF6, IRAK1 and IRAK4. I- experimentation enzulu yaveza i-up-regulation of NRF2 kanye ne-concominent elevation ye- antioxidant enzyme SOD2 kanye ne-autophagy/mitophagy markers okukhombisa ukuthi u- FA uqalisa uhlelo lwe-NRF2 cytoprotective Kanye nokususwa kwama-mitochondria a- damaged kumaseli. Lemiphumela ilethe ulwazi kuma-novel mechanism e-immune dysregulation ebangwa ngu-FA.

Ukuzivumelanisa nezimo kwe-metabolic ikona okucacisa umthamo weseli ekuzinakekeleni kuzinguquko kwi-status zezakhi. Ama-mitochondria ibona abahleli ababalulekile ekuzivumelaniseni kwe-metabolic Kanti i-dysfunction ibandakanyeka nokungazivumelanisi kwe-metabolic Kanye ne-pathology. Ama-toxins a-foodborne avamise ukuzibeka ukuba angaba nomuthelela kwi-metabolic toxicity. Kwazise, igalelo lika-FA kwi-homeostasis yamandla esibindini somuntu (HepG2) esi-cultured icwaningiwe. Amaseli e-HepG2 alungele uhlelo lwe-oxidative Kanye ne-glycolytic metabolism aqhathwe nama-range amanani ahlukene ka-FA (4, 63 and 250 µg/mL) kumahora awu-6. I-data ihlanganisa i-constitutive expression ka-HIF-1α ukuziphendulela ku-FA ecindazela u-pyruvate decarboxylation nge- up-regulation ka-PDK-1 kanye ne-phosphorylation ka-Pyruvate Dehydrogenase E1α subunit. Kwabakhona ukulahleka kuka-ATP Kanye ne-metabolite shunt uku-lactate umkhiqizo kumaseli ka-HepG2 a-oxidatively poised. Lokhu kwaqinisekiswa ukunyuka kokuvelela kuka PKM2 kanye no-LDHa ngaphezukwalokho, i-potential ka-FA ukuqala i-glucose “addiction”.

Kanye ne-phenotype reminiscent ye-Warburg effect iveziwe kafushane. Lemiphumela inikeza ulwazi olunzulu ngegalelo lale-foodborne mycotoxin enganakiwe kwi-dysregulation yamandla e-metabolism.

I-Fusaric acid iyi-compound ehlobene ne-niacin kuphinde nje sekwafakaziswa ukuthi i-chelator ama-divalent cations. Le-mycotoxin iyi-putative mitochondrial toxin phinde isukele ukuqala kuka-oxidative stress. Ama-Mitochondrial sirtuins angama-deacetylases angaphansi kwa-NAD phinde adlala igalelo elibaluleke kakhulu kwi-cytoprotection ngokumelene ne- oxidative Kanye ne-mitochondrial stressors phinde futhi igcine ukuzivumelanisa nezimo ze- metabolic. Igalelo lika-FA kwi-dysfunction ka-mitochondrial SIRTs (3 and 5) laphenywa kulesi-study. I-molecular docking iveze i-ligand affinity eqinile ka-FA kubona bonke o- SIRTs be-mitochondria, kodwa ke u-SIRT3 iwona otshengise izinga eliphezulu le-binding affinity. Imiphumela itshengisa ukuthi u-FA uya-bind kwindawo e-active ka-SIRT3 avimbe i-binding ka-NAD (co-factor). Bonke o-SIRT baba-down regulated kwizinga le-protein kodwa batshengisa ukunyuka ngezinga kwi-mRNA expression. I-expression profile yama- transcription factor ajwayelekile, PGC-1α atshengise i-induction enamandla kwizinga lama protein okusho ukuthi u-FA u-affect inhlelo zika-SIRT translation. I-status se- phosphorylation ka eif2α (an indicator of translational inhibition) sikhuphukile. Kuphinde nje, i-expression profile ka miRNA 30c, ekuyiyo ecindazela i-SIRT translation yanyuka ngezinga eli-significantly. Lesi-study siveza ukuthi u-FA ucindezela ama-activity ka-SIRT; phinde futhi le-mycotoxin i-hinder i-SIRT expression ngokuthi i-interfere kwinhlelo ze- translational naze-transcriptional phinde ivimbe ukususwa kwama-zinc ions njengoba kudingeka ukugcina i-stability sika-SIRT. Ukuvala nje, u-FA ulawula ama-cytoprotective mechanisms aqalwe i-mitochondrial stress kwazise lezinhlelo zi-perturb i-cellular homeostasis eholela kuma-aberrant inflammatory processes Kanye namandla emetabolism. Ama-toxins a-foodborne anomthelela kwi- mitochondrial toxicity futhi

anomthelela kwizimo zezifo ezinjengo mdlavuza Kanye nezifo sikashukela okuyisisuka nge-oxidative stress, aberrant immune regulation Kanye ne-metabolic inflexibility.

CHAPTER 1

1. Introduction

1.1. Background

Toxigenic fungi parasitise agricultural commodities and produce a plethora of mycotoxins (Pitt, 2000, Tournas and Niazi, 2018). Chronic and acute exposure to these secondary metabolites is often associated with adverse outcomes in animals and humans (Zain, 2011). Mycotoxin research interest increased following the discovery of aflatoxin B_1 (AFB_1) as the aetiological agent of Turkey X disease (Bradburn et al., 1994, Spensley, 1963). Ensuring feed and food security has significant implications for health and economic development, particularly in Africa where environmental factors contribute to accelerated fungal growth and mycotoxin production (Wagacha and Muthomi, 2008, Udomkun et al., 2017).

From an African perspective, *Aspergillus* and *Fusarium*-derived aflatoxins and fumonisins, are of major concern. These two classes of mycotoxins are wide spread in dietary staples, particularly maize (Fandohan et al., 2003, Bankole et al., 2006, Wagacha and Muthomi, 2008). Recently, there is a growing interest in the potential toxicity of fusariotoxins (secondary toxic metabolites produced by *Fusarium* species) (Nedělník, 2002). An often neglected fusariotoxin is fusaric acid (FA, 5-butylpicolinic acid). This highly ubiquitous toxin is an established phytotoxin (Löffler and Mouris, 1992, Venter and Steyn, 1998, Wu et al., 2008, Stipanovic et al., 2011a) but was considered to be of moderate toxicity in mammals (Wang and Ng, 1999). Fusaric acid exhibits pharmacological effects on the cardiovascular, nervous and immune systems (Wang and Ng, 1999). The toxic effects of FA in combination with co-produced, better-known mycotoxins have revealed synergistic and additive effects in animal models (Bacon et al., 1995, Smith et al., 1997, Fairchild et al., 2005). However, studies focusing on single toxin analysis are more suited to the correlation of exposure and toxic outcomes.

The canonical mechanism of FA toxicity is associated with oxidative stress through excessive reactive oxygen species (ROS) production and depletion of intrinsic antioxidant systems (Kuźniak, 2001, Hirai et al., 2005, Singh and Upadhyay, 2014, Devnarain et al., 2017). Fusaric acid is an efficient chelator of divalent cations and subsequent inactivation of metalloenzymes (Stack Jr et al., 2004, Yin et al., 2015). Recently, FA was shown to induce DNA damage (Ghazi et al., 2017) as well as aberrant signalling cascades and cell death in several human cell lines (Dhani et al., 2017, Devnarain et al., 2017).

Fusaric acid is a putative mitochondrial toxin, which decreases adenosine triphosphate (ATP) levels in plant and animal models (D'Alton and Etherton, 1984, Telles-Pupulin et al., 1998, Pavlovkin et al., 2004, Singh and Upadhyay, 2014, Abdul et al., 2016). However, knowledge on the molecular mechanisms

underlying this mitochondrial dysfunction is lacking. The effect of FA on hepatic energy metabolism was assessed in rat liver mitochondria (Telles-Pupulin et al., 1998) and an *in vitro* human liver model (Abdul et al., 2016). Fusaric acid inhibited the activity of key tricarboxylic acid (TCA) cycle enzymes and oxidative phosphorylation (Telles-Pupulin et al., 1998). Further, FA induced mitochondrial stress via downregulation of key mitochondrial stress response proteins as well as inhibited mitochondrial biogenesis (Abdul et al., 2016).

Mitochondria regulate and maintain several aspects of cell homeostasis, including energy substrate flux, apoptosis, inflammation and cell signalling. Mitochondrial dysfunction is associated with pathogenesis of cancer and insulin resistance through aberrant metabolic programming (Modica-Napolitano and Singh, 2004, Singh, 2004, Kim et al., 2008, Sorrentino et al., 2018).

Studies have revealed the importance of mitochondria in coping with cellular stressors. Mitochondrial stress responses including the mitochondrial unfolded protein response (UPR^{mt}), antioxidant defences, and mitophagy are induced to maintain cellular integrity and organism homeostasis (Kanamaru et al., 2012, Lin et al., 2018). The sirtuin (SIRT) family of nicotinamide adenine dinucleotide (NAD) dependent enzymes is strongly linked to mitochondrial stress responses (Kumar and Lombard, 2015, Lin et al., 2018). Post translational modifications (PTMs), such as lysine acetylation, ADP ribosylation, malonylation, glutarylation, and succinylation on various mitochondrial proteins has emerged as a mechanism to fine tune mitochondrial responses. These modifications are directly regulated by members of the SIRT family. Recent studies in SIRT biology have uncovered their key roles in regulating multiple mitochondrial pathways and dysregulation is often associated with aberrant metabolic homeostasis and disease initiation and progression (Ahuja et al., 2007, Lombard et al., 2007, Du et al., 2011, Kumar and Lombard, 2015).

Compromised mitochondria are recognised arbitrators for the pro-inflammatory response. They act by controlling innate immunity via ROS sensitive inflammatory pathways and activation of nuclear factor κB (NFκB) (García-Ruiz et al., 1995, Amma et al., 2005) or direct priming and activation of inflammasomes (Zhou et al., 2011, Nakahira et al., 2011, Won et al., 2015). These pathways often work together to process and activate pro-inflammatory cytokines necessary to mount an immune response (López-Armada et al., 2013).

The inflammasome is a multi-protein complex that is activated by caspase 1 and subsequently cleaves pro-interleukin 1β (IL-1β) into its biologically active form (Franchi et al., 2009, López-Armada et al., 2013). Recent studies have highlighted mitochondria as important and direct activators of the nucleotide-binding oligomerization domain like receptor protein 3 (NLRP3) through ROS and damage associated

molecular pattern (DAMP) generation (Zhou et al., 2011, Nakahira et al., 2011, Kepp et al., 2011, Tschopp, 2011). The NLRP3 inflammasome has been implicated in several pathologic states (Menu and Vince, 2011, Vandanmagsar et al., 2011, Wen et al., 2012) and is becoming the subject of intense research. Indeed, substantial evidence supports mitochondrial dysfunction and the development of an inflammatory phenotype. Mitophagy (removal of damaged mitochondria via autophagy) can prevent and control inflammasome activation by preventing ROS and DAMPs accumulation (Goldman et al., 2010, Nakahira et al., 2011, Kim et al., 2016).

Mitochondria are hubs of metabolism that interlink several biochemical processes for efficient energy substrate utilisation(Houten and Wanders, 2010, Cheng and Ristow, 2013, Liesa and Shirihai, 2013). This makes these organelles integral regulators of metabolic flexibility (capacity to switch between energy substrates) (Gao et al., 2014). The metabolic program is an active regulator of cell phenotype and changes to the metabolic program results in aberrant energy substrate regulation (Metallo and Vander Heiden, 2010). Mitochondrial dysfunction is strongly linked to metabolic inflexibility. The pathologic potential of metabolic inflexibility is seen in diabetes and insulin resistance (Galgani et al., 2008, Corpeleijn et al., 2009), while a metabolic switch from mitochondrial oxidative phosphorylation (OxPhos) to aerobic glycolysis (Warburg effect) is strongly associated with a cancer cell phenotype and aberrant proliferation (Seyfried and Shelton, 2010, Liberti and Locasale, 2016).

Dietary bioactive compounds are known to have profound therapeutic effects by mitigating inflammation and metabolic inflexibility (Kris-Etherton et al., 2004, Serrano et al., 2016, Souilem et al., 2017), however very little is known regarding common foodborne toxins and their roles in inflammation and metabolic disease initiation and progression.

1.1.1. Problem statement

The majority of the South African population relies heavily on maize as part of their staple diet. *Fusarium* species parasitise agricultural commodities especially maize and are known producers of FA (Bacon et al., 1996). This toxin is highly prevalent, with levels of up to 13,593 µg/kg sample maize being reported in the literature (Streit et al., 2013). Although oxidative stress and inhibition of metalloenzymes are canonical mechanisms of FA toxicity, little work has been done to explore the mitotoxic potential of FA and downstream implications at the molecular level.

1.1.2. Hypothesis

It was hypothesised that FA induced mitochondrial stress that is associated with aberrant inflammatory responses and metabolic flexibility in addition to energy substrate regulation.

1.1.3. Aim and Research questions

This study aimed to determine the mitotoxic effects of FA and subsequent dysregulation of molecular pathways related to inflammation and energy substrate utilisation in a human liver cell line. The following research questions were posed:

- Does FA induce mitochondrial stress?
- Is FA involved in the dysregulation of the NLRP3 inflammatory response?
- Does FA bring about metabolic inflexibility and aberrant energy processing?
- Can FA disrupt mitochondrial protein PTMs?

1.1.4. Objectives

The mitotoxic effects of FA in human hepatoma (HepG2) cells were investigated by measuring mitochondrial output and determining cellular adaptations to mitochondrial dysfunction. Several aspects of inflammation and metabolic flexibility in addition to the molecular mechanisms involved were examined. The specific objectives of the study were to investigate the effects of FA on:

- Mitochondrial stress and biogenesis and its implications on cell death and oxidative stress.
- The role of mitochondrial dysfunction and release of DAMPs in the priming and activation of the NLRP3 inflammasome. .
- Cytoprotective responses, specifically mitophagy and antioxidant stress responses.
- Metabolic flexibility and utilisation of energy substrates.
- Mitochondrial mediators (SIRTs) of PTMs

1.1.5. Experimental approach

Primary human hepatocytes (PHH) are regarded as the benchmark for toxicity studies due to their physiologic similarities to liver tissue. However, isolation from whole livers unsuitable for transplant or from healthy sections after surgery can damage cellular contents and membranes (Guillouzo, 1998, Guguen-Guillouzo and Guillouzo, 2010). Furthermore, inter-variability between donors, loss of

phenotype and scarcity of donors limit the use of PHH for toxicity studies (Heslop et al., 2017). Therefore, the use of hepatoma derived cell lines such as HepG2 is often preferred as they offer the advantage of homogenous cell populations and unlimited sub-cultivation potential.

The HepG2 cell line has been extensively used in toxicity studies (Westerink and Schoonen, 2007, Kim et al., 2009, Gerets et al., 2012, Ramirez et al., 2018), but it is important to consider the bioenergetic phenotype of cells when studying mitochondrial and metabolic effects of toxins. The HepG2 cell line originated from the hepatocellular carcinoma of a 15 year old Caucasian male (ATCC), and as such anaerobic metabolism and glycolysis predominate energy metabolism due to the Warburg and Crabtree effects (Dell'Antone, 2012). Consequently, cell lines originating from tumours are more resistant to mitochondrial toxins. By substituting galactose for glucose in cell culture media, HepG2 cells become aerobically poised and are forced to undergo OxPhos to maintain ATP levels (Figure 1.1). This bioenergetic modification has been reported to sensitise cells to mitochondrial toxins and identify compounds that induce mitochondrial dysfunction (Marroquin et al., 2007, Kamalian et al., 2015, Plecitá-Hlavatá et al., 2015). Consistent with this methodology, chapters 3 and 4 sought to determine the underlying molecular mechanisms of FA on energy metabolism and mitochondrial SIRTs dysfunction in HepG2 cells.

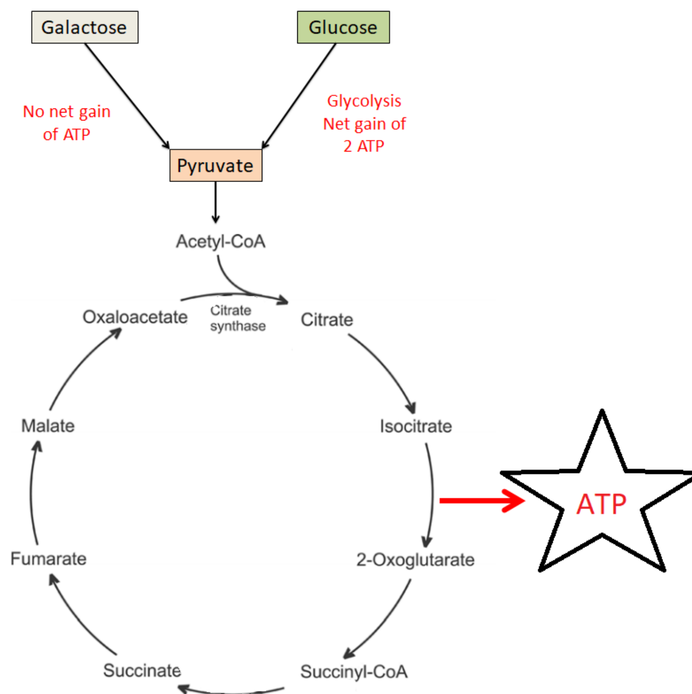


Figure 1.1: Glucose metabolism to pyruvate via glycolysis yields a net gain of 2 ATP. The metabolism of galactose to pyruvate is inefficient and no substantive ATP is produced. Substituting galactose for glucose

forces cells to undergo to use the TCA cycle and OxPhos to maintain ATP levels. Figure adapted from (Ostergaard et al., 2007).

1.2. Literature Review

1.2.1. Food security and mycotoxins

The Food and Agriculture Organization (FAO) defines food security as a ‘‘situation that exists when all people, at all times, have physical, social, and economic access to sufficient, safe, and nutritious food that meets their dietary needs and food preferences for an active and healthy life’’ (FAO, 2014). Developing countries, particularly those in Africa are faced with a multitude of socio-economic struggles making it one of the most food insecure regions in the world. Efforts to improve food security in developing countries such as South Africa, must aim to expand regulatory structures for safe consumption and alleviate poverty through trade of food products that meet stringent international standards (Udomkun et al., 2017, Ferrão et al., 2017).

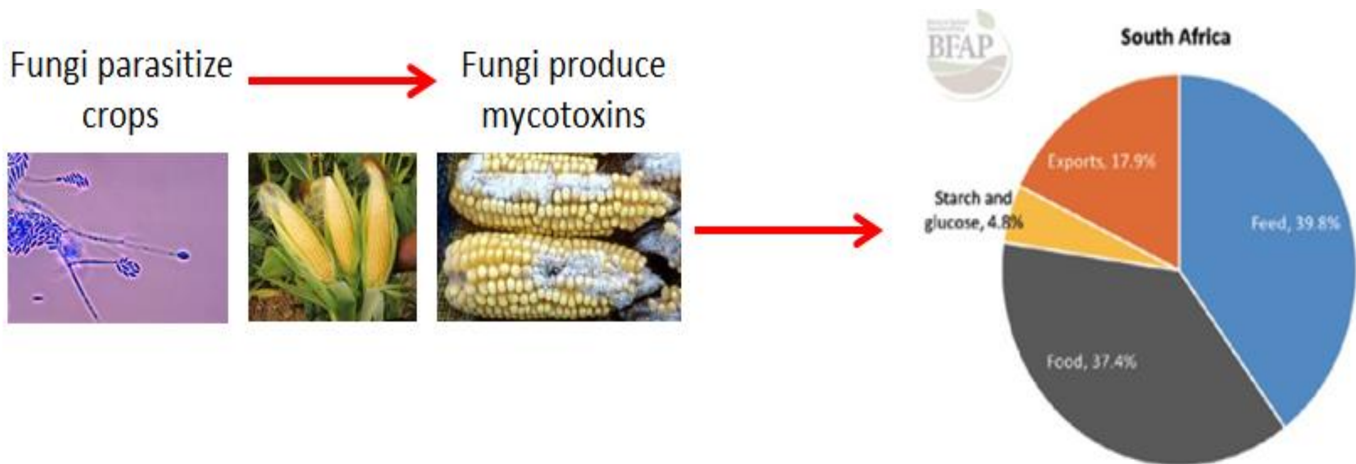
Enhanced food security is often considered vital to improving health and nutrition status of a country through reducing incidents of food and waterborne diseases. Mycotoxins pose a serious threat to food security. It is estimated that about a quarter of the world’s agricultural produce is contaminated with these ubiquitous toxins (Outlook, 2015). This is of particular concern to South Africa where many low income consumers rely on staple diets and traditional food processing that are highly susceptible to mycotoxin contamination (Shephard et al., 2002, Odhav and Naicker, 2002, Isaacson, 2005, Shephard et al., 2005) and can be linked to aetiology of cancer particularly in rural populations (Sydenham et al., 1990, Isaacson, 2005).

1.2.2. Mycotoxins

1.2.2.1. Mycotoxin overview

Mycotoxins are secondary metabolites of filamentous fungi which parasitise agricultural commodities especially grain and wheat (Figure 1.2). One species of fungi is capable of producing a plethora of mycotoxins and one type of mycotoxin can be produced by several fungal species, (Pitt et al., 2000, Pitt, 2000, Hussein and Brasel, 2001), suggesting that co-production and contamination by several mycotoxins is highly probable. The fungal species belonging to the *Aspergillus* and *Fusarium* genera are considered the most important to Africa (Fandohan et al., 2003, Bankole et al., 2006, Wagacha and Muthomi, 2008).

The severity of contamination is linked to environmental factors as well as pre-harvest and post-harvest (storage of commodities) conditions which contribute to fungal growth and mycotoxin contamination (Magan et al., 2003, Chulze, 2010, Magan et al., 2011, Bernhoft et al., 2012). Furthermore, mycotoxins are not completely removed by food processing and can be found as contaminants in finished food products (Karlovsky et al., 2016, Tibola et al., 2016).



<u>Figure 1.2</u>: Scheme of fungal contamination and mycotoxin production in maize destined for consumption in South Africa. Image adapted from (AgTag, 2015).

The contamination of agricultural commodities by mycotoxins is a global problem (Wild and Gong, 2009). A highly concentrated and inhomogeneous distribution of a toxin can spoil an entire batch of crops (Rivas Casado et al., 2009). This is of particular concern given the open and international trade of food and feed. Such massive import and export of agricultural commodities requires stringent guidelines and regulatory measures. Legislation exists for metabolites regarded as “regulated toxins” and mycotoxins with guidance levels. These two categories include the aflatoxins, some type A and B trichothecenes, zearalenone (ZEN), fumonisins and ochratoxin A (OTA), due to their established acute and chronic toxicity; values specified in feed are either maximum allowed levels or guidance values (Kovalsky et al., 2016).

In the European Union, differences in food/feed commodity, degree of processing (e.g., maize, finished feed) and intended consumers (e.g., animal feed) lead to variable maximum allowable concentrations. Lowest levels are in place for aflatoxin with a maximum limit of 20 µg kg^{-1}. Fumonisin levels in animal feed have a set guidance level of 60 mg kg^{-1}, but with lower values for pigs, poultry and young animals. Guidance values for deoxynivalenol (DON) (0.9–12 mg kg^{-1}), ZEN (0.1–2 mg kg^{-1}) and OTA (0.1–0.25 mg kg^{-1}) are significantly lower. The upper level provides a general value with exceptions applying for

animals showing high sensitivity. Lowest values are typically established for swine, poultry and young animals. Recommended values for the sum of T-2 and HT-2 toxins are lowest with 250 µg kg^{-1} for compound feed, 500 µg kg^{-1} for cereal products and 2,000 µg kg^{-1} for oat milling products (Kovalsky et al., 2016).

The analysis of worldwide mycotoxin occurrence data is of interest, as it can identify geographical areas, which are highly contaminated due to climate change or conditions that influence contamination rates. This can affect international trade of agricultural commodities (Paterson and Lima, 2010, Magan et al., 2011).

1.2.2.2. Mycotoxin toxicity and their biological effects

Exposure to mycotoxins induces numerous acute and chronic effects in animals and humans, such as genotoxicity, hepatotoxicity, nephrotoxicity, immunosuppression and teratogenic effects (Veselý and Vesela, 1995, Peraica et al., 1999, Hussein and Brasel, 2001). These toxic metabolites are established potent mutagens and carcinogens in human and animal models (Sydenham et al., 1990, Gelderblom et al., 1988, Chuturgoon et al., 2014b, Chawanthayatham et al., 2017). Strong correlations can be drawn between carcinogenesis and mycotoxin adducts with DNA. Aflatoxin B_1 (AFB_1) and its derivatives bind covalently to DNA and often cause mutations leading to cancer (Weng et al., 2017). Fumonisin B_1 (FB_1), a common fusariotoxin, may lead to liver cancer through interference with sphingolipid metabolism (Garcia-Ruiz et al., 2015, Burger et al., 2018). Recent studies by Chuturgoon *et al* have highlighted DNA modifications, inhibition of apoptosis and epigenetic aberrations favouring cancer phenotypes (Chuturgoon et al., 2014b, Chuturgoon et al., 2014a, Chuturgoon et al., 2015). Mycotoxins dysregulate protein synthesis by inhibiting translation (Bin-Umer et al., 2014) and up-regulating micro-RNAs (miRNAs) that degrade target mRNA preventing translation (Bin-Umer et al., 2014, Chuturgoon et al., 2014b).

Oxidative stress – a phenomenon classified by excessive generation of free radicals, is a common and plausible mechanism for general mycotoxin toxicity. Oxidative stress causes DNA damage, oxidation of macromolecules and cell death (Da Silva et al., 2018). Intracellular antioxidants prevent damage to cellular components as a result of oxidative stress. Patulin, a mycotoxin found on apples and some grains, has a strong affinity for sulfhydryl groups, especially glutathione (GSH) – an intracellular antioxidant (Jin et al., 2016). Several studies have indicated that mycotoxins dysregulate antioxidant responses through aberrant control of nuclear factor erythroid 2-related factor 2 (NRF2) (Da Silva et al., 2018), a crucial transcription factor for antioxidant enzymes (Liufang et al., 2016).

Mitochondria are major sites of ROS production due to electron leakage from the electron transport chain (ETC) (Sullivan and Chandel, 2014, Zorov et al., 2014). Mycotoxins contribute to excessive ROS production by inhibiting mitochondrial function and consequently damaging mitochondrial DNA. The mitochondrial alterations associated with mycotoxin toxicity is the uncoupling of OxPhos, induction of mitochondrial permeability and inhibition of mitochondrial stress response proteins (Abdul et al., 2016, Da Silva et al., 2018) resulting in decreased ATP synthesis. Mitochondrial dysfunction is linked to inflammation which can be attributed to the activation of NF-κB and transcription of inflammatory genes (Kastl et al., 2014). Several mycotoxins can modulate the inflammatory response by activating or inhibiting NF-κB signalling (Tsai et al., 2016, Adesso et al., 2017, Da Silva et al., 2018).

The variable molecular structures of mycotoxins account for their wide range of toxic mechanisms of action. Fungal secondary metabolites can cause adverse health effects in many whole organ systems. Literature on mycotoxin-related human diseases reveals strong correlation between ingesting mycotoxin-contaminated food and illness, especially hepatic, gastrointestinal, renal, hormonal and carcinogenic diseases (Peraica et al., 1999, Fung and Clark, 2004). A summary of the toxic effects of mycotoxins is shown in figure 1.3.

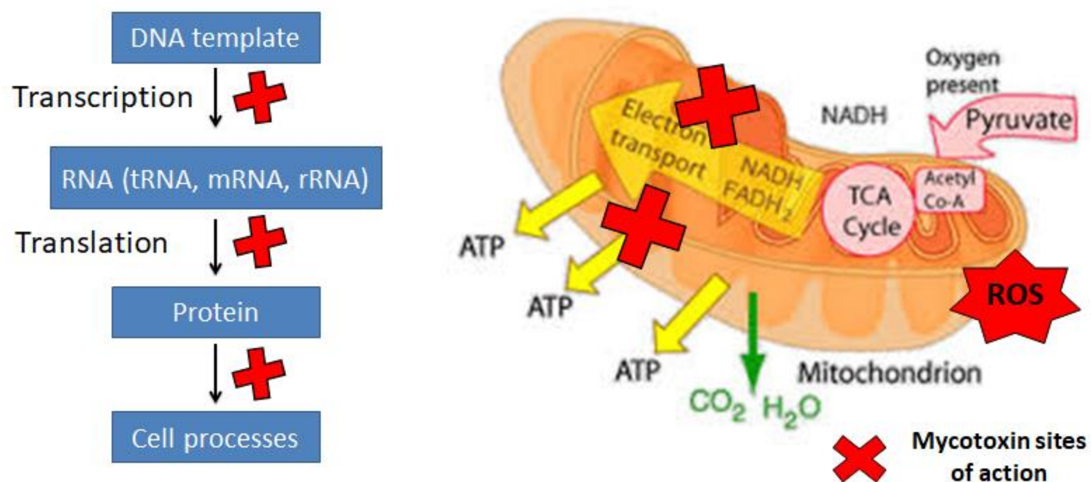


Figure 1.3: Mycotoxins exert a variety of biological activities and inhibit various cellular processes in animals and humans. Prepared by author.

1.2.2.3. Emerging mycotoxins

A multitude of fungal metabolites in food and feed can now be identified and measured with modern analytical techniques (Streit et al., 2013, Gruber-Dorninger et al., 2018). In addition to the extensively studied and established mycotoxins, emerging mycotoxins are shown to occur ubiquitously in agricultural produce (Meca et al., 2010, Zinedine et al., 2011, Vaclavikova et al., 2013). Emerging mycotoxins are defined as "mycotoxins, which are neither routinely determined, nor legislatively regulated; however, the evidence of their incidence is rapidly increasing" (Vaclavikova et al., 2013, Gruber-Dorninger et al., 2016). Under this definition, several fungal metabolites with suspected toxicity would fall under the category of emerging mycotoxins. The use of liquid chromatography tandem mass spectrometry (LC–MS/MS) provides new insights into the structure and chemical classification of these novel toxins. Risk assessments are underway should legislature regarding regulation of these emerging mycotoxins need to be implemented (Vaclavikova et al., 2013).

Many of these newly discovered secondary metabolites are irrelevant to food and feed safety, but the occurrence data collected on a large scale, prior to the assessment of toxicity, can lead to "active" research rather than "reactive" research. This can contribute greatly to avoiding catastrophic outbreaks as was the case with Turkey X disease. While it is imperative to assess both occurrence and toxicity to draw up holistic risk assessments, the need to prioritise research of highly ubiquitous toxins (Figure 1.4) over the most bioactive compounds is now conceivable (Gruber-Dorninger et al., 2016).

Figure 1.4: Feed contaminated with mycotoxins sensitised scientists to the possibility that these secondary metabolites might be deadly to organisms. This increased active research into mycotoxins. Prepared by author.

1.2.3. Fusaric acid

Fusaric acid is one of the most widely distributed fusariotoxins, produced by several *Fusarium* species including *F. fujikuroi, F. oxysporum*, *F. moniliforme*, *F. subglutinans*, *F. verticillioides*, *F. crookwellense, F. napiforme* and *F. proliferatum*. A study by Bacon *et al.* (1996) estimated that 11,665 species of plants (hundreds of which are agriculturally important) may serve as hosts of *Fusarium* spp, suggesting that human exposure and contact with FA is considerably high (Bacon et al., 1996). Occurrence data for FA suggest that this mycotoxin is a common contaminant of maize (Bacon et al., 1996, Streit et al., 2013) with an average and maximum of 643 and 13,593 µg/kg sample, respectively (Streit et al., 2013).

Fusaric acid is a polyketide derived secondary metabolite (Brown et al., 2015) and is formed from a polyacetate unit and aspartic acid or closely related metabolites (Hill et al., 1966, Stipanovic et al., 2011b). A study investigating the biosynthesis of FA used ^{13}C-labeled substrates such as [1, 2-^{13} C2] acetate along with ^{13}C- and ^{15}N-labelled aspartate and [^{15}N] glutamine. The integration of labelled substrates is consistent with the biosynthesis of FA from three acetate units at C5-C6, C7-C8, and C9-C10, the remaining carbons are derived from aspartate via oxaloacetate and the TCA cycle. Furthermore, this study proved that the nitrogen in FA is derived from an endogenous pool to which both glutamine and aspartate contribute, with the former making a larger contribution (Stipanovic et al., 2011b).

Fundamentally, FA is classified as a pyridine derivative and is a niacin (nicotinamide, NAM) related compound. Fusaric acid is made up of a pyridine ring and a butyl group at position C5 (Stack et al., 2014) and bares structural similarities to its parent compound, picolinic acid (PA) (Figure 1.5) (Ogata et al., 2001). Picolinic acid (PA) is synthesised endogenously by the liver, kidney and brain (Grant et al., 2009); PA is produced in a side pathway of NAD biosynthesis in animals (Ogata et al., 2001). Tryptophan can be metabolised via the kyneurinine pathway which oxidatively degrades this amino acid to yield kynurenic acid, PA and NAD (Figure 1.6) (Grant et al., 2009). Picolinic acid is also produced as a toxin by the mould species *Magnorpathegrisea* and *Fusarium* spp contributing to their pathogenesis (Zhang et al., 2004). Structural similarities to biologically relevant molecules contributes significantly to mycotoxin mechanisms of action and toxicity (Zain, 2011). Similarities between FA, PA and niacin are depicted in figure 1.6.

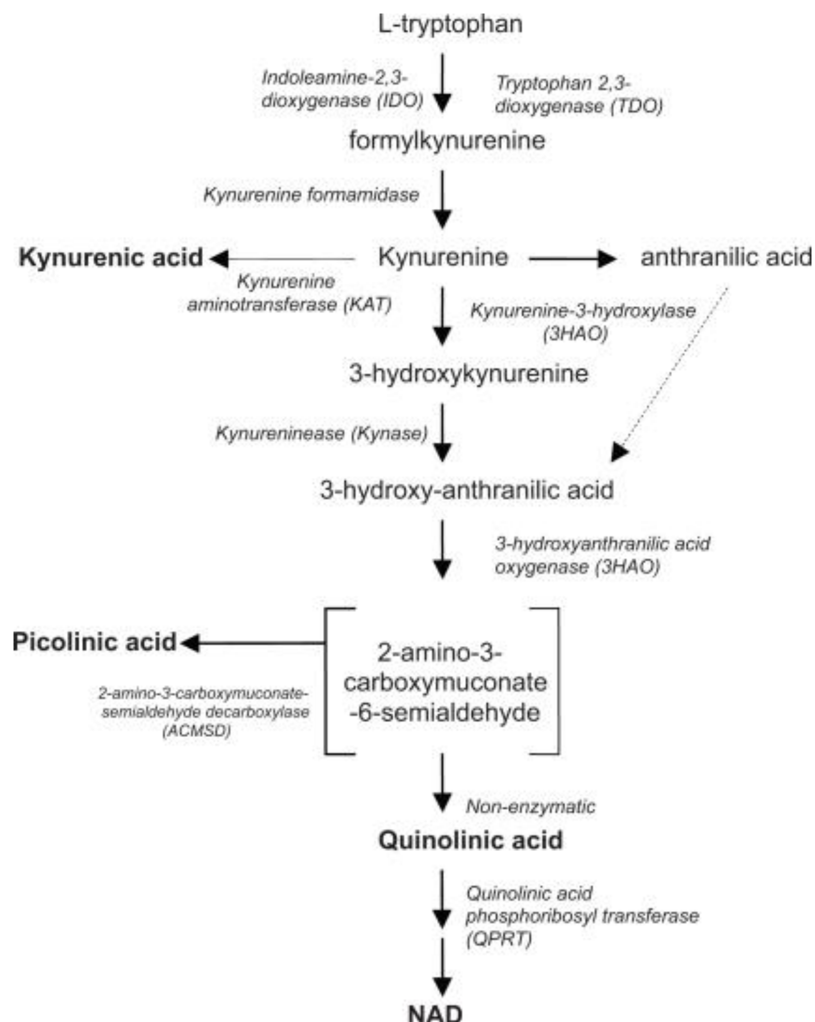


Figure 1.5: Endogenous synthesis of PA via the kyneurine pathway in the human brain (Grant et al., 2009).

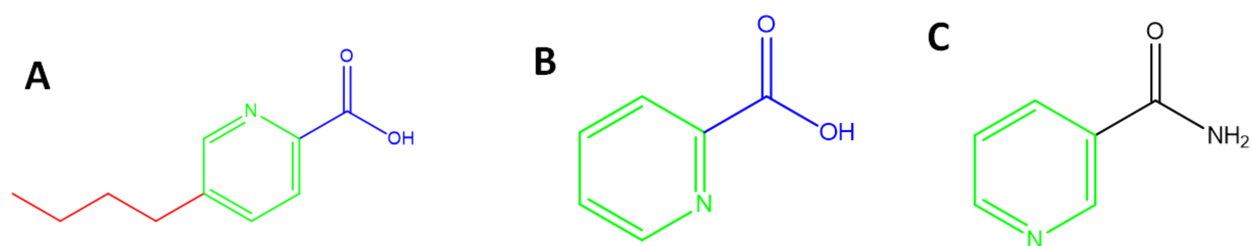


Figure 1.6: Structures of FA (**A**), PA (**B**) and NAM (**C**). All three molecules contain pyridine rings (green) however FA has a butyl group at position 5 (red). Both PA and FA are acidic (blue). Prepared by author.

1.2.3.1. Pharmacological use of FA

Fusaric acid is the precursor of the beta blocker, bupicomide (Velasco et al., 1975); FA potently inhibits dopamine-β-hydroxylase (DBH, catalyses the conversion of dopamine to norepinephrine) in plasma causing endogenous norepinephrine levels to drop significantly and was considered a unique, hypotensive drug (Terasawa and Kameyama, 1971, Matta and Wooten, 1973). The long term hemodynamic effect of an FA calcium salt in 10 elderly hypertensive patients showed that in the first year both the systolic and diastolic blood pressures were lowered, with no apparent adverse effects. There were no consistent changes in heart rate or plasma volume. It was concluded that the hypotensive response was attained by reduction of the total peripheral vascular resistance index (Terasawa et al., 1976). A study in rabbits showed that FA can induce relaxation of isolated arterial preparations, likely through chelation of calcium (Hidaka and Asano, 1976).

The effects of FA on DBH extend to the nervous system where it affects neurotransmitter levels. Experiments in a geriatric patient population showed FA improved the mental state of patients already on neuroleptics. Furthermore, FA significantly relieved oro-facial dyskinesia, tremor, and rigidity (Viukari and Linnoila, 1977). The effects of FA on thyroid stimulating hormone (TSH) were clearly demonstrated in patients with hypothyroidism. Alterations in brain dopamine, epinephrine and norepinephrine (neuro amines) may directly or indirectly suppress pituitary TSH secretion (Yoshimura et al., 1977). The use of FA as a treatment for Parkinson's disease due to its DBH inhibitory activity has also been proposed but not fully explored (Matta and Wooten, 1973). These very early studies highlighted the bio-activity of FA in humans.

In more recent studies FA was shown to be a promising tumoricidal agent in several *in vitro* models. Work by Stack Jr *et al.* (2004) presented FA as a potential therapeutic agent for targeting head and neck squamous cell cancer. Many metalloproteins are involved in DNA repair and promotion of on-going cell growth and proliferation, chelation may be an alternate means of inducing cell cycle arrest or apoptosis. The potential anti-tumour action of FA may be brought on in part by its ability to chelate divalent cations from metalloproteins such as metallopanstimulin-1, which is a zinc finger protein involved in DNA repair and protein synthesis (Stack Jr et al., 2004).

Ruda *et al.* (2006) demonstrated that oral administration of FA inhibits tumour growth in an animal model. Following tumour cell inoculation mice were treated with FA or saline. This study revealed that mice treated with FA had considerably slower tumour growth rates as well as reduced tumour mass when compared to control mice. This suggests a suppressive effect of FA on head and neck squamous cell

cancer xenografts (Ruda et al., 2006). Consequently, experiments with FA in combination with two of the most currently used chemotherapeutic agents - paclitaxel and carboplatin showed better efficacy and tumoricidal potential than when these drugs were used as single agents. Data from this study suggested that these combinations may provide an improved anti-tumour effect. This type of adjuvant therapy was touted as being a paradigm change in chemotherapy (Jaglowski and Stack Jr, 2006, Ye et al., 2013).

1.2.3.2. Toxicity of Fusaric Acid

1.2.3.2.1. Plant toxicity

The phytotoxic potential of FA is well established as it has been identified as the causal agent of wilt disease (Gaumann, 1957, Toyoda et al., 1991, Bacon et al., 1996). Fusaric acid accelerates the development of wilt disease by causing non-stomatal water loss as a result of membrane damage to leaf cells (Wang et al., 2015a). This phytotoxin inhibits photosynthesis by reducing chlorophyll mass and inducing cellular necrosis in leaves (Wu et al., 2008). Experiments by Diniz and Oliveira showed that the development of corn seedlings is adversely affected by FA. Fusaric acid interferes with energy metabolism, thus inhibiting the ATP dependent process of root hair elongation (Diniz and Oliveira, 2009). It is plausible that FA is a putative mitochondrial toxin as it impairs the flow of electrons between succinate dehydrogenase and coenzyme Q, inhibits : (1) ATPase/ATP-synthase activity, (2) α-ketoglutarate dehydrogenase (Telles-Pupulin et al., 1996) and (3) some metalloenzymes (e.g., cytochrome oxidase) leading to respiratory impairment (Singh and Upadhyay, 2014). Mitochondrial dysfunction is necessary for the programmed cell death observed in cells treated with FA. These cells exhibit a loss in mitochondrial membrane potential, depleted ATP content and antioxidant activities as well as elevated levels of hydrogen peroxide (H_2O_2) and lipid peroxidation (LPO) (Samadi and Behboodi, 2006, Jiao et al., 2014, Singh and Upadhyay, 2014).

1.2.3.2.2. Animal Toxicity

Since FA is considered moderately toxic, its effects in animals and mechanisms of toxicity are not well studied. The overall toxicity of FA is grossly underestimated as only a few feed and food products have been screened for FA despite its ubiquitous presence (Smith and Sousadias, 1993, Porter et al., 1995, Streit et al., 2013). Toxic interactions of FA and FB_1, two of the most common mycotoxins produced by *Fusarium*, confirmed in a chicken egg model experiment that FA can enhance toxicity in mammalian systems if it is consumed with other mycotoxins (Bacon et al., 1995).

Porter and colleagues showed that FA is co-synthesised with other fusariotoxins (Porter et al., 1995) and these toxins exhibit synergistic and additive effects. Experiments conducted in immature swine showed that by feeding a blend of FA and deoxynivalenol, feed intake and weight gain were drastically decreased (Smith et al., 1997, Swamy et al., 2002). Fusariotoxin blends were found to alter neurotransmitter levels and brain regional neurochemistry (due to DBH inhibition), thus providing a potential mechanistic basis for the feed refusal (Swamy et al., 2002). Chickens and turkeys fed FA-contaminated diets (up to 150 mg FA/kg diet), on the other hand, did not show any abnormalities in behaviour, feed intake, weight gain, and appearance of visceral organs or lesions (Chu et al., 1993, Fairchild et al., 2005), this suggests that the effect of FA is species dependent.

The immunotoxicity of diets co-contaminated with FA and other fusariotoxins has been verified by decreased cell-mediated immunity but enhanced the humoral response in chickens (Chu et al., 1993), and elevated serum immunoglobulin concentrations in pigs (Swamy et al., 2002). Further research is needed to determine the aetiology of the elevated serum immunoglobulin concentrations and its subsequent pathophysiology.

Studies focusing on FA alone are likely to show a better correlation between its concentration and potential mechanisms of action. It is acknowledged that FA is of relatively low acute toxicity with lethal dose (LD_{50}) values in mice of 80 and 100 mg/kg body weight for intra peritoneal and intra venous administration respectively. Death caused by the LD_{50} is believed to be due to the hypotensive effect of FA (Hidaka et al., 1969). The bioavailability (25 mg/kg) in Sprague−Dawley rats of FA was calculated to be 58% (Stack et al., 2014) while its half-life in human plasma was approximated to be 12 hours (Matta and Wooten, 1973).

Oral dosing of swine with FA had profound changes on behaviour and neurochemistry. Vomiting was noted in more than half of the treated animals. The major changes in brain tissue of sacrificed pigs were elevated concentrations of tryptophan, 5-hydroxytryptamine and serotonin. Dosed pigs appeared lethargic; the authors suggested that lethargy was due to increased serotonin synthesis (Smith and MacDonald, 1991).

An *in vivo* rat model further confirmed that FA induced neurochemical alterations. Rats were administered FA intraperitoneally and showed elevated brain levels of serotonin, 5-hydroxy-indoleacetic acid, tyrosine, and dopamine, while norepinephrine was decreased. Dopamine in the pineal gland increased with a concomitant decrease to norepinephrine. Pineal N-acetyl-serotonin was increased whereas pineal serotonin and its two major metabolites 5-hydroxyindoleacetic acid and 5-hydroxytryptophol decreased. This was hypothesised to be a result of partial inhibition of tyrosine-

hydroxylase and DBH by FA (Porter et al., 1995). It is well known that tryptophan is needed for the synthesis of neurotransmitters and is a precursor of serotonin. A previous study revealed FA administration in rats increased both free circulating tryptophan and tryptophan levels in the brain suggesting that FA affects tryptophan disposition. This may occur through two mechanisms as *in vivo* peripheral alterations in catecholaminergic metabolism and by FA chemical structure since *in vitro* experiments showed that FA displaced tryptophan binding to albumin, thus increasing the plasma free tryptophan pool (Chaouloff et al., 1986). These studies suggest possible mechanisms for the altered neurochemistry induced by FA.

The embryotoxic and teratogenic effects of FA have been assessed in mice (Reddy et al., 1996) and zebrafish (Yin et al., 2015) models respectively. During pregnancy, FA increased the maternal corticosterone levels of dams (CD-1 mice) which can lead to cleft palate in the offspring via reduced catecholaminergic activity. This may be due to reduced catecholamine synthesis via FA dependent DBH inhibition (Reddy et al., 1996). Yin et al. investigated the teratogenicity of FA in zebrafish and found that FA caused an undulated notochord in zebrafish development as a result of copper chelation and subsequent inhibition of lysyl oxidase (Yin et al., 2015).

Fusaric acid was measured in colostrum of rat pups and was directly proportional to the amount of FA consumed by nursing dams indicating lactational passage of FA. Further FA was found to decrease pineal serotonin and tyrosine in nursing pups. The data also suggest limited neonate weight gains may be related to either decreased milk production in dams or mycotoxin effects on the neonate (Porter et al., 1996). In summary, FA is active in the neurochemical, cardiovascular and reproductive systems, but most *in vivo* data indicate relatively low toxicity.

1.2.3.2.3. In vitro toxicity

Several *in vitro* studies have revealed a toxic profile of FA, this coupled with the use of advanced molecular techniques, helped elucidate some of its molecular mechanisms of toxicity in human cell models. Toxicity data generated from cell viability assays indicate that the effects of FA are cell line dependent. Recent studies (on human cancer cell lines) revealed that FA decreased cell viability at higher concentrations (24 hrs; IC_{50} = 78 µg/ml, 107.7 µg/ml and 104 µg/ml for SNO (oesophageal cancer), THP-1 (leukaemic) and HepG2 (human liver) cells respectively) (Abdul et al., 2016, Devnarain et al., 2017, Dhani et al., 2017). These cytotoxicity studies provide evidence that FA can be considered to be toxic in humans.

Understanding the molecular mechanisms underlying the potential toxicities of FA is paramount to risk assessment. The overt production of ROS is often associated with FA toxicity. Elevated levels of malondialdehyde (MDA) - a marker for lipid peroxidation and oxidative stress- have been detected in human cell lines (Abdul et al., 2016, Dhani et al., 2017, Devnarain et al., 2017). Fusaric acid increased the production of ROS through enhancement of the Fenton reaction (Iwahashi et al., 1999, Hirai et al., 2005). The nitrogen atom in the pyridine ring and the oxygen atom in the carboxyl group seem to participate in the chelation of the Fe^{2+} ion (Figure 1.7) (Hirai et al., 2005) thereby increasing hydroxyl radical formation. Fusaric acid induced the expression of NRF2, the key antioxidant transcription factor, suggesting cell defence systems are up-regulated to cope with oxidative stress (Abdul et al., 2016).

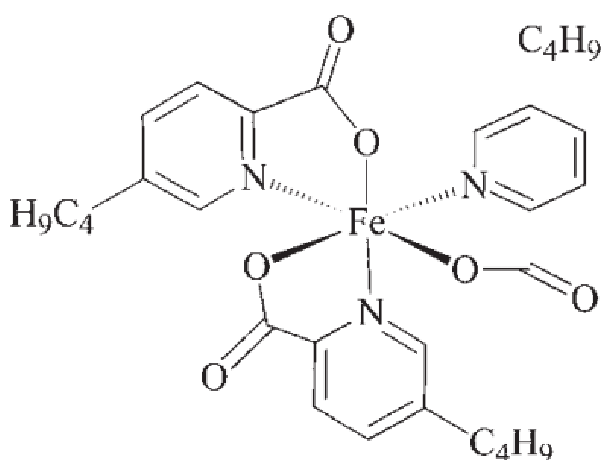


Figure 1.7: Ferrous iron in complex with FA (Hirai et al., 2005).

The induction of cell death by FA and its implications for cytotoxicity have been established in several human cell lines. Activation of caspase 3 (executioner caspase) was elevated in human liver (Abdul et al., 2016), oesophageal (Devnarain et al., 2017) monocytic (Dhani et al., 2017) and promyelocytic (Ogata et al., 2001) cell lines. Further investigation into apoptosis initiation showed increased caspase 8 and 9 activities, suggesting that FA is capable of up-regulating both the extrinsic and intrinsic apoptotic pathways (Dhani et al., 2017, Devnarain et al., 2017). Mechanistic insights into the induction of caspase activation are yet to be fully elucidated. Work by Dhani et al (2017) and Devnarain et al (2017) showed that downregulation of anti-apoptotic B-cell lymphoma 2 (Bcl-2) and upregulation of BCL2-Associated X Protein (BAX) proteins (Devnarain et al., 2017, Dhani et al., 2017) are common upstream events in caspase mediated cell death. Fusaric acid is a genotoxic agent capable of inducing DNA fragmentation in HL-60 cells (Ogata et al., 2001). A later study by Ghazi et al (2017) validated the DNA damaging effect of FA and proposed that PTMs to the tumour suppressor protein, p53 (the guardian of the genome) leads to apoptosis in liver derived cells (Ghazi et al., 2017).

Fusaric acid, considered as a putative mitochondrial toxin (Arias, 1985, Telles-Pupulin et al., 1998), is a major disruptor of cell energy metabolism. Several *in vitro* studies have shown FA to dysregulate NAD/NADH metabolism and decrease ATP levels in mammalian cells (Telles-Pupulin et al., 1998, Abdul et al., 2016, Dhani et al., 2017, Devnarain et al., 2017). Work by Telles-Pupulin et al (1998) has demonstrated that FA inhibited succinate-dehydrogenase, α-ketoglutarate-dehydrogenase and OxPhos. The inhibition of OxPhos is the result of direct action on the ATP-synthase/ATPase complex without significant inhibition of the ATP/ADP exchange in isolated perfused rat liver. Oxygen uptake and gluconeogenesis from pyruvate are both inhibited by FA. Although the same enzymes are inhibited in maize seedlings, rat mitochondria are more sensitive (Telles-Pupulin et al., 1998). Furthermore, chain elongation of FA is catalysed in mitochondria to yield CoA esters of FA with acetyl-CoA utilised as a source of C_2 (Mizugaki et al., 1986).

Cells have developed a network of regulated stress-response mechanisms. Fusaric acid was shown to abrogate mitochondrial responses to oxidative stress by decreasing the expression of SIRT3 and PGC-1α. The accumulation of toxic, oxidised and misfolded mitochondrial proteins can be enhanced by FA via down-regulation of LON protease (LON-P) and heat shock protein 70 (HSP70) (Abdul et al., 2016). Increased oxidative stress due to mitochondrial dysfunction often enhances mitochondrial biogenesis (Lee and Wei, 2005). The process of generating new and functional mitochondria relies on the coordinated transcription of nuclear and mitochondrial genes. Fusaric acid downregulated the expression of hepatic nuclear respiratory factor 1 (NRF1) and PGC-1α, key co-transcription factors required for expression of mitochondrial specific proteins (Abdul et al., 2016). Furthermore LON-P deficient cells switched to necrosis as a result of ATP depletion caused by mitochondrial defects (Bota et al., 2005). Lactate dehydrogenase (LDH) release was increased by FA- an indication of increased necrotic cell death (Abdul et al., 2016, Devnarain et al., 2017). Taken together these studies showed that mitochondrial function and biogenesis is impaired by FA and this has implications on cell death and viability. An overview of FA mediated toxicity is shown in figure 1.8.

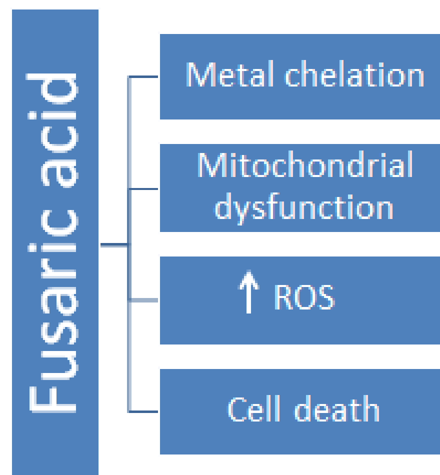


Figure 1.8: Major mechanisms of FA mediated toxicity in cells. Prepared by author.

1.2.4. Mitochondria

Mitochondria are double membrane organelles that fulfil cellular energy needs through the conversion of macromolecules (carbohydrates, fatty acids, and amino acids) into ATP – the energy currency of the cell. Therefore, tissues with high metabolic rates like muscles, liver, brain, and heart have the highest density of mitochondria (Kakkar and Singh, 2007). These organelles auto-replicate and possess their own genome (Jornayvaz and Shulman, 2010). Mitochondrial DNA is circular and encodes 13 mRNAs, 22 tRNAs and 2 rRNAs. All 13 mRNAs encode proteins necessary for OxPhos. The remaining mitochondrial proteins are encoded by the nuclear genome (Yoboue and Devin, 2012). The coordinated synthesis and import of proteins encoded by the nuclear genome is required for correct mitochondrial biogenesis (Jornayvaz and Shulman, 2010). Because a major portion of mitochondrial proteins are encoded by nuclear DNA, several mechanisms exist to target, import, and correctly assemble these proteins thereby ensuring proper mitochondrial function and morphology (Ventura-Clapier et al., 2008).

1.2.4.1. Mitochondria and ATP synthesis

Mitochondria are divided into four compartments: an inner membrane, an outer membrane, a matrix space surrounded by the inner membrane, and an inter-membrane space between the outer and inner membranes. The TCA cycle occurs in the matrix of the mitochondria and OxPhos occurs on the inner membrane (Kakkar and Singh, 2007).

The TCA cycle is a central pathway that connects several individual metabolic pathways. It is considered the most important metabolic pathway for energy supply as it integrates carbohydrate, fatty acid, and amino acid metabolism. The TCA cycle is responsible for the production of several intermediates needed for anabolic processes as well as NADH and flavin adenine dinucleotide ($FADH_2$) which are fed forward into the ETC (Figure 1.9) (Akram, 2014).

Production of ATP relies on electron transfer at the ETC. This chain is comprised of multi-enzyme complexes that are embedded in the inter-mitochondrial membrane: complex I: NADH CoQ reductase, complex II: succinate-CoQ reductase, complex III: reduced CoQ cytochrome c reductase, complex IV: cytochrome c oxidase and complex V: ATP synthase which is made up of the F_1 and F_0 units (Kakkar and Singh, 2007).

The ETC generates an electrochemical proton gradient across the inner membrane which drives OxPhos. Complex 1 oxidises NADH to NAD^+ and initiates the process of electron flow. Electrons sequentially flow through complex III, cytochrome c and lastly through complex IV where bound oxygen is reduced to water. During the process of electron flow from NADH to molecular oxygen, each of the three complexes (I, III, and IV) catalyses the translocation of protons across the inner mitochondrial membrane. During OxPhos the F_0 sub-unit conducts energy from the electrochemical proton gradient to the F_1 unit promoting ATP synthesis. This gradient facilitates the entry of adenosine diphosphate (ADP) and inorganic phosphate (Pi) into the matrix space. The ATP synthase complex then binds ADP to Pi producing ATP (Figure 1.9) (Kakkar and Singh, 2007).

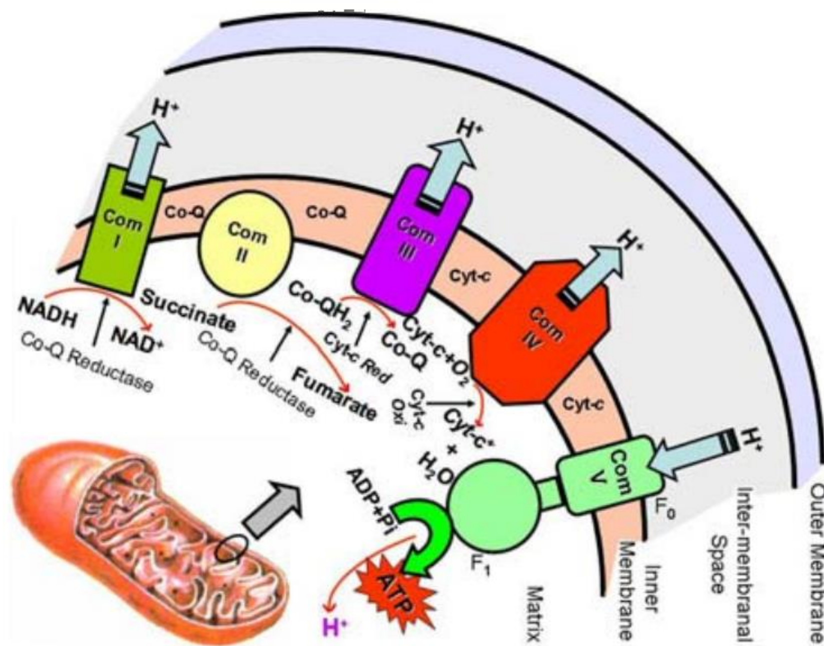


Figure 1.9: Representation of the various complexes involved in the ETC and OxPhos (Kakkar and Singh, 2007).

1.2.4.2. Mitochondria and redox signalling

Mitochondria are also involved in redox signalling pathways by fine tuning NAD^+/NADH redox coupled reactions and generating superoxide (O_2^-) together with H_2O_2 (Yin and Cadenas, 2015). In mitochondria, the flow of electrons down the ETC to complex IV results in their deposition onto molecular oxygen to form water. However, electrons can also react with oxygen at sites in the ETC to form ROS. Complexes I and III are associated with the majority of mitochondrial ROS (mtROS) generation (Shadel and Horvath, 2015).

To be effective cell signalling molecules, these oxygen species need to reversibly modify target proteins to alter their activity. Generally, this is through modification of a thiol group on a cysteine residue. All of these post-translational modifications can potentially act as "redox switches" altering protein function, thereby, enabling it to respond appropriately to the reduction potential of a particular redox couple, or to the generation of a particular ROS. However, if an active-site thiol is modified, then the protein loses its functionality (Collins et al., 2012, Schulz et al., 2014).

1.2.4.3. Mitochondria and cell death

Mitochondria are key regulators of cell death (Liu et al., 2018). Apoptosis is a programmed and controlled mechanism that allows for the active removal of damaged and dying cells while allowing healthy cells to survive (Henson and Hume, 2006). The mitochondrial permeability transition pore (MPTP) which is found at exchange points between the outer and inner mitochondrial membrane, consists of a complex made of the voltage dependent anion channel (VDAC), the adenine nucleotide translocase and cyclophilin-D (CyP-D) proteins (Baines et al., 2005). Under normal physiological conditions the pore is closed, however events such as elevated ROS production and BAX expression can lead to an increase in the permeability of the mitochondrial membrane to molecules with a molecular weight less than 1.5kDa. The subsequent entry of protons into the mitochondria causes expansion of the matrix and rupture leading to the release of pro-apoptotic factors, cytochrome c and apoptosis-inducing factor (AIF) (Green and Reed, 1998, Harris and Thompson, 2000).

Caspases are intracellular cysteine proteases that cleave protein substrates after aspartic acid residues and are responsible for the distinctive morphological features associated with apoptosis. Cytochrome c release is a major trigger for the activation of the mitochondrial (intrinsic) apoptotic pathway (Figure 1.10). Once

in the cytoplasm, cytochrome c binds its cytosolic partner apoptotic protease activating factor-1 (Apaf-1), and induces the oligomerisation of Apaf-1 cytochrome c complex in a dATP/ATP-dependent manner. This multimeric complex known as the apoptosome then recruits the initiator caspase, caspase-9, to the complex and induces procaspase-9 activation. The activated caspase-9 then cleaves and activates downstream caspases such as caspase-3 and caspase-7 that constitute major caspase activity in apoptotic cells (Hu et al., 1999, Jiang and Wang, 2000).

As apoptosis is an ATP requiring process, should MPTP occur in a small number of cells, sufficient ATP will direct the pathway in favour of apoptosis. Conversely, if a majority of cells undergo MPTP opening with a concomitant decrease in OxPhos, then necrosis will be the overriding mechanism of cell death. Thus, the mechanism of cell death via either apoptosis or necrosis is dependent on mitochondrial function (Kroemer et al., 1998, Susin et al., 1998, Lekshmi et al., 2018).

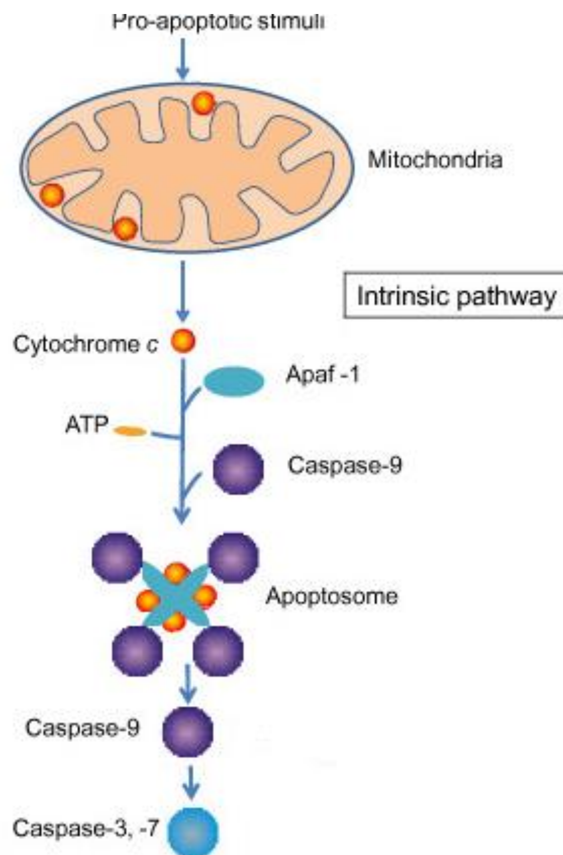


Figure 1.10: Generalised schematic for the mitochondrial apoptotic pathway. Adapted from (Li and Sheng, 2012).

1.2.4.4. Mitochondria and inflammation

Inflammation is a basic biological response to alleviate invading pathogens and contribute to tissue repair and preventing further cell injury (Medzhitov, 2008). Several recent studies have begun to highlight the intricate role of mitochondrial dysfunction in inflammatory responses through two established pathways (Figure 1.11). Mitochondrial dysfunction may generate low-grade inflammatory responses through ROS generation and NF-κB activation (García-Ruiz et al., 1995, Gloire et al., 2006).

The NLRP3 inflammasome forms cytosolic oligomers with apoptosis-associated speck-like protein (ASC) in cells triggering autocatalytic activation of caspase-1. Caspase-1, in turn, cleaves pro-IL-1β to its biologically active form (Bauernfeind et al., 2009, Mankan et al., 2012, Karmakar et al., 2015). The NLRP3 inflammasome undergoes bipartite activation (Latz, 2010). Often activation of NF-κB is the first signal (signal 1), and is needed for the transcriptional activation of pro-IL-1β and NLRP3. The second signal activates the NLRP3 inflammasome. Recently, two studies have reported on the important role for mitochondria in NLRP3 inflammasome activation (Nakahira et al., 2011, Zhou et al., 2011). Specifically, Zhou et al. (2011) found that mtROS is critical for NLRP3 activation; whereas Nakahira et al. (2011) reported that oxidised mitochondrial DNA (mtDNA) plays an important role. Moreover, impairment of the ETC with known inhibitors such as rotenone confer a selective priming signal for NLRP3 activation (Won et al., 2015).

Excess ROS drives oxidation of macromolecules, mtDNA is especially vulnerable to oxidative damage because of its close proximity to the site of ROS production and lack of protective histones (Bogenhagen, 2012). Mitochondrial dysfunction drives mitochondrial genome mutagenesis, affecting genes encoding ETC complexes and compromising the efficiency of OxPhos, which may lead to further mtDNA mutations (Yakes and Van Houten, 1997, Indo et al., 2007) thereby causing a vicious cycle and perpetual inflammasome activation.

An alternative pathway by which defective mitochondria evoke inflammatory responses is by the release of their contents into the cytoplasm and/or extracellular environment. DAMPs are macromolecules capable of eliciting a strong local inflammatory response, and are released by host cells during severe injury induced by infection or stress (Chen and Nuñez, 2010). Generally, depending on the specific circumstances, cells can suffer from several stress pathways that promote specific inflammatory responses *viz* DAMP-induced inflammation or sterile inflammation. Taken together mitochondrial dysfunction is essential to the development of the inflammatory phenotype (López-Armada et al., 2013).

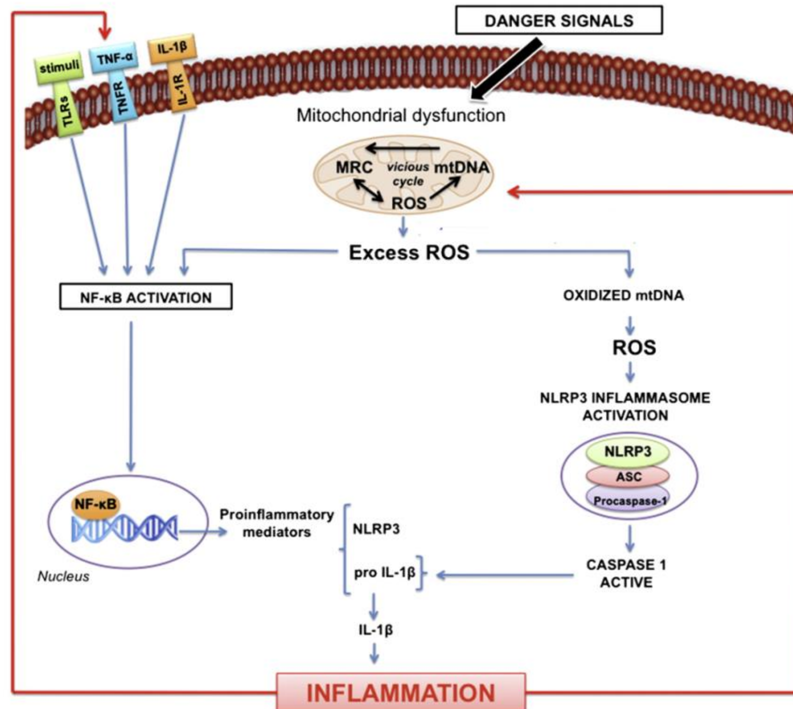


Figure 1.11: The interrelation between mitochondrial dysfunction and inflammatory response. Adapted from (López-Armada et al., 2013).

1.2.5. Mitochondrial dysfunction – metabolic diseases

Metabolic dysfunction in common diseases are rarely caused by isolated genetic defects; rather, they derive from the interaction of polygenic stimuli and influencing environmental factors resulting in impaired metabolism and signs of aberrant cell homeostasis. It comes as no surprise that the regulatory networks that govern mitochondrial function also play important roles in several diseases (Galgani et al., 2008).

1.2.5.1 Metabolic flexibility and insulin resistance

Metabolic flexibility is the capacity for the organism to adapt fuel oxidation to fuel availability (Galgani et al., 2008). Normal energy metabolism requires periodic switching between glucose and fat oxidation by the mitochondria to regulate "carbon combustion" of available fuel sources according to physiological and nutritional situations. Mitochondria are ideally situated to monitor and sense changes to nutrient status throughout the metabolic network and suggests that shifts in energy flux control are integrated a executed by functional mitochondria. Intricate metabolic and cell signalling cascades enable coordinated transitions between fuel sources to maintain energy homeostasis. Metabolic diseases are now being

recognised as disorders of metabolic inflexibility associated with impaired fuel switching and energy dysregulation (Muoio, 2014).

Within the environment of chronic overfeeding, competition between energy substrates is escalated and there is persistent oxidation of all major fuels since mitochondria are left in a state of indecision. The capacity of mitochondria to switch between fuels is coupled to changes in cellular and/or tissue functions, such as insulin action, glucose disposal, lipolysis and lipid storage as well as inflammation (Koves et al., 2008, Meex et al., 2009, Liu et al., 2012). Aberrant fuel switching and selection precedes and contributes to insulin resistance. *In vivo* rodent models have shown that obesity-induced perturbations in substrate switching are evident and metabolic inflexibility is an early event in the course of glucose intolerance (Noland et al., 2009, Muoio and Neufer, 2012, Muoio et al., 2012).

1.2.5.2. Metabolic aberrations in cancer cells

Aberrant metabolism is a hallmark of cancer cells (Hanahan and Weinberg, 2011). Most cancer cells are reliant on aerobic glycolysis; a phenomenon termed the "Warburg effect" (Warburg, 1956). Aerobic glycolysis is an inefficient way to produce ATP; however, this type of metabolism is adapted for rapid cell growth and division by facilitating the uptake and incorporation of nutrients as building blocks (e.g., nucleotides, amino acids, and lipids) needed to produce a new cell (Vander Heiden et al., 2009). Originally, defective mitochondria were thought to be the key determinant in facilitating a switch from OxPhos to aerobic glycolysis as an adaptive mechanism (Warburg, 1956). However, several recent publications have suggested that mitochondria remain functional in cancer cells (Wallace, 2012, Shen et al., 2018, Jia et al., 2018, Rinaldi et al., 2018).

During the development of oncogenesis, cancer cells acquire an adaptive phenotype that is beneficial and necessary for cell survival and proliferation (Hanahan and Weinberg, 2011). The classical metabolic profile of cancer is increased glucose consumption and lactate production. Under this model the majority of glucose undergoes glycolysis to produce two molecules of pyruvate. The pyruvate is then diverted from the mitochondrial OxPhos pathway toward lactate production via lactate dehydrogenase A (LDH-A) (Olson et al., 2016). Pyruvate can be considered as the link between cytosolic and mitochondrial metabolism. Pyruvate is synthesised as the final step of glycolysis by pyruvate kinase (PK). This enzyme exists as either the M1 or M2 isoform, with M2 being more prevalent in cancer cells (Demaria and Poli, 2012, Graziano et al., 2017). Cells with an elevated PKM2/PKM1 ratio can slow the production of pyruvate in response to proliferation signals. This phenomenon likely enables the preferential use of

glycolytic intermediates for the biosynthesis of cellular building blocks (Anastasiou et al., 2012, Lunt et al., 2015).

In oxidative cells, pyruvate is transported into mitochondria where it is converted to acetyl-coA in the mitochondrial matrix by the pyruvate dehydrogenase (PDH) complex. Several lines of evidence suggest post-translational regulation of PDH in cancer by inhibitory phosphorylation. PDH kinase 1 (PDK1) phosphorylates and inactivates PDH. This kinase is frequently overexpressed in cancer cells and has been strongly implicated in oncogenesis (Kikani et al., 2012, Tan et al., 2013, Emmanouilidi and Falasca, 2017). Acetyl-coA (produced by PDH) is then condensed with oxaloacetate to form citrate using the enzyme citrate synthase in the TCA cycle. In some situations, cancer cells produce acetyl-coA from scavenged acetate via acetyl-coA synthetase 2 (ACSS2), in order to maintain proliferation during metabolic stress (Mashimo et al., 2014, Schug et al., 2015).

The metabolic inflexibility and glucose reliant nature of cancer cells is considered largely irreversible (Olson et al., 2016). Some of these phenotypes, associated with the Warburg effect, are the products of altered transcriptional programs brought on by mutations and/or tumour cell environment (Lengauer et al., 1998, Harris, 2002, Gatenby and Gillies, 2004). Hypoxia is recognised as an important feature of tumours and plays a fundamental role in several cellular processes. These responses to hypoxia are coordinated by activation of the hypoxia-inducible factors (HIFs) that plays critical roles in the adaptation of tumour cells to a hypoxic microenvironment (Ruan et al., 2009). Activation of glycolytic genes by HIF-1 is important for metabolic adaptation of cancer cells to hypoxia through increased conversion of glucose to pyruvate and subsequently to lactate (Figure 1.12). HIF-1 is a heterodimeric transcription factor, consisting of HIF-1α and HIF-1β subunits. HIF-1α actively suppresses metabolism at the TCA cycle by directly activating the gene encoding PDK1. A hypoxia-induced metabolic switch shunts glucose metabolites from the mitochondria to glycolysis to maintain ATP production and prevents toxic ROS production (Kim et al., 2006). A summary

Since Warburg’s initial observations, many of the molecular pathways of how glucose metabolism is altered by specific signalling pathways during tumorigenesis have been elucidated, however, foodborne agents and their role in up-regulating a phenotype reminiscent of the Warburg effect have not been determined.

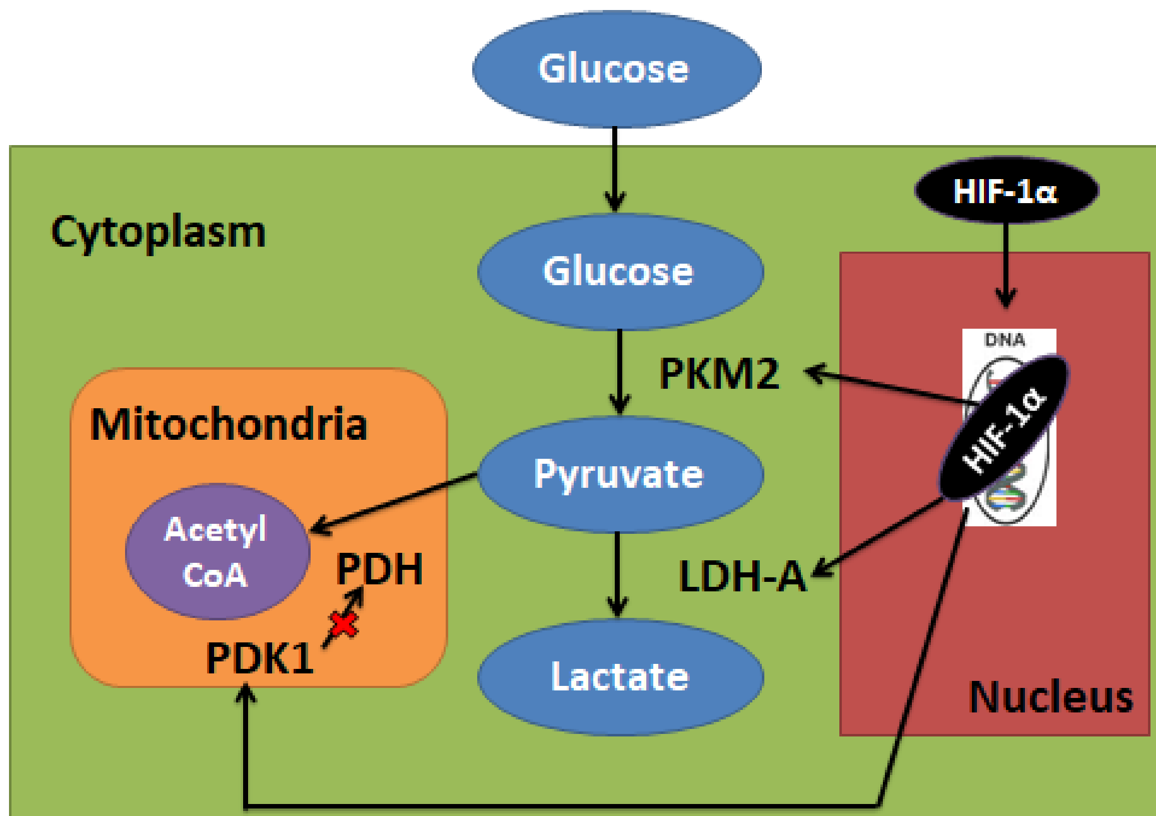


Figure 1.12: Schematic representation of glucose shunting mediated by HIF-1α in cancer cells. Prepared by author

1.2.6. Cytoprotection in response to mitochondrial stress

Mounting appropriate responses to stress conditions are imperative for maintaining a functional mitochondrial network. Due to their role in energy metabolism and ATP production, mitochondria are exposed to high levels of ROS, making them susceptible to mtDNA mutations and protein miss folding. Mitochondria are formed from proteins encoded by both nuclear and mitochondrial genomes; therefore, additional regulatory mechanisms are required for the proper coordination of protein synthesis and the mitochondrial import of nuclear-encoded proteins. For these reasons, mitochondria have developed several systems of quality control to ensure that the number of functional and healthy mitochondria is present to meet the demands of the cell. Mitochondrial quality control processes are multi-tiered, operating at the protein, organelle and cell levels (Pickles et al., 2018).

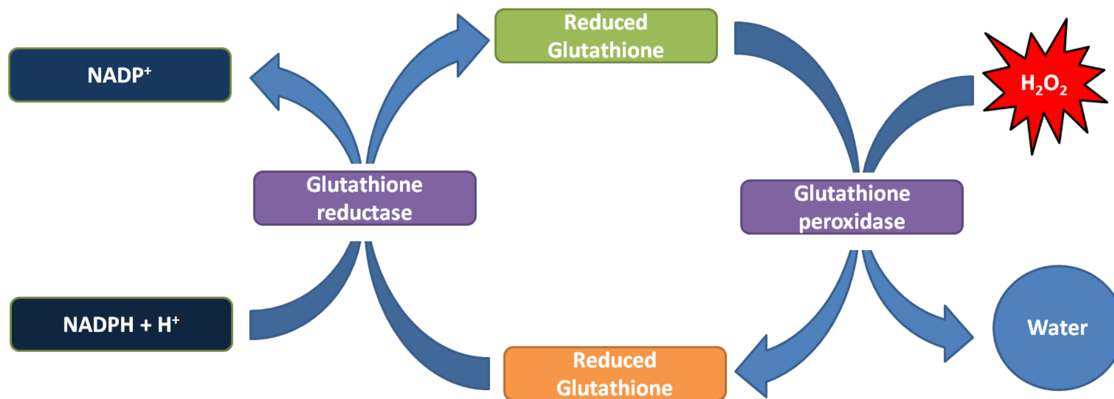
1.2.6.1. NRF2 and mitochondrial homeostasis

The transcription factor NRF2 is the master regulator of cellular redox homeostasis. A large network of antioxidant enzymes, proteins involved in cell repair and damage control, xenobiotic detoxification as well as anti-inflammation and other transcription factors are regulated at the transcription level by NRF2 (Holmström et al., 2016). In most cells, mitochondrial OxPhos generates the majority of ROS (St-Pierre et al., 2002, Zorov et al., 2014). Several disease states have implicated excess ROS and oxidative stress as a common feature (Pham-Huy et al., 2008, Kawagishi and Finkel, 2014). Although traditionally viewed as a toxic by-product of respiration, ROS is now being appreciated as a powerful signalling molecule (Ray et al., 2012, Zhang et al., 2016).

Mitochondrial ROS production can be counterbalanced by the activation of NRF2, a cap 'n' collar basic leucine zipper transcription factor. Under basal conditions, NRF2 is sequestered in the cytoplasm by Kelch-like ECH-associated protein 1 (Keap1). This complex directs NRF2 polyubiquitination and degradation by functioning as an adaptor of the Cul3 based E3 ligase (Dinkova-Kostova et al., 2005). Upon oxidative stress, NRF2 is liberated from Keap1 and enters the nucleus, to stimulate the expression of antioxidant response element (ARE) genes (Thimmulappa et al., 2002, Cho et al., 2005). A study by Dinkova-Kostova *et al*. (2005) determined the kinetics and stoichiometry of Keap1 that regulates its susceptibility to oxidant or electrophile mediated modifications. The results revealed that zinc was bound to the reactive cysteine thiols of Keap1 and established that inducers displace this metal. Thus, regulation of the phase II response involves chemical modification of critical cysteine residues of Keap1, whose reactivity is modulated by zinc binding. Keap1 is a zinc-thiol protein enabled with a switch controlled by both metal-binding and thiol reactivity. Under non-stressed conditions Keap1 binds zinc that is coordinated in part by reactive cysteine residues. In this conformation, Keap1 binds NRF2 and marks the transcription factor for degradation. Upon inducer sensing, the zinc is released and the reactive cysteine residues are modified by alkylation, oxidation, or thiol-disulphide interchange, leading to a conformational change that separates the Kelch domains and releases NRF2 allowing its nuclear translocation and enhanced expression of phase 2 response genes (Dinkova-Kostova et al., 2005).

Reduced glutathione is the primary small molecule antioxidant in the mammalian cell and a product of several of the downstream target genes of NRF2. NRF2 regulates the expression of γ-glutamyl cysteine ligase catalytic and modulatory subunits, glutathione reductase (Holmström et al., 2016), along with the nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) producing enzymes (Chan and Kwong, 2000) all of which are involved in the biosynthesis and maintenance GSH. Glutathione cycles between

reduced and oxidised forms to neutralise H_2O_2 (Figure 1.13), thus, counterbalancing the production of ROS (Holmström et al., 2016).



<u>Figure 1.13</u>: Glutathione cycling in cells. Prepared by author.

It has been established that the NRF2 pathway is initiated in response to mitochondrial toxins (Calkins et al., 2005, Shih et al., 2005) and several mitochondria associated diseases show dysregulation of the NRF2 pathway (Chen et al., 2009, Tufekci et al., 2011), suggesting an additional role to the detoxification of ROS. The mitochondrial membrane potential ($\Delta\psi_m$) is considered an indicator of the metabolic state and overall mitochondrial health of cells. In a healthy cell, $\Delta\psi_m$ is maintained by the mitochondrial respiratory chain. NRF2 has been shown to activate transcription of the mitochondrial electron transport chain component NDUFA4 and down-regulate cytochrome c oxidase subunits COX2 and COX4 (Agyeman et al., 2012). The basal $\Delta\psi_m$ is higher when NRF2 is constitutively up-regulated (Holmström et al., 2013). Taken together these studies show that respiration is affected by the activity of NRF2. Mitochondrial substrate production is regulated by NRF2 and is considered the most profound way that this transcription factor affects mitochondrial function. The NADH redox index within mitochondria (the balance between consumption of NADH by complex I and production of NADPH in the TCA cycle) is significantly lower in cells showing NRF2 knockout (KO) cells in comparison to their wild type (WT) counterparts (Mitsuishi et al., 2012). By estimating the ratio of ADP to oxygen as a measure of ATP synthesis it was found that NRF2 deficiency results in a decreased efficiency of oxidative phosphorylation, whereas activation of NRF2 has the opposite effect. Furthermore, Nrf2 deficiency leads to ATP production primarily through glycolysis, and this ATP is then used F_1F_0-ATPase to maintain the $\Delta\psi_m$ (Holmström et al., 2013).

Recent evidence has suggested that NRF2 links mitochondrial biogenesis to oxidative stress. The role of PGC-1α in ROS metabolism was previously explored (St-Pierre et al., 2006). The expression of mitochondrial ROS-detoxifying enzymes including superoxide dismutase 2 (SOD2) increased with PGC-1α (St-Pierre et al., 2006). The PGC-1 family of coactivators plays a central role in a regulatory network governing the transcriptional activation of mitochondrial biogenesis and respiratory function. These coactivators target multiple transcription factors including nuclear respiratory factor 1 (NRF1) and the orphan nuclear hormone receptor, ERRα, among others to trigger expression of mitochondrial proteins (Wu et al., 1999, Schreiber et al., 2004, Scarpulla, 2011). Activation of NRF2 with sulforaphane increased mitochondrial mass and induction of PGC1α and PGC1β (Brose et al., 2012) as well as NRF1 (Piantadosi et al., 2008). Taken together, these results suggest that the role of NRF2 in maintaining the levels of both NRF1 and PGC1α is complex and becomes prominent under conditions of oxidative stress.

The role of NRF2 in removal of damaged/dysfunctional mitochondria represents another layer of the broad cytoprotective mechanisms orchestrated by this transcription factor through mediating expression of mitophagy and autophagy associated genes including PTEN-induced putative kinase 1 (PINK1) and p62 (Jain et al., 2010, Komatsu et al., 2010, Jiang et al., 2015, Murata et al., 2015). These observations suggest that the Keap1/NRF2 pathway regulates both mitochondrial homeostasis and ROS production.

1.2.6.2. Autophagy/mitophagy

Autophagy is a catabolic process that is responsible for the bulk removal of cytoplasmic components including damaged organelles, miss-folded proteins and elimination of intracellular pathogens (Mizushima, 2007, Mizushima et al., 2008, Jiang et al., 2015). This degradation process is needed to preserve cellular homeostasis and defend against oxidative or proteotoxic stress. Signals of extracellular and intracellular origin can trigger autophagy, such signals include oxidative stress (Kiffin et al., 2006), endoplasmic reticulum stress (Yorimitsu et al., 2006), or nutrient deprivation (Yang and Klionsky, 2010). Mitophagy is the selective autophagic clearance of dysfunctional mitochondria and can be regarded as a targeted defence against oxidative stress (Lemasters, 2005, Zhang, 2013).

In mammals, the autophagic removal of mitochondria is dependent on two genes. The PTEN-induced putative kinase 1, which codes for a kinase localised to the mitochondrial membrane, and *PARK2*, whose protein product, Parkin, is a cytosolic E3 ubiquitin ligase (Narendra et al., 2008, Geisler et al., 2010). Under basal conditions, the translocase of the outer membrane (TOM) and translocase of the inner membrane (TIM23) complexes allows for PINK1 to be imported into mitochondria in a process dependent on a mitochondrial targeting sequence (Lazarou et al., 2012, Pickles et al., 2018). Upon entry

and at the outer mitochondrial membrane, PINK1 is processed by the matrix peptidase (MPP) and the inner membrane protease PINK1/PGAM5- associated rhomboid-like protease (PARL) (Jin et al., 2010). The resulting cleaved product with an amino-terminal is released into the cytoplasm and degraded (Figure 1.14A) (Yamano and Youle, 2013).

Compromised mitochondrial protein import causes stabilisation of PINK1 on the outer mitochondrial membrane (OMM) (Narendra et al., 2008) in a complex composed of TOM20, TOM22, TOM40 and TOM70 (Lazarou et al., 2012, Okatsu et al., 2013). PINK1 kinase activity leads to Parkin translocation from the cytoplasm to the PINK1-bound organelle, and is therefore essential for Parkin recruitment (Vives-Bauza et al., 2010, Okatsu et al., 2012). In addition to auto-phosphorylation, PINK1 can phosphorylate Parkin on Serine 65 of its ubiquitin-like domain, thereby increasing its E3 ligase activity to recruit additional proteins necessary for removal of damaged mitochondria (Figure 1.14B) (Kane et al., 2014).

Proteins belonging to the family of autophagy related genes (ATGs) coordinate the expansion of the phagophore to form the autophagosome (Burman and Ktistakis, 2010). Autophagy receptor proteins bind ubiquitinated cargo through their ubiquitin-binding domains and connect them to the autophagosome to promote autophagy. Sequestosome 1 (SQSTM1) or p62 has been proposed to act as a receptor linking depolarised mitochondria to microtubule-associated protein 1A/1B-light chain 3 (LC3) on autophagosomes to (Ashrafi and Schwarz, 2013). Furthermore, p62 is required for the perinuclear clustering of depolarised mitochondria (Okatsu et al., 2010).

Both autophagy and mitophagy can mitigate the potential cellular damage caused by dysfunctional mitochondria through down regulation of the NLRP3 inflammasome activation and apoptosis as well as preventing the generation of excess ROS by removing these damaged organelles (Kim et al., 2007, Zhou et al., 2011, Kim et al., 2016).

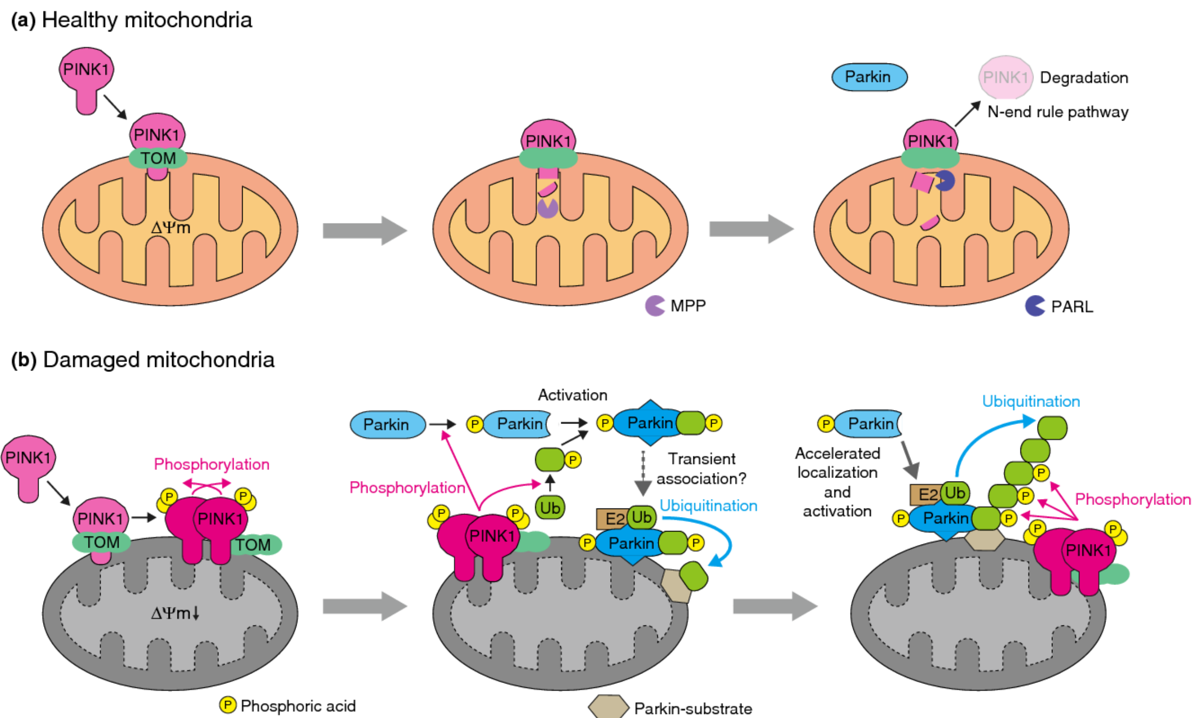


Figure 1.14: PINK1 and Parkin co-operate to induce mitophagy. (A) Under basal conditions PINK1 undergoes proteolytic cleavage by the matrix protein MPP and by the inner mitochondrial membrane protein PARL. Processed PINK1 is then targeted to the proteasome for degradation. (B) Upon damage, PINK1 import is compromised and is stabilised on the OMM, where it phosphorylates both Parkin and ubiquitin. Parkin is then recruited to phosphorylated ubiquitin to signal for autophagic clearance of the damaged organelles (Eiyama and Okamoto, 2015).

1.2.6.3. Sirtuins

To protect cells from stress induced damage and maintain homeostasis, mitochondria upregulate responses that are referred to as mitochondrial stress responses, these include the UPR^{mt}, antioxidant defences and mitophagy (Barbour and Turner, 2014). Sirtuins have received considerable attention as mediators of cytoprotective adaptations to mitochondrial stress as well as several roles in the regulation of metabolism and related diseases (Lin et al., 2018).

Sirtuins are a family of highly conserved NAD^+ dependent deacetylases (Saunders and Verdin, 2007). All SIRTs contain a highly conserved catalytic core which has a structurally homologous and large NAD^+/NADH binding Rossmann-fold domain, zinc-binding domain and numerous loops that form a cleft. This cleft attaches the two domains where the NAD^+ and acetyl lysine containing protein substrates enter and bind to the enzyme for deacetylation. The mammalian SIRT homologs (SIRT1-7) display a wide range of tissue specificity, subcellular localization; enzymatic activity and protein targets (table

1).The mitochondrial specific SIRTs (SIRT 3, 4 and 5) can directly influence mitochondrial activity and stress responses (Lin et al., 2018).

Table 1: Localization and function of the mammalian SIRT homologs

SIRTUIN	Intracellular Location	Primary Enzymatic Function
SIRT1	Nucleus Cytosol	Deacetylase
SIRT2	Nucleus Mitochondria Cytosol	Deacetylase
SIRT3	Mitochondria Nucleus	Deacetylase
SIRT4	Mitochondria	ADP-ribosyltransferase
SIRT5	Mitochondria Cytosol	Deacetylase Demalonylase Desuccinylase
SIRT6	Nucleus	Deacetylase ADP-ribosyltransferase
SIRT7	Nucleolus	Deacetylase

1.2.6.3.1. Mitochondrial SIRTs and oxidative stress

Sirtuins are regarded as energy sensors due to their dependence on NAD^+ (Li and Kazgan, 2011). The three mitochondrial sirtuins: SIRT3, SIRT4, and SIRT5 are mainly found in the matrix due to the presence of a mitochondrion targeting sequence in their N terminals (Haigis and Guarente, 2006). Sirtuins participate in antioxidant defence to combat mitochondrial stress. The SOD2 antioxidant enzyme is the primary scavenger of superoxide in the mitochondria (Kokoszka et al., 2001, Indo et al., 2015). Sirtuin 3 is indispensable for cellular antioxidant defence by activating SOD2 activity via deacetylation of lysine 68 (Chen et al., 2011) and by indirectly enhancing SOD2 expression via the deacetylation of FOXO3A (Zhang et al., 2014). This deacetylase also increases the transcriptional activation of PGC-1α to suppress oxidative stress and increase mitochondrial biogenesis (Kong et al., 2010).

Sirtuin 4 demonstrates a negative role in mitochondrial function and promotes ROS production. Mitochondrial depolarization is promoted by elevated expression of SIRT4 as well as loss of mitochondrial function and reduced mitochondrial oxygen consumption. It was recently shown that SIRT4 disrupts PDH activity, thus inhibiting ATP production (Mathias et al., 2014).

Sirtuin 5 elicits antioxidant potential via two mechanisms. First, SIRT5 protects cells from apoptosis and this involves the forkhead box O3 (FOXO3A) induced clearance of ROS by SOD2 in cells treated with cigarette smoke extract (Wang et al., 2015b). Second, SIRT5 promotes the desuccinylation and deglutarylation of isocitrate dehydrogenase 2 (IDH2) and glucose-6-phosphate dehydrogenase (G6PD) respectively, increasing their catalytic activities, maintaining NADPH homeostasis and redox potential and contributing to oxidative stress resistance (Zhou et al., 2016).

1.2.6.3.2. Mitochondrial SIRTs and Mitophagy

Mitophagy is critical for maintaining mitochondrial homeostasis in response to stress. Sirtuin 3 facilitates mitophagy by deacetylating FOXO3A which is indispensable for inducing mitophagy (Tseng et al., 2013). Moreover, SIRT3 is required for enhancing the lipidation and transcript levels of LC3B which promotes mitophagy (Liang et al., 2013). Sirtuin 4 was observed to downregulate mitophagy. The translocation of Parkin to the mitochondria was decreased in SIRT4-overexpressing cells, indicating that Parkin-associated mitophagy was reduced when SIRT4 was overexpressed (Lang et al., 2017). An increasing number of studies support the regulatory role of SIRT5 in mitophagy. Polletta et al. (2015) showed that mitophagy markers (Pink1 and Parkin) increased when SIRT5 was silenced or pharmacologically inhibited, while these same markers decreased in cells overexpressing SIRT5 (Polletta et al., 2015). These results indicate that SIRT5 is involved in repressing mitophagy. SIRT5 may also increase resistance to mitophagy by inhibiting mitochondrial fission. The mitochondrial degradation increased after SIRT5 knockout and was blocked after autophagy inhibitor treatment, indicating the repressive role of SIRT5 in mitophagy (Guedouari et al., 2017). The repressed mitophagy may be attributed to SIRT5-induced inhibition of mitochondrial fission. Taken together, SIRT5 may have both direct and indirect impacts on mitophagy.

1.2.6.3.3. Mitochondrial SIRT3 and SIRT5 regulation

The transcriptional co-activator PGC-1α is essential for maintaining mitochondrial function and protection through up-regulation of SOD2 (Leick et al., 2010, Lu et al., 2010). PGC-1α is considered the master regulator of mitochondrial biogenesis and respiration (Ventura-Clapier et al., 2008, Fernandez-

Marcos and Auwerx, 2011). The elucidation of the transcriptional regulation of diverse metabolic pathways have led to the discovery of mitochondrial SIRTs (3 and 5) as putative downstream targets of PGC-1α (Kong et al., 2010, Buler et al., 2014).

The mitochondrial SIRTs are also regulated at the epigenetic level by microRNAs (miRNAs) (Zhang et al., 2018). These are small, non-coding RNA molecules which act as post-transcriptional regulators of gene expression. Individual miRNAs can regulate the expression of multiple genes and individual genes can be regulated by several miRNAs (He and Hannon, 2004). MicroRNAs regulate gene expression by acting as RNA silencers or posttranscriptional gene regulators (Figure 1.15). During the first step of protein synthesis, the DNA which codes for the protein of interest is synthesised into mRNA (transcription). In the absence of miRNA, the mRNA is converted into protein (translation). In the presence of miRNA with near-perfect, partial complementarity to the mRNA of interest, miRNA binds in the three prime untranslated region (3′ UTR) and represses translation, thus inhibiting protein synthesis. In the presence of miRNA with perfect complementarity, miRNA binding in the 3′ UTR is thought to degrade mRNA and inhibit protein synthesis. In humans, perfect complementarity is rare, with varying degrees of partial complementarity the predominant situation (Romaine et al., 2015).

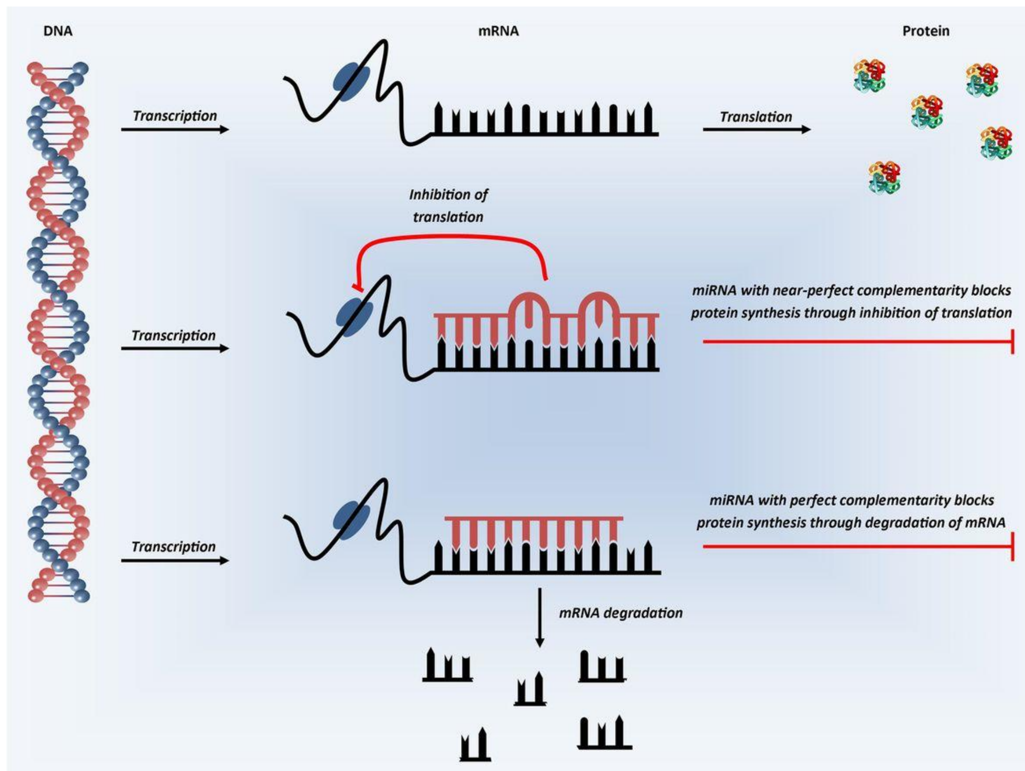


Figure 1.15: Representation of MiRNA mechanism of action (Romaine et al., 2015)

Mycotoxins exert a plethora of effects in organisms that may lead to adverse health effects. Although common contaminants of food sources, they receive minimal attention, particularly in terms of mechanistic data. There is a need to better understand the toxicological profile of these foodborne toxins, thus improving regulations and laws to protect consumers. This study examined the effects of a neglected fusrariotoxin and its implications for humans through the use of an *in vitro* metabolic model.

Fusaric acid is regarded as a putative mitochondrial toxin; however, there remains a dearth of knowledge regarding the molecular events that contribute to mitochondrial dysfunction and its links to aberrant metabolism and inflammatory responses. This study aimed to integrate the mechanisms of mitochondrial dysfunction and downstream implications to provide relevant insight into the toxic potential of this neglected mycotoxin.

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Chapter 2

Fusaric acid induces mitochondrial stress in human hepatocellular carcinoma (HepG2) cells.

Fusaric acid is a ubiquitous but neglected mycotoxin and is considered toxicologically significant only when in the presence of co-produced mycotoxins such as FB1. Fusaric acid is described as a putative mitochondrial toxin, however exact mechanisms remain elusive. This paper established mechanisms of mitochondrial toxicity induced by FA in HepG2 liver cells and its implications on cell viability, mitochondrial biogenesis and cell death.

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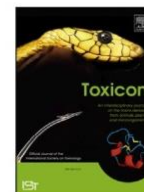

Fusaric acid induces mitochondrial stress in human hepatocellular carcinoma (HepG2) cells

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Fusarium spp are common contaminants of maize and produce many mycotoxins, including the fusariotoxin fusaric acid (FA). FA is a niacin related compound, chelator of divalent cations, and mediates toxicity via oxidative stress and possible mitochondrial dysregulation. Sirtuin 3 (SIRT3) is a stress response deacetylase that maintains proper mitochondrial function. We investigated the effect of FA on SIRT3 and oxidative and mitochondrial stress pathways in the hepatocellular carcinoma (HepG2) cell line. We determined FA toxicity (24 h incubation; $IC_{50} = 104$ μg/ml) on mitochondrial output, cellular and mitochondrial stress responses, mitochondrial biogenesis and markers of cell death using spectrophotometry, luminometry, qPCR and western blots. FA caused a dose dependent decrease in metabolic activity along with significant depletion of intracellular ATP. FA induced a significant increase in lipid peroxidation, despite up-regulation of the antioxidant transcription factor, Nrf2. FA significantly decreased expression of SIRT3 mRNA with a concomitant decrease in protein expression. Lon protease was also significantly down-regulated. FA induced aberrant mitochondrial biogenesis as evidenced by significantly decreased protein expressions of: PGC-1α, p-CREB, NRF1 and HSP70. Finally, FA activated apoptosis as noted by the significantly increased activity of caspases 3/7 and also induced cellular necrosis. This study provides insight into the molecular mechanisms of FA (a neglected mycotoxin) induced hepatotoxicity.

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1. Introduction

Fusaric acid (FA, 5-butylpicolinic acid) is an often neglected mycotoxin (Nedělník, 2002). It is a widely distributed secondary metabolite of the genus *Fusarium* (Bacon et al., 1996) with an average of 643 μg/kg sample of FA being reported as a natural contaminant in the literature (Streit et al., 2013). FA exhibits low to moderate toxicity and has several pharmacological properties (Wang and Ng, 1999; Stack et al., 2004). FA acts in synergism with co-produced mycotoxins thereby augmenting overall toxicity as evidenced by studies conducted in swine (Malovrha and Jakovac-Strajn, 2010) and turkey poults (Fairchild et al., 2005). Analysis focusing on a single toxin is likely to show better correlation between its concentration and induced toxicity.

FA is a known phytotoxin implicated in the pathogenesis of wilt disease (Gäumann, 1957) and necrotic and apoptotic cell death in plant models (Wu et al., 2008; Samadi and Behboodi, 2006). Previous studies demonstrate the potent anti-proliferative and cytotoxic activity of FA toward a variety of cell lines citing interference with catalytic metalloproteins, enhancement of DNA damage and apoptosis as probable mechanisms (Ogata et al., 2001; Ye et al., 2013; Jaglowski and Stack, 2006; Ruda et al., 2006; Stack et al., 2004; Fernandez-Pol et al., 1993). Several studies on FA showed it to induce oxidative stress by down regulating anti-oxidant enzymes and increasing the production of reactive oxygen species (ROS) (Singh and Upadhyay, 2014; Jiao et al., 2014; Iwahashi et al., 1999). Excess ROS induces oxidative modifications to macromolecules, inhibits protein functions, promotes cell death (Circu and Aw, 2010) and has been implicated in disease initiation and progression (Qiu et al., 2010). Although ROS can be generated in many cellular compartments, the mitochondria is the major contributor (Balaban et al., 2005). ROS formation occurs when unpaired electrons escape the electron transport chain (ETC) and react with molecular oxygen (St-Pierre et al., 2006). FA was shown to dysregulate mitochondrial

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output and metabolic processes in animal (Telles-Pupulin et al., 1998) and plant models (Telles-Pupulin et al., 1996; Jiao et al., 2014). Although the effects of FA on mitochondrial and oxidative stress are documented little is known about the underlying molecular mechanisms of FA induced oxidative stress and mitochondrial dysfunction.

Sirtuin 3 (SIRT3) is an important regulator of mitochondrial lysine acetylation (Lombard et al., 2007). SIRT3 deacetylase activity is stimulated by its co-factor, nicotinamide adenine dinucleotide (NAD^+) and inhibited by its reaction product, nicotinamide (NAM) (Guan et al., 2015). SIRT3 influences homeostasis by targeting enzymes that regulate key mitochondrial processes including the Kreb's cycle (Finley et al., 2011) and oxidative phosphorylation (OXPHOS) (Ahn et al., 2008). SIRT3 also maintains mitochondrial integrity by regulating the stress response protein LON protease (LON) at the post-translational level (Gibellini et al., 2014a). LON is an ATP dependent protease that catalyses the degradation of oxidatively damaged proteins in the mitochondrial matrix. LON can also act as a chaperone protein, independent of its proteolytic activity and promotes the assembly of ETC subunits (Bota et al., 2005).

The primary defence against oxidative stress are endogenous anti-oxidant enzymes that scavenge excess ROS. The transcriptional co-activator, peroxisome proliferator-activated receptor gamma co-activator α (PGC-1α) interacts with various transcription factors to regulate biological programs (Lin et al., 2005). PGC-1α regulates ROS metabolism by mediating the expression of anti-oxidant enzymes catalase and superoxide dismutase (SOD) (St-Pierre et al., 2006). Oxidative stress increases the expression of both PGC-1α, and SIRT3 through activation of oestrogen-related receptor α (ERR-α). In turn, SIRT3 stimulates PGC-1α expression via phosphorylated cAMP response element binding protein (pCREB), thereby forming a positive feedback loop and increasing anti-oxidant defences (Kong et al., 2010). SIRT3 is also known to enhance SOD2 activity (the primary mitochondrial superoxide detoxification enzyme) by deacetylation (Tao et al., 2010; Qiu et al., 2010).

The induction of the transcription factor nuclear factor-erythroid 2-related factor (Nrf2) and subsequent mediation of phase 2 response is an important cellular reaction to oxidative stress and is dependent on the release of Nrf2 from its repressor Kelch-like ECH-associated protein 1 (Keap1), a zinc thiol protein (Dinkova-Kostova et al., 2005). It has been suggested that Nrf2 is a transcriptional regulator of SIRT3 (Flick and Lüscher, 2012) and LON (Ngoa et al., 2013).

Mitochondrial biogenesis is a mechanism developed by cells to prevent mitochondrial lesions and maintain mitochondrial integrity (Esposti et al., 2012). PGC-1α is the master regulator of mitochondrial biogenesis and respiration (St-Pierre et al., 2006) by mediating the activity of several transcription factors including nuclear respiratory factor 1 (NRF-1) (Yoboue and Devin, 2012). Furthermore, SIRT3 was shown to mediate the effects of PGC-1α on mitochondrial biogenesis (Kong et al., 2010).

The liver undergoes first pass metabolism which facilitates the uptake of toxins along with it being the major site for detoxification reactions make it highly susceptible to mycotoxin toxicity (Fenton, 2002). Furthermore, this organ is highly metabolic and possesses a high density of mitochondria which regulate a host of hepatic functions including cell survival (Esposti et al., 2012). The liver derived HepG2 cell line is widely used as an *in vitro* toxicity model owing to the inducible expression of detoxifying enzymes (Mersch-Sundermann et al., 2004).

Although a common contaminant of agricultural produce little is known about the underlying molecular mechanisms of FA induced oxidative stress and mitochondrial dysfunction. We investigated the effects of this niacin related mycotoxin on SIRT3 and its ability to induce oxidative and mitochondrial stress in the HepG2 liver cell line.

2. Materials and methods

2.1. Materials

The HepG2 cell line was acquired from Highveld Biologicals (Johannesburg, South Africa). Cell culture reagents were purchased from Whitehead Scientific (Johannesburg, South Africa). Western blot reagents were purchased from BioRad (Hercules, CA, USA). All other reagents and consumables were purchased from Merck (Darmstadt, Germany), unless otherwise stated.

2.2. Cell culture

HepG2 cells were cultured in minimum essential media (10% foetal calf serum, 1% penstrepfungizone, 1% L-glutamine) at 37 °C in a humidified incubator (5% CO_2). Cells were allowed to reach 80% confluency in 25 cm^3 culture flasks before treatment with FA and the SIRT3 inhibitor NAM [(10 mM) which was used as the positive control].

2.3. Cell viability assay

To determine the effect of FA on mitochondrial output, the methyl thiazol tetrazolium (MTT) assay was used. Approximately 20,000 cells were seeded into a 96-well microtitre plate and then treated with FA (0–500 μg/mL) in triplicate for 24 h (h). The 24 h time period was selected to evaluate the adverse effects of acute exposure. Treatments were removed and the cells incubated with an MTT salt solution [5 mg/mL in 0.1 M phosphate-buffered saline (PBS)] and media (4 h, 37 °C). Following incubation, the supernatants were aspirated and dimethyl sulfoxide was added (100 μL/well) and incubated at 37 °C for a further 1 h. Optical density of the formazan product was measured by a microplate reader (BioTek μQuant, Winooski, VT, USA) at 570 nm with a reference wavelength of 690 nm. The half maximal inhibitory concentration (IC_{50}) was determined. All subsequent assays were carried out after 24 h incubation with the IC_{50} value (104 μg/mL).

2.4. Intracellular ATP quantification

The luminometric Cell Titer-Glo® assay was used to determine levels of intracellular ATP. Cells were aliquoted in a white microtitre plate (20,000 cells in 50 μL 0.1 M PBS in triplicate) to which the ATP Cell Titer-Glo® Reagent (Promega, Madison, USA) was added (20 μL). The plate was incubated for 30 min at room temperature (RT) in the dark. Luminescence was measured on a Modulus™ microplate luminometer (Turner BioSystems, Sunnyvale, USA). Luminescence is proportional to ATP levels and expressed as relative light units (RLU).

2.5. Lipid peroxidation assay

Oxidative damage was assessed using the thiobarbituric acid assay which quantifies the levels of malondialdehyde (MDA), a by-product of lipid peroxidation. Supernatants from the FA, NAM and untreated control flasks were added to test tubes (200 μL) followed by the addition of 2% H_3PO_4 (200 μL), 7% H_3PO_4 (200 μL) and thiobarbituric acid/butylated hydroxytoluene solution (400 μL). A positive control of MDA was prepared. All samples were adjusted to pH 1.5 and boiled (15 min). Once cooled, butanol (1.5 mL) was added to each test tube, vortexed and allowed to separate into distinct phases. The upper phase (100 μL) from each sample was dispensed into a 96-well microtitre plate in five replicates. The optical density was measured on a spectrophotometer at 532 nm with a reference wavelength of 600 nm. The mean optical density of

five samples per treatment was calculated and divided by the absorption coefficient (156 mM^{-1}). Results were expressed as MDA concentration (μM).

2.6. Quantitative PCR

RNA was isolated from control, FA and NAM treated HepG2 cells (flasks) using an in house protocol (Chuturgoon et al., 2014). Total RNA was quantified (Nanodrop 2000) and standardized (1000 ng/mL).

Standardized RNA samples were converted to 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 μL 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 30 min, 85 °C for 5 min and a final hold at 4 °C.

Gene expression of *SIRT3* (Sense 5′-GCATTCCAGACTTCAGATCGC-3; Anti-sense 5′-GTGGCAGAGGCAAAGGTTCC-3′) was assessed using the iQ™ SYBR® Green PCR kit (BioRad; 170-880) and carried out using CFX Touch™ Real Time PCR Detection System (BioRad, Hercules, CA, USA). The PCR was initiated with the following thermocycler profile: An initial denaturation for 8 min at 95 °C followed by 39 cycles of 95 °C, denaturation for 15 s, annealing for 1 min at 50 °C, and extension at 72 °C for 30 s. A final extension at 60 °C was performed for 31 s. Each measurement was done in triplicate and normalized against β-actin which was run under the same conditions as the housekeeping gene. Data was analysed using the method described by Livak and Schmittgen (2001) and represented as fold change relative to the housekeeping gene ($2^{-\Delta\Delta C}$t) (Livak and Schmittgen, 2001). *β-actin* (Sense5′-TGACGGGTCACCCACACTGTGCCCAT-3'; Antisense 5′CTAGAAGCATTTGCGGTGGACGATGGAGGG-3′).

2.7. Protein expression analysis

Western blot was run to determine protein expression of SIRT3, Nrf2, PGC-1α, NRF1, LONP1, pCREB and HSP70. Crude protein from control, FA and NAM treated HepG2 cells was isolated using Cytobuster™ (Novagen, San Diego, CA, USA) supplemented with phosphatase and protease inhibitors (Roche, Mannheim, Germany). Cytobuster (200 μL) was added to flasks and incubated on ice for 10 min before being mechanically lysed. Cell lysates were decanted into 1.5 mL microcentrifuge tubes and centrifuged (12,000*g*, 10 min) to obtain crude protein. The bicinchoninic assay was used to quantify protein, which was subsequently standardized to 1.5 mg/mL.

Samples were prepared in Laemmli buffer ([dH_2O, 0.5 M Tris-HCl (pH 6.8), glycerol, 10% SDS, β-mercaptoethanol, 1% bromophenol blue]) and electrophoresed (150 V, 1 h) in sodium-dodecylsulphate polyacrylamide gels (4% stacking, 10% resolving) using BioRad compact power supply. Protein was then transferred onto nitrocellulose membranes using the Trans-Blot® Turbo Transfer system (BioRad) (400 mA, 45 min). Following transfer, membranes were blocked with 5% non-fat dry milk (NFDM) made up in Tris-buffer saline (TTBS) [0.5% Tween20, dH_2O, KCl, Tris, NaCl, pH 7.4] for 1 h at RT. Membranes were then immune-probed with primary antibody (1:1000 dilution in 5% NFDM) against Nrf2 (ab31163, Abcam), PGC-1α (ab72230, Abcam), SIRT3 (ab86671, Cell Signalling Technology, Beverly, MA. USA), pCREB (9191, Cell Signalling Technology), HSP70 (4876, Cell Signalling Technology), NRF1 (12381, Cell Signalling Technology) and LONP1 (HPA002192, Sigma-Aldrich, St Louis, MO) for 1 h at RT then overnight at 4 °C. Membranes were washed with TTBS (5 times, 10 min) and then incubated with secondary antibody conjugated to HRP [goat anti-mouse (31,800); goat anti-rabbit (ab6112) 1:10,000 in 1% BSA] for 1 h at RT. Membranes were washed with TTBS (5 times, 10 min). Protein bands were visualized using Clarity Western ECL Substrate (BioRad) detection reagent. Images were captured using gel documentation system Alliance 2.7 (UViTech, Cambridge, UK). UViTech Alliance Analysis software was used to analyse protein expression.

Membranes were stripped with 5% hydrogen peroxide, incubated in blocking solution (5% NFDM; 1 h; RT), rinsed thrice in TTBS and probed with HRP-conjugated antibody for the house-keeping protein, β-actin (A3854 Sigma-Aldrich). The relative band intensity was normalised against β-Actin. Results were expressed as relative band density (RBD).

2.8. LDH activity

The LDH cytotoxicity detection kit (Roche, Mannheim, Germany) was used to measure cell death/damage. To measure LDH activity, supernatant (100 μL) was transferred into a 96-well microtitre plate in triplicate. Thereafter, substrate mixture (100 μL) containing catalyst (diaphorase/NAD^+) and dye solution (INT/sodium lactate) was added to the supernatant and allowed to react at RT for 25 min. Optical density was measured at 500 nm (microplate reader - Bio-Tek μQuant). Results are presented as mean optical density.

2.9. Activity of caspases (9,8 and 3/7)

Caspase 9,8 and −3/−7 activity was detected with Caspase-Glo® assay (Promega, Madison, USA). As per manufacturer's protocol, Caspase-Glo® reagent was reconstituted and added to wells of a 96-well white microtitre plate (20 μL of reagent per 50 μL of 20,000 cells/well in triplicate). Samples were incubated in the dark (30 min, RT). The luminescent signal was measured on a Modulus™ microplate luminometer (Turner Biosystems, Sunnyvale, USA) Caspase activity expressed as relative light units (RLU).

2.10. Statistical analysis

Data was analysed using GraphPad Prism V5.0 software (GraphPad Software Inc., La Jolla, USA) and considered 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.

3. Results

3.1. Mitochondrial output

To determine the effect of FA on mitochondrial output, cell viability (MTT assay) and intracellular ATP levels were assessed.

FA decreased metabolic activity in a dose dependent manner with an IC_{50} of 104 μg/mL (used in all subsequent experiments; Fig. 1A). FA significantly ($p = 0.0062$) decreased the intracellular ATP levels (luminometric assay) ($0.815 \pm 0.0682 \times 10^6$ RLU) when compared to the control ($2.044 \pm 0.1582 \times 10^6$ RLU; Fig. 1B).

3.2. Oxidative stress and detoxification

Lipid peroxidation was used as a measure of FA induced oxidative stress. MDA levels were significantly increased in FA treated cells when compared to control cells ($p = 0.0002$; 95% CI, -14.02×10^{-3} to -6.644×10^{-3}). Higher ROS levels were also observed in FA treated cells relative to NAM treated cells

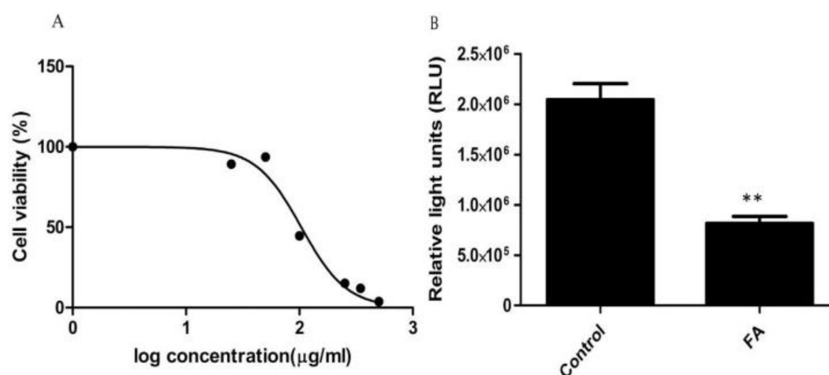

Fig. 1. Metabolic activity as a measure of cell viability decreased in a dose dependent manner following FA treatment with a range of concentrations (0–500 μg/mL) using the MTT assay. Drastically high cell mortality rates were observed at higher concentrations (A). Intracellular ATP levels (B) of HepG2 cells significantly decreased following treatment with FA following for 24 h (**p = 0.0062).

(p = 0.0002; 95% CI: 5.978 × 10^{-3} to 13.36 × 10^{-3}). NAM treatment did not significantly influence ROS levels when compared to control cells (Fig. 2A).

Increased oxidative stress by FA may interfere with cellular antioxidant systems such as Nrf2 expression. FA significantly increased Nrf2 expression (western blot) when compared to controls ($p < 0.0014$; untreated vs. FA, 95% CI, 3.058 × 10^{-2} RBD to −0.367 × 10^{-2} RBD; FA vs. NAM, 95% CI 1.493 × 10^{2} RBD to 3.9 × $^{-2}$ RBD) but no significant changes were observed between untreated cells and NAM treated cells (Fig. 2B).

3.3. Mitochondrial stress

We then measured changes in expression of SIRT3 (both protein and gene), a key regulator of mitochondrial function, in FA and NAM treated cells relative to untreated cells.

FA significantly decreased SIRT3 protein expression as compared to both controls (p = 0.0012; untreated vs. FA, 95% CI, 0.2884 × 10^{-3} RBD to 0.7275 × 10^{-3} RBD; FA vs. NAM, 95% CI -0.5893 × 10^{-3} RBD to −0.1502 × 10^{-3} RBD, Fig. 3A).

To validate the reduced expression of SIRT3 protein levels, we investigated *SIRT3* gene expression by qPCR. *SIRT3* mRNA levels were significantly reduced by FA but not NAM when compared to untreated cells (Fig. 3B) (p = 0.0007; untreated vs. FA, 95% CI, 0.7160 to 1.473; FA vs. NAM, 95% CI -1.164 to −0.4069).

The down-regulation of SIRT3 has implications for mitochondrial stress responses. We measured protein expression of LON, a key mitochondrial stress response protein. FA significantly decreased expression of LON in the untreated control (p = 0.0044; untreated vs. FA, 95% CI, 0.1997 RBD to 1.287 RBD; untreated vs. NAM, 95% CI 0.4179 RBD to 1.411 RBD). The difference in LON expression induced by FA compared to NAM was not significant (Fig. 3C).

3.4. Mitochondrial biogenesis

Both SIRT3 and LON are involved in mitochondrial biogenesis and the effect of FA on this process was then examined.

The effect of FA on PGC-1α protein expression, the master regulator of mitochondrial biogenesis was determined. Both FA and NAM significantly decreased PGC-1α protein expression as compared to the untreated control (p = 0.0005; untreated vs. FA, 95% CI, 0.09168 RBD to 0.2127 RBD; untreated vs. NAM, 95% CI 0.09206 RBD to 0.2274 RBD; Fig. 4A).

Also, the expression of the mitochondrial biogenesis regulatory protein p-CREB was significantly decreased by FA and NAM (p = 0.0008; untreated vs. FA, 95% CI, 0.361 × 10^{-3} RBD to 1.362 × 10^{-3} RBD; untreated vs. NAM, 95% CI 0.6213 × 10^{-3} RBD to 1.622 × 10^{-3} RBD, Fig. 4B).

Mitochondrial biogenesis is regulated at the transcriptional level by NRF1. Both FA and NAM significantly decreased NRF1 expression in comparison to controls (Fig. 4C; p = 0.0004; untreated vs. FA, 95% CI, 0.1124 RBD to 0.2121 RBD; untreated vs. NAM, 95% CI 0.1177 RBD to 0.2270 RBD).

Finally, FA significantly decreased expression of HSP70 when compared to untreated and NAM stimulated cells (p = 0.0102;

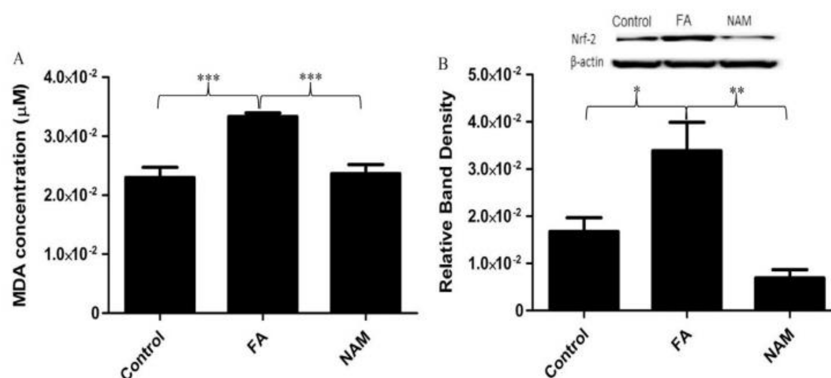

Fig. 2. FA increased ROS production in HepG2 cells as measured by MDA (a by-product of lipid peroxidation). FA significantly increased MDA levels (A, ***p = 0.0002) with a concomitant increase in relative protein expression of the phase II detoxification transcription factor Nrf-2, when compared to control cells (B, */**p < 0.0014).

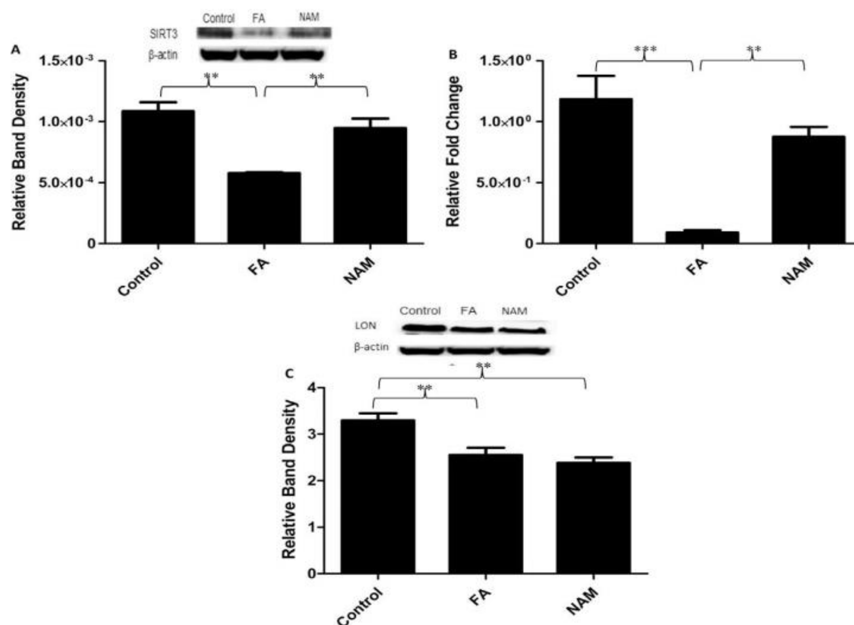

Fig. 3. FA significantly decreased expression of both SIRT3 protein (A, **: p = 0.0012) and SIRT3 mRNA levels (B, ***/**p = 0.0007) as compared to controls. Also LON protein expression was significantly down-regulated (C, **: p = 0.0044) in both FA and NAM treated cells.

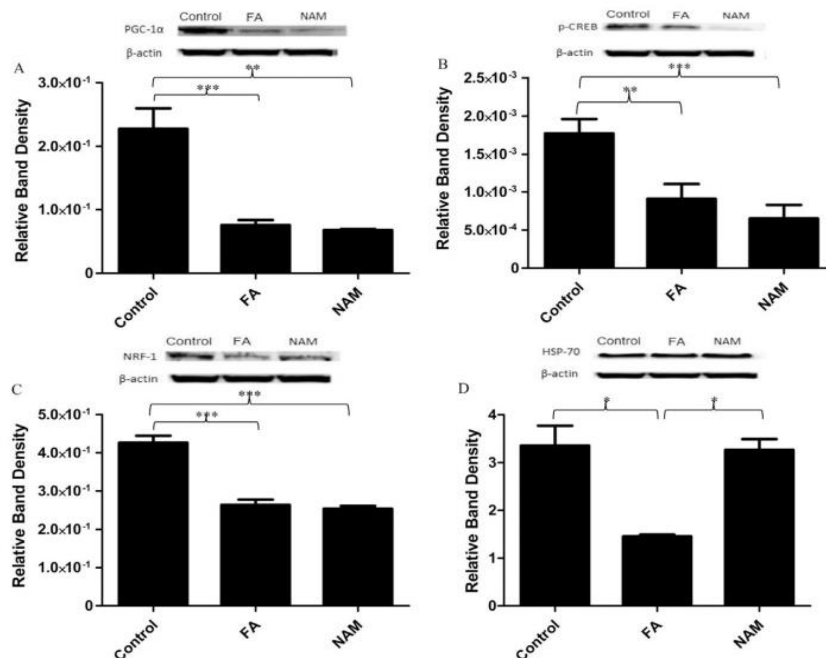

Fig. 4. FA induced aberrant expression of proteins involved in mitochondrial biogenesis and electron transport chain assembly after 24 h incubation as determined by western blot analysis. FA significantly decreased protein expression of: PGC-1α (A, **/***: p = 0.0005), pCREB (B, ***/**: p = 0.0008), NRF1 (C, ***: p = 0.0004) and HSP70 (D, *p = 0.0102) when compared to untreated control cells.

untreated vs. FA, 95% CI, 0.5672 RBD to 3.234 RBD; FA vs. NAM, 95% CI 3.142 RBD to −0.4758 RBD; Fig. 4D).

3.5. Cell death

Since both mitochondrial and oxidative stress result in cell death we investigated the effect of FA on caspase activity and cell membrane integrity as a measure of necrosis.

The caspases are the molecular machinery of apoptosis and the activities of key initiator caspases (−8 and −9) and executioner caspases (−3 and −7) in HepG2 cells after treatment with FA was determined (Fig. 5A). Our results show a decrease in the activity of caspase 9 and a significant ($p < 0.0001$) decrease in caspase 8 activity. Interestingly, despite the decrease in initiator caspase

activity, caspase 3 and 7 activity was found to be significantly increased in response to FA treatment ($p = 0.002$).

FA induced significant cell membrane damage when compared to control cells as measured by increased LDH activity and is an indication of necrotic cell death ($p < 0.0001$, 2.902 ± 0.1385 vs. 0.8328 ± 0.06286; Fig. 5B).

4. Discussion

FA is produced by *Fusarium* spp, a ubiquitous soil fungus, and contaminates many agricultural products (Bacon et al., 1996), to date the belief was that it possessed low to mild toxicity (Wang and Ng, 1999). The liver is highly susceptible to toxic insult (Fenton, 2002). Hepatocytes are densely populated with mitochondria which serve as integrators for several metabolic pathways and regulators of hepatocyte survival. Hepatocyte damage is strongly associated with mitochondrial dysfunction coupled with increased ROS production (Esposti et al., 2012).

Previous studies have implicated ROS generation as a major contributor to FA induced toxicity (Hirai et al., 2005; Iwahashi et al., 1999; Jiao et al., 2014), whilst other studies showed FA chelation as a toxic mechanism by interfering with catalytic metalloproteins (Ruda et al., 2006; Stack et al., 2004) and enhancing DNA damage (Jaglowski and Stack, 2006; Ogata et al., 2001). Studies in plant and animal models have implicated mitochondrial dysfunction (Telles-Pupulin et al., 1996; 1998; Jiao et al., 2014) and increased recruitment of apoptotic machinery (Samadi and Behboodi, 2006; Ogata et al., 2001).

The molecular mechanisms of FA toxicity, however, are not fully elucidated. SIRT3 regulates many aspects of mitochondrial function including ATP generation and stress responses (Chen et al., 2014). SIRT3 is ideally positioned to function as a mitochondrial fidelity protein and a loss of its function can result in cellular damage and eventual death.

Our results show that FA decreased cell viability in a dose dependent manner, with a drastic decrease at higher concentrations (Fig. 1A). The MTT assay measures cell viability based on the generation of reducing equivalents by metabolically active cells (Nikzad et al., 2014). FA impedes oxidative reactions of the Kreb's cycle by inhibiting α-ketoglutarate dehydrogenase and succinate dehydrogenase (Telles-Pupulin et al., 1996; 1998). α-Ketoglutarate dehydrogenase regulates metabolic flux via the Kreb's cycle and catalyses the conversion of α-ketoglutarate to succinyl-CoA producing $NADH_2$ and directly provides electrons for the respiratory chain. Succinate dehydrogenase oxidizes succinate to fumarate producing $FADH_2$ and transfers electrons from succinate to ubiquinone thus playing a role in the Kreb's cycle and ETC (Champe et al., 2005). Our results seem to be in agreement with these studies that show FA interferes with reducing equivalent metabolism as indicated by the MTT results.

SIRT3 down-regulation at both the protein and gene levels (Fig. 3A and B) shows that FA may selectively target the mitochondrion. Also, FA is a weak acid and this may also explain its mitochondrial toxicity (weak acids disrupt the hydrogen gradient across the mitochondrial membrane and decreases ATP production). The decreased SIRT3 protein expression can be attributed to its decreased gene transcript levels. It is also possible that FA, an efficient chelator of zinc (Stack et al., 2004), directly down regulated SIRT3 protein expression. SIRT3 contains a conserved enzymatic core with two domains, including a large Rossmann fold domain that binds NAD^+ and a small domain formed by two insertions of the large domain binding to a zinc atom (Nogueiras et al., 2012). FA may have caused loss of functional stability of SIRT3 through removal of the zinc ion.

FA severely depleted intracellular ATP levels (Fig. 1B). Ahn et al. (2008) showed decreased respiration and ATP levels in SIRT3 double knock-out mice livers (Ahn et al., 2008), and is in agreement with our human *in vitro* model data. SIRT3 targets NADH dehydrogenase and succinate dehydrogenase and stimulates their activity (Finley et al., 2011; Chen et al., 2014). FA is structurally similar to NAM and may inhibit SIRT3 activity through competitive binding at the NAD^+ site. Furthermore, these complexes contain iron-sulphur clusters (Champe et al., 2005; Atamna et al., 2002); FA can chelate iron *in vitro*, and this would prevent the transfer of electrons in the ETC.

Furthermore, it was shown that FA directly suppressed the ETC by inhibiting ATP synthase activity (Telles-Pupulin et al., 1998). Thus FA may directly or indirectly inhibit mitochondrial enzymes as well as reducing equivalent metabolism and decrease ATP synthesis.

FA significantly elevated levels of MDA, a by-product of lipid peroxidation and marker of oxidative stress (Fig. 2A). This is in agreement with Jiao et al. (2014) (Jiao et al., 2014). Although ROS is damaging to a variety of cellular macro-molecules, membrane lipids are especially sensitive to free radicals (Tretter and Adam-Vizi, 2005). The inhibition of the ETC by FA is a likely cause of the highly oxidative environment. It was shown that inhibition of the ETC, particularly at complex I and II leads to the increased generation of ROS (Chen et al., 2007). FA itself is thought to induce oxidative stress through chelation of iron and enhancement of the

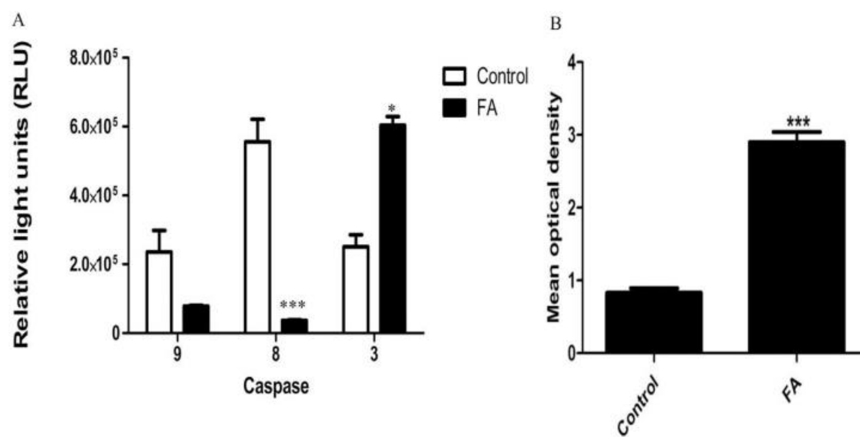

Fig. 5. Apoptotic cell death markers were significantly influenced by FA. Activity of initiator caspase 8 was significantly downregulated (A, *** < 0.0001) whilst the executioner caspases 3/7 activities were significantly increased (A, *p = 0.002). Also, FA induced significant LDH leakage from HepG2 cells indicative increased necrosis (B, ***p < 0.0001).

Fenton reaction (Iwahashi et al., 1999; Hirai et al., 2005). The nitrogen atom in the pyridine ring and the oxygen atom in the carboxyl group, may act as a chelator and enhance the Fenton reaction leading to formation of the hydroxyl radical (Iwahashi et al., 1999).

Induction of the phase 2 response is an important cellular defence to oxidative stress and is dependent on the release of Nrf2 from its repressor KEAP-1, a zinc thiol protein. Zinc is bound to reactive cysteine thiols of Keap1 and is displaced by electrophiles. Release of zinc alters the conformation of Keap1 thereby allowing translocation of Nrf2 to the nucleus (Dinkova-Kostova et al., 2005). Our results show a significant increase in Nrf2 expression (Fig. 2B). However, given that KEAP-1 is a zinc thiol protein it may be possible that FA chelates this ion and enhances the release of Nrf2. Despite elevated Nrf2 levels, the increased oxidative stress in the HepG2 cells still persisted. This may be due to the chelation of zinc, a critical ion involved in the transcriptional function of Nrf2 (Li et al., 2014).

SIRT3 acts to dampen oxidative stress through enhanced expression and activity of SOD2 (Qiu et al., 2010; Tao et al., 2010). SIRT3 expression is normally up-regulated by oxidative stress (Flick and Lüscher, 2012), however our results show that FA decreased its expression at the gene level (Fig. 3B). Kong et al. (2010) showed that PGC-1α stimulated SIRT3 expression at the gene and protein levels (Kong et al., 2010) via CREB phosphorylation (Flick and Lüscher, 2012) and SIRT3 in turn up-regulated PGC-1α expression —these proteins are regulated by a positive feedback loop (Chen et al., 2014). Our results supports this as we show that FA disrupts this feedback loop by down regulating SIRT3, PGC-1α and p-CREB (Fig. 3A, B, 4A, 4B). Although no direct regulation of PGC-1α by SIRT3 has been reported; a study showed that SIRT3 expression is required for the induction of oxidative responses by PGC-1α (Kong et al., 2010). Interestingly inhibition of SIRT3 by NAM resulted in similar reductions to PGC-1α and p-CREB protein expression when compared to FA treated cells, thus supporting the hypothesis that FA can dysregulate SIRT3 activity through competitive inhibition.

Despite FA and NAM inhibiting SIRT3 and resulting in similar reductions to PGC-1α protein expression, no significant elevation in MDA levels was observed in NAM treated cells as compared to controls. There was also no difference in the expression of Nrf2 by NAM. The results are supported by a study in SIRT3 knock-out mouse embryonic fibroblasts that showed oxidative stress to only be significantly elevated upon induction of cellular stress (Kim et al., 2010).

The removal of oxidatively damaged proteins via proteolytic degradation is an important cell defence to high oxidative stress. Oxidized mitochondrial proteins must be removed rapidly to prevent their aggregation, cross linking and toxicity. LON, an ATP dependent protease, catalyses the degradation of oxidatively damaged proteins in the mitochondrial matrix (Bota et al., 2005). LON expression was decreased after treatment with both FA and NAM (Fig. 3C). Given that Nrf2 expression was up-regulated and SIRT3 expression down regulated by FA, it is likely that FA induced LON disruption at the post-translational level. This is supported by inhibition of SIRT3 with NAM showing similar reductions in LON expression. Furthermore the depletion of ATP by FA will prevent

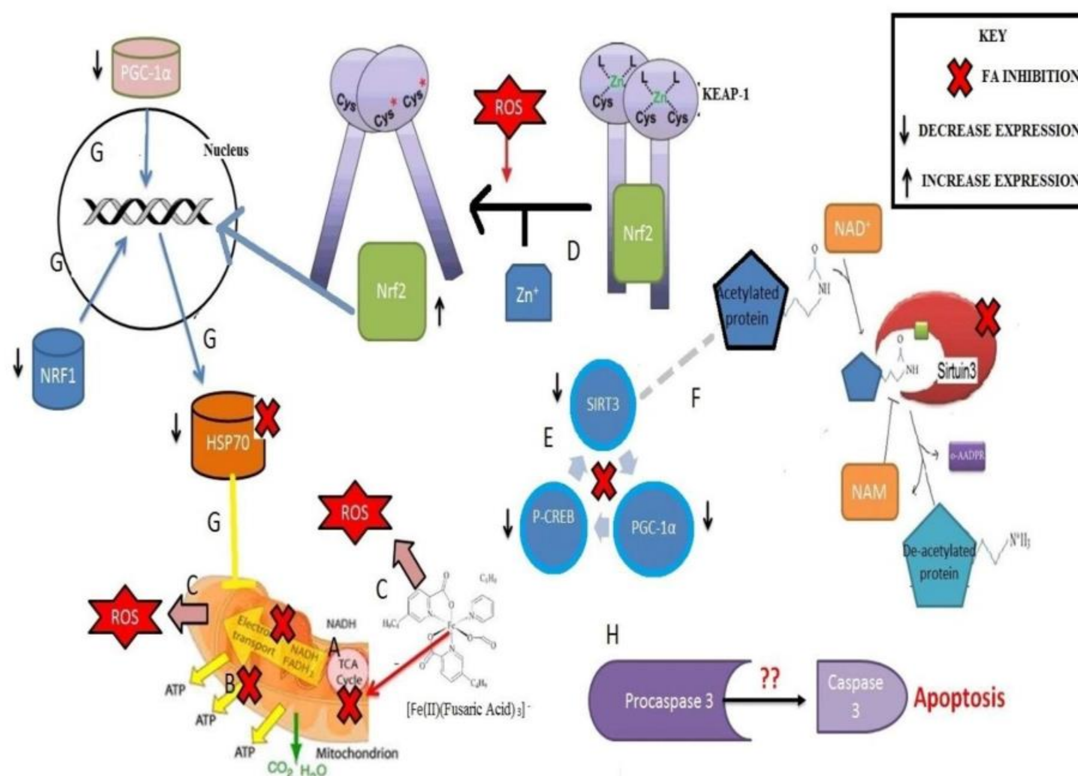

Fig. 6. Illustration of the investigated mitochondrial and oxidative stress pathways dysregulated by FA. A) FA inhibits Krebs cycle and the ETC resulting in aberrant reducing equivalent metabolism leading to decreased ATP synthesis. B) FA dissipates the mitochondrial proton gradient and inhibits ATP synthase decreasing ATP production. C) Mitochondrial dysfunction and FA mediated chelation of iron causes oxidative stress. D) Release of Nrf2 from KEAP-1 due to chelation of zinc and oxidative stress. E) Decreased expression of SIRT3, PGC-1α and p-CREB due to dysregulation of positive feedback loop between these 3. F) FA pyridine structure interferes with mitochondrial protein acetylation by inhibiting SIRT3 activity. G) Aberrant mitochondrial biogenesis due to decreased transcription (loss of NRF1 and PGC-1α) and translocation/assembly (loss of HSP70 and LON) of mitochondrial proteins. H) Increased activity of executioner caspases through novel initiator caspase independent pathway.

catalytic activity of LON inhibiting proteolytic degradation of oxidized proteins. FA induced mitochondrial stress in HepG2 cells, is further aggravated by loss of LON function.

Since FA induced both oxidative and mitochondrial stress, we then determined its effect on mitochondrial biogenesis. Increased mitochondrial biogenesis aids cells in preventing mitochondrial lesions and maintaining mitochondrial integrity (Esposti et al., 2012). PGC-1α is known to enhance NRF1 dependent gene expression by acting as a co-activator. NRF1 (a transcription factor) activates the expression of oxidative phosphorylation components and the expression of Tfam resulting in increased expression of nuclear mitochondrial genes and mitochondrial DNA replication (Piantadosi and Suliman, 2006). We noted a decrease in NRF1 expression (Fig. 4C). Kong et al. (2010) found that inhibition of SIRT3 expression diminished the PGC-1α induction of mitochondrial biogenesis. They proposed that NRF1 is a substrate of SIRT3 however further studies are required to determine the molecular mechanism by which SIRT3 controls mitochondrial biogenesis (Kong et al., 2010).

Most genes encoding mitochondrial proteins are located in the nucleus rather than in the mitochondrial genome, these proteins must be imported into the mitochondria. This involves complex folding and assembly processes to ensure proper enzyme activity (Voos, 2013).

HSP70 is involved in mitochondrial protein homeostasis and mediating translocation and folding reactions (Voos, 2013). We showed a decreased expression of HSP70 by FA (Fig. 4D). HSP70 coordinates two calcium ions one of which contributes to protein stability while the other is needed to carry out ATP hydrolysis (Sriram et al., 1997), thus FA may affect both stability and activity of HSP70 through chelation of calcium. Additionally FA mediated depletion of ATP will impede folding reactions of HSP70 since ATP hydrolysis is coupled with its molecular chaperone function (Voos, 2013). LON can also act as a chaperone, independent of its proteolytic activity and promote the assembly of cytochrome *c* oxidase subunits. Thus down regulation of LON results in impaired mitochondrial and respiratory function (Bota et al., 2005; Gibellini et al., 2014b).

Plant based studies have concluded that FA causes cell death through oxidative stress and mitochondrial dysfunction (Jiao et al., 2014; Singh and Upadhyay, 2014). Experiments by Ogata et al. (2001) showed that FA induced apoptosis effectively in HL60 cells (Ogata et al., 2001) confirming the apoptotic inducing potential of FA in a human *in vitro* model. Our results are in agreement as we show increased activity of the key executioner caspases 3 and 7, a marker of the apoptotic process (Fig. 5A). Bota et al. (2005) showed activation of executioner caspase 3 and apoptotic cell death as a result of LON down-regulation (Bota et al., 2005).

Given that mitochondrial stress was induced by FA it seemed logical to assume that the enhanced activity of caspase 3 and 7 would be attributed to the intrinsic apoptotic pathway. However, the data suggest that caspase 9 is not a good candidate for the activation of executioner caspases since this initiator caspase is decreased in FA treated cells when compared to control cells, although not significantly. In the cytoplasm, cytochrome *c* binds to the APAf-1 and forms the apoptosome to induce the activation of pro-caspase 9 and initiates an enzymatic reaction cascade leading to the execution of apoptosis via caspase 3 activity (Dai et al., 2014). Cytochrome *c* is an iron metalloprotein and an absolute requirement for caspase 9 activity, hence the possible chelation of iron from cytochrome *c* by FA prevents the proper formation of the apoptosome leading to decreased activity of caspase 9. Furthermore, our results are in agreement with previous studies that showed Apaf-1 transformed ATP into ADP and is an ATPase; ATP is essential for Apaf-1 binding to cytochrome *c* (Hu et al., 1999; Chiarugi, 2005). Thus FA may have prevented activation of caspase 9 by depletion of intracellular ATP levels. Further analysis of initiator caspase activity revealed drastically decreased activity of caspase 8- the initiator of the extrinsic apoptotic pathway. This may be explained by the enhanced permeability of FA through the cell membrane due to the fat soluble side chain (butyl group) on the pyridine ring preventing activation of death receptors in this pathway. Studies have alluded to early activation of caspase 3 independent of caspase 9 and 8 in cells undergoing oxidative stress (Varghese et al., 2003; Tartier et al., 2000) and seem to be in agreement with our data. Roy et al. (2001) demonstrated caspase 3 proenzyme is auto-catalytically competent (Roy et al., 2001) and can undergo self-activation *in vitro* (MacCorkle et al., 1998). Data from our study indicate that FA induced apoptosis is not mediated by conventional initiator caspases and may occur via a novel pathway that involves activation of executioner caspases independent of apical initiator caspases.

Furthermore LON deficient cells switch to necrosis as a result of ATP depletion caused by mitochondrial defects (Bota et al., 2005). LDH release was increased (Fig. 5B) - an indication of increased necrotic cell death. Thus, while apoptosis may be initiated after FA treatment the switch to necrotic cell death may be as a result of depleted ATP. An overview of FA toxicity is presented in Fig. 6.

5. Conclusion

Taken together our data shows that mitochondrial function and biogenesis is impaired by FA. Our results point to loss of SIRT3 in response to FA as a possible mechanism of action culminating in cytotoxicity in the HepG2 cell line (Fig. 6). This study highlights FA as a possible inducer of hepatotoxicity and may aid in understanding and predicting the contamination risks of FA. This is of particular importance to developing countries where the risk of mycotoxin exposure is high due to consumption of often contaminated agricultural produce.

Ethical statement

We declare that we followed strict guidelines in adhering to ethics. This study was a cell culture (*in vitro*) project and as such required no Institutional Ethical Approval. We declare that this manuscript has not been submitted elsewhere for consideration and has not been previously published.

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Transparency document

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CHAPTER 3

Fusaric acid upregulates NRF2 as a Cytoprotective Response to Prevent NLRP3 activation in HepG2 liver derived cells

In chapter 2 the mitotoxic potential of FA in a human derived liver cell model was established. This chapter further explored the implications of FA induced mitochondrial and oxidative stress on the inflammatory pathways with particular focus on IL-1β processing. Fusaric acid repressed NLRP3 priming and activation through mitophagic clearance of damaged mitochondria. This paper highlights FA as an immune suppressor despite mitochondrial toxicity.

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Fusaric acid Induces NRF2 as a Cytoprotective Response to Prevent NLRP3 activation in the liver derived HepG2 cell line

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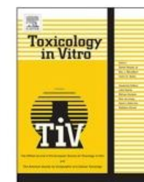

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ABSTRACT

Fusaric acid (FA) is a neglected fusarium mycotoxin despite its ubiquitous presence. FA is a niacin related compound and mediates toxicity *via* oxidative stress and mitochondrial dysfunction. The NLRP3 inflammasome is a multiprotein scaffold that plays a key role in IL-β maturation. We investigated the effects of FA on IL-1β processing, NRLP3 inflammasome priming and activation along with the potential of FA to initiate cytoprotective mechanisms using spectrophotometry, luminometry, qPCR and western blots in the HepG2 liver cell line. FA disrupted synthesis and maturation of IL-1β by inhibiting NRLP3 priming and activation. Further experimentation revealed an up-regulation of NRF2 with concomitant elevation in the anti-oxidant enzyme SOD2 and autophagy markers suggesting that FA induces NRF2 cytoprotective programs in these cells. We conclude that FA attenuates inflammasome priming and activation and sheds light on the immunosuppressive potential of FA in liver cells.

1. Introduction

The inflammasome is a multiprotein complex that is primed and activated upon recognition of pathogen or damage associated molecular patterns (DAMPs). Assembly of the inflammasome complex results in activation of caspase 1 and subsequent maturation of the potent pro-inflammatory cytokine, IL-1β (Latz et al., 2013; He et al., 2016). The nucleotide-binding domain (NOD)-like receptor protein 3 (NLRP3) inflammasome has gained considerable attention due to its potentiating role in several disease states including cancer (Wei et al., 2014) and metabolic disorders (Jiang et al., 2018).

The nuclear factor κB (NFκB) is essential for NLRP3 priming through transcriptional activation of inflammasome components and IL-1β (Connat et al., 2018; Bauernfeind et al., 2009). Recently, several studies have highlighted the anti-inflammatory functions of NFκB in preventing excessive and premature NRLP3 inflammasome activation through promotion of autophagy and mitophagy (Zhong et al., 2016; Nakahira et al., 2011; Zhou et al., 2011). Mitophagy is the selective degradation of damaged mitochondria mediated by the PTEN-induced putative kinase 1 (PINK1) (Lazarou et al., 2015). Mitochondria play supplementary roles in the innate immune system. Signals such as oxidized mitochondrial DNA (mtDNA) and mitochondrial reactive oxygen species (mtROS) are established inflammasome activators (Zhong et al., 2016; Nakahira et al., 2011; Zhou et al., 2011; Won et al., 2015).

Oxidative stress is the over-production of reactive oxygen species (ROS) which can lead to cell damage. Cells are capable of eliciting several adaptive mechanisms to cope with oxidative stress. An oxidative conditioning effect is often observed when low levels of oxidants induce a hormetic response encompassing several cell survival mechanisms. Such a response is often the result of nuclear factor erythroid derived 2-like 2 (NRF2) transcriptional activation (Maher and Yamamoto, 2010; Luna–López et al., 2014). This transcription factor induces the expression of several antioxidant (Gwarzo, 2009; Vnukov et al., 2015) and autophagy genes (Jain et al., 2010; Murata et al., 2015) preventing cellular damage.

Fusaric acid (FA) is a common foodborne mycotoxin produced by several *fusarium* species (Bacon et al., 1996) with a reported contamination level of up to 643 μg/kg sample (Streit et al., 2013). It has been reported that FA displays strong synergistic effects with co-produced mycotoxins such as Fumonisin B1 and deoxynivalenol (Bacon et al., 1995; Smith et al., 1997). There is increasing human *in vitro* data indicating the potential genotoxic, cytotoxic and mitotoxic effects of FA (Ghazi et al., 2017; Abdul et al., 2016; Dhani et al., 2017; Devnarain et al., 2017). Accumulating evidence highlights FA as a putative mitochondrial toxin that deregulates energy processes and stress responses as well as biogenesis (Abdul et al., 2016; Telles-Pupulin et al., 1998) and induction of oxidative stress (Abdul et al., 2016; Dhani et al., 2017; Devnarain et al., 2017). The immunotoxicity of FA is not well

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documented. The available data indicate that FA alters lymphocyte proliferation and apoptosis (Dhani et al., 2017; Ogata et al., 2001).

There is considerable interest surrounding mitochondrial dysfunction, and its links to oxidative stress and inflammasome activation. Following ingestion of food, the liver is exposed to several foodborne toxins including FA. Frequently, low doses of toxins activate an adaptive stress response whereas high doses activate acute responses like cell death. The aim of this study was to evaluate the priming and activation of the NLRP3 inflammasome as a consequence of the established mitotoxicity of FA in HepG2 liver cells. Furthermore, we address the complex crosstalk between the induced mitochondrial dysfunction accompanied by mitophagy as a cytoprotective mechanism against the mitochondrial toxin FA. Aberrant crosstalk of these transregulatory networks in immune dysregulation can lead to disease initiation and progression.

2. Methods and materials

2.1. Reagents

The HepG2 human liver cell line was purchased from Highveld Biologicals (Johannesburg, SA). Cell culture reagents were purchased from Lonza Biowhittaker (Basel, Switzerland). Western blot reagents were purchased from Bio-Rad (Hercules, CA, USA). FA isolated from *Gibberella fujikuroi* (F6513) rotenone (R8875) and antimycin A (A8674) were acquired from Sigma-Aldrich (St Louis, MO, USA). All other reagents and consumables were purchased from Merck (Darmstadt, Germany), unless otherwise stated.

2.2. Cell culture and treatment protocol

HepG2 cells were cultured in Eagle's minimum essential media (10% foetal calf serum, 1% penstrepfungizone, 1% L-glutamine, CCM) at 37 °C in a humidified incubator (5% CO_2). Cells were allowed to reach 80% confluency in 25 cm^3 culture flasks prior to treatment with FA.

FA was dissolved in sterile 0.1 M PBS to make a stock concentration of 2 mg/mL. This was then diluted in CCM to final concentrations of 25, 50, and 104 µg/mL (0 µg/mL FA in control). These concentrations were selected based on a hypothesised hormetic cellular response around the reported IC_{50} concentration (104 µg/mL) in the HepG2 cell line for 24 h (Abdul et al., 2016). Rotenone (ROT; 10 mM) and antimycin A (AA; 10 mM) was prepared in Dimethyl Sulphoxide (DMSO) and a final concentration of 5 µM was used for all subsequent experiments (0.1% v/v – benchmark DMSO concentration for *in vitro* experiments). All experiments were repeated three times (independently) in triplicate to validate results.

2.3. Endogenous antioxidant status

The GSH-Glo™ (V6912, Promega, Madison, USA) was used to quantify reduced glutathione as a measure of oxidative challenge in FA treated HepG2 cells. Cells were transferred to a white microtitre plate (50 µL of 20,000 cells/ well; 3 replicates). GSH standards (0–5 µM) were prepared from a stock solution diluted in deionized water (5 mM). The 2X GSH-Glo™ Reagents were prepared according to the manufacturer's instructions, added to the experimental wells (50 µL/well), and incubated (RT, 30 min). Reconstituted Luciferin Detection Reagent (50 µL) was added to each well and incubated (RT, 15 min) before the luminescence was measured (Modulus™ microplate luminometer, Turner Biosystems, Sunnyvale, USA). A standard curve was constructed using GSH standards and GSH concentrations (µM) in control and FA treated cells were extrapolated.

2.4. Detection of caspase 1 activity

Caspase-1 activity was measured using the Caspase-Glo® 1 Inflammasome Assay (G9951, Promega, Madison, USA). Briefly, 50 µL of cell suspension (20,000 cells/well in 0.1 M PBS) was seeded into a white microtitre plate in triplicate. As per manufacturer's guidelines, Caspase-Glo® 1 reagents were reconstituted and added to each well (50 µL). Thereafter, the plate was incubated (dark, 1 h, RT). Following incubation, luminescence was detected using a Modulus™ microplate luminometer (Turner BioSystems, Sunnyvale, USA). Results are expressed as relative light units (RLU).

2.5. Cytokine quantification

Supernatants of control and FA treated cells were analyzed for pro-inflammatory IL-1β by enzyme-linked immunosorbent assays (ELISA) using a commercially available kit (cat# BD 557966), BD Biosciences, USA] according to manufacturer's instructions. Briefly, 96-well microtitre plates were coated with capture antibody diluted in coating buffer and incubated at 4 °C overnight. The wells were then washed three times (300 µL/well) and residual buffer was removed. Plates were blocked with assay diluent (200 µL/well, RT for 1 h), thereafter assay diluent was removed and plates were washed thrice. Recombinant human IL-1β standards and experimental samples were added to each well (100 µL) and incubated (2 h, RT). Plates were washed five times. Detection antibody was added (100 µL) and incubated at RT for 1 h; the plates were then washed five times. Diluted enzyme reagent (horseradish peroxidase [(HRP)-conjugated Streptavidin (Sav-HRP)] was added (100 µL), incubated at RT for 30 min, then removed and washed seven times. Substrate solution was then added (100 µL) and incubated at RT for 30 min in the dark. Following this incubation, stop solution was added to each well (50 µL) and the plate was read using a Bio-Tek µQuant MQx200 spectrophotometer after 30 min at 450 nm with a reference wavelength of 570 nm.

Data was analyzed against the linear portion of the generated standard curve. Results were expressed as picograms (pg) of cytokine/mL.

2.6. Mitochondrial depolarisation

The loss of mitochondrial membrane potential is a hallmark of mitochondrial dysfunction. HepG2 cells were seeded in a 6-well plate and exposed to FA (0, 25, 50 and 104 µg/mL) for 24 h. The cells were transferred to a black plate ((20,000 cell/well) and incubated with JC-1 (BD biosciences) staining solution (5 µg/mL) for 20 min at 37 °C. After the cells were washed twice with the JC-1 staining buffer, the fluorescence densities of the JC-1 monomers (λ excitation = 488 nm, λ emission = 529 nm) and JC-1 aggregates (λ excitation = 524 nm, λ emission = 594 nm) (Zheng et al., 2013) were detected using a luminometer with fluorescent detection capabilities.

2.7. Western blot

Protein expression was quantified using western blots. Briefly, Cytobuster™ Reagent (250 µL) (Novagen, San Diego, CA, USA, catalogue no. 71009) was used for protein extraction. Protein samples were quantified with the bicinchoninic acid (BCA) assay and standardized to 1 mg/mL. The samples were boiled (5 min) in Laemmli Sample buffer [dH20, 0.5 M Tris-HCl (pH 6.8), glycerol, 10% SDS, β-mercaptoethanol, 1% bromophenol blue]. Prepared protein samples were electrophoresed in sodium dodecyl sulphate polyacrylamide (SDS-PAGE) gels (4% stacking gel, 7.5% resolving gel) for 1.5 h at 150 V (Bio-Rad Mini PROTEAN Tetra-Cell System). The separated proteins were electrotransferred onto nitrocellulose membranes using the Trans-Blot Turbo Transfer system (standard mixed protein program, Bio-Rad). The membranes were blocked with 5% bovine serum albumin (BSA) in Tris

Table 1
List of antibodies used to perform western blot experiments.

Antibody (catalogue number)
Heat Shock Protein 60 (BD 611563) Mouse
Phospho-IKKβ (Ser176/180) (16A6) Rabbit mAb
Phospho-NF-κB p65 (Ser536) (93H1) Rabbit mAb
IRAK1 (D51G7) #4504 Rabbit mAb
IRAK4 Antibody #4363 Rabbit mAb
UCP2 Antibody (N-19): sc-6526 Goat polyclonal
TRAF6 (D21G3) #8028 Rabbit mAb
TFAM (D5C8) #8076 Rabbit mAb
NRF2 (D1Z9C) XP® #12721 Rabbit mAb
NRF2 (phospho S40) (ab76026) Rabbit mAb
SQSTM1 / p62 (ab56416) mouse mAb
PINK1 antibody (ab23707) Rabbit mAb
SOD2 antibody (SAB2701618 SIGMA)
Anti-rabbit IgG, HRP-linked Antibody #7074
Anti-mouse IgG, HRP-linked Antibody #7076
Donkey anti-goat IgG-HRP: sc-2020
Monoclonal Anti-β-Actin−Peroxidase antibody A3854 SIGMA

Buffer Saline with tween 20 (TTBS- NaCl, KCl, Tris, dH_2O, 0.5% tween 20, pH 7.5) or 5% Non-Fat Dry Milk (NFDM) in TTBS for phospho- and non-phospho- antibodies respectively. Membranes were incubated with primary antibody (Table 1) overnight (4 °C). The membranes were incubated (RT) with HRP-conjugated secondary antibody for 1 h (1:5000 dilution). Protein bands were detected using the Clarity western ECL chemiluminescent substrate and images were captured with the ChemiDoc™ XRS+ Molecular Imaging System (Bio-Rad). Protein expression was analyzed using Image Lab Software version 5.0 (Bio-Rad) and the results were expressed as relative band density (RBD). Protein expression was normalized against house-keeping protein, HRP-conjugated β-actin. Proteins upstream of NF-κB activation and mtDNA protection were evaluated only at the IC50 as this was considered a generalized response to FA toxicity.

2.8. Isolation of RNA and qPCR

RNA was isolated from control and FA treated HepG2 cells using an in house protocol (Chuturgoon et al., 2014). Total RNA was quantified (Nanodrop 2000, ThermoFischer) and standardized (1000 ng/mL). Standardized RNA samples were converted to complementary DNA (cDNA) using a commercially available kit (iScript™ cDNA Synthesis kit, Bio-Rad; catalogue no 107–8890). A 20 μL reaction volume containing 1 μL RNA template, 4 μL 5× iScript™ reaction mix,1 μL iScript reverse transcriptase and nuclease free water was prepared. Thermocycler conditions were 25 °C for 5 min, 42 °C for 30 min, 85 °C for 5 min and a final hold at 4 °C.

mRNA levels of genes related to anti-oxidant response, inflammation and mitophagy was assessed (Table 2) using the Sso Advanced™ Universal SYBR® Green Supermic (Bio-Rad, catalogue no. 172-5271) on the CFX Touch™ Real Time PCR Detection System (Bio-Rad, Hercules, CA, USA). Conditions for PCR reactions: Initial denaturation (95 °C, 8 min), denaturation (95 °C, 30 s), annealing (variable: Table 2, 40 s) and extension (72 °C, 30 s). Each measurement was done in triplicate and normalized against GAPDH which was run under the same conditions. Data was analyzed using the method described by Livak and Schmittgen (2001) and represented as fold change relative to the housekeeping gene (2 − ΔΔCt) (Livak and Schmittgen, 2001).

2.9. Statistical analysis

Statistical analysis was performed using GraphPad Prism v5.0 software (GraphPad Software Inc., La Jolla, USA). A one-way ANOVA followed by Bonferroni test for multiple group comparison was used where more than two concentrations were being compared. An unpaired *t*-test with Welch's correction was used to assess the differences between means for control *vs.* IC_{50}. Results are expressed as mean with standard deviation (SD). Level of significance was set at $p < .05$.

Table 2
Primer sequences and conditions used for gene expression studies.

Sequence (5′-3′)	Annealing temperature
SOD2	57 °C
Forward: GAGA TGTT ACAC GCCC AGAT AGC	
Reverse: AATC CCCA GCAG TGGA ATAA GG	
IL-1β	58 °C
Forward: TGAA AGCT CTCC ACCT CCAG	
Reverse: CACG CAGG ACAG GTAC AGAT	
Caspase 1	58 °C
Forward: GCTT TCTG CTCT TCCA CACC	
Reverse: CATC TGGC TGCT CAAA TGAA	
NF-κB p65	58 °C
Forward: ACCA ACAA CAAC CCCT TCCA	
Reverse: GTAG TCCC CACG CTGC TCTT	
NLRP3	55 °C
Forward: AAGG AAGT GGAC TGCG AGAA	
Reverse: CCCT CGAA TTTG CCATA	
ATG5	60 °C
Forward: GCAG ATGG ACAG TTGC ACAC A	
Reverse: TTTC CCCA TCTT CAGG ATCA A	
LC3	60 °C
Forward: GAGA AGCA GCTT CCTG TTCT GG	
Reverse: GTGT CCGT TCAC CAAC AGGA AG	
ATG7	58 °C
Forward: CAGT TTGC CCCT TTTA GTAG TGC	
Reverse: CCTT AATG TCCT TGGG AGCT TCA	
GAPDH	Variable
Forward: TCCA CCAC CCTG TTGC TGTA	
Reverse: ACCA CAGT CCAT GCCA TCAC	

3. Results

3.1. FA attenuates NLRP3 inflammasome activation

Mitochondrial dysfunction is linked to NLRP3 inflammasome mediated activation of caspase 1 (He et al., 2016). Cells treated with ROT and AA was used as positive controls, as these mitochondrial inhibitors are inducers of caspase 1 activation (Fig. 1A). FA activates caspase 1 at a concentration of 25 μg/mL but strongly inhibits activity at higher concentrations (Fig. 1A). Further analysis of mRNA levels for inflammasome components revealed FA significantly downregulated gene expressions of *caspase 1* (Fig. 1B) and *NLRP3* (Fig. 1C) in a dose dependent manner and the levels of pro-*IL-1β* (mRNA expression) at all tested concentrations (Fig. 1D). To confirm that FA did not induce pro-IL-1β maturation, an ELISA was performed for IL-1β (biologically active form). IL-1β was significantly decreased in the supernatant of FA stimulated cells at the IC_{50} concentration. Taken together these results indicate that IL-1β processing is dependent on NLRP3 activity in hepatocytes incubated with FA.

3.2. FA inhibits NF-κB activation

NF-κB regulates the expression of pro-inflammatory mediators. When analyzed by western blot, the phosphorylated and transcriptionally active p65 subunit was found to be significantly decreased at the IC_{50} concentration (Fig. 2A). In contrast FA significantly elevated p65 transcript levels at 50 μg/mL and 104 μg/mL μg/mL (Fig. 2B). These results strongly suggest that FA affects post-translational activation of the NF-κB p65 subunit. IKK dependent phosphorylation is essential for NF-κB release and transcriptional activation. Using an antibody targeted against the phosphorylated activation loop (Ser177 and Ser181) we show FA decreased the protein expression of activated IKKβ (Fig. 2C). Since FA inhibited the expression of activated IKKβ we next investigated the effects of FA up-stream of IKKβ phosphorylation. FA (IC50) significantly reduced the relative protein expression of TRAF6 –

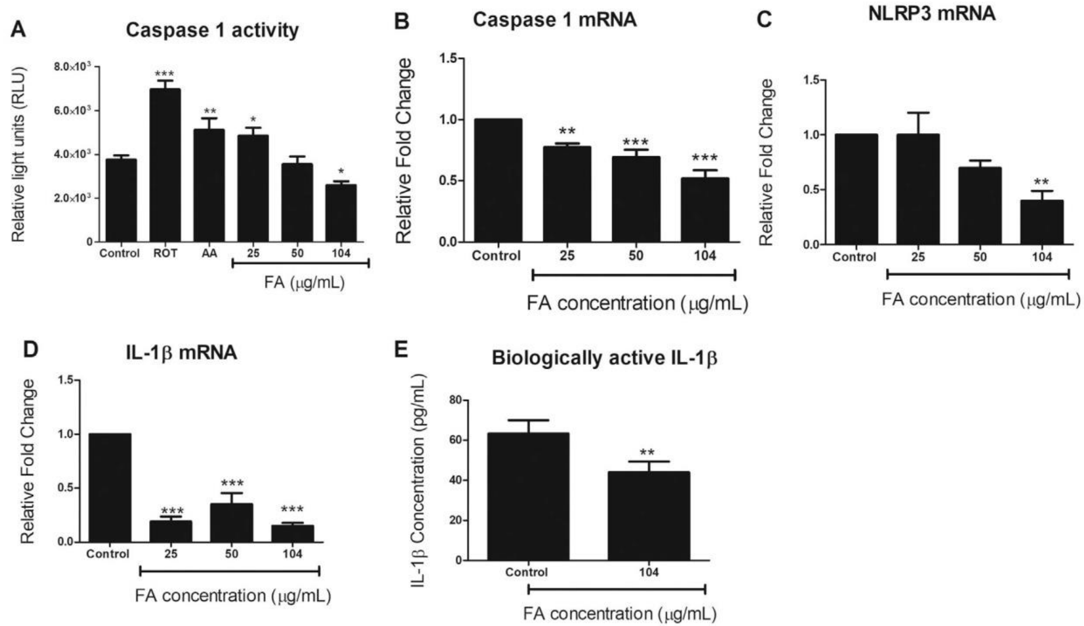

Fig. 1. FA suppresses NLRP3 dependent caspase 1 activation and inhibits IL-1β processing. FA reduced caspase 1 activity (A). The mRNA levels of *caspase 1* (B) and *NLRP3* (C) were significantly decreased. Furthermore, FA prevented IL-1β production (D) and maturation to the biologically active form (E). Results are expressed as mean and SD (n = 3). * - control *vs* FA μg/mL p = .01–0.05, ** - control *vs* FA μg/mL p 0.001–0.05, *** - control *vs* FA μg/mL p < .001.

an adaptor molecule necessary for innate immune signalling (Fig. 2D); down-regulated the expression of IRAK-1 (Fig. 2E) and IRAK4 (Fig. 2F) both critical kinases required for IKKβ phosphorylation.

3.3. Markers of mitochondrial dysfunction and mtROS production are elevated by FA

Mitochondrial dysfunction is associated with NLRP3 activation; we assessed the protein levels of the mitochondrial chaperone heat shock

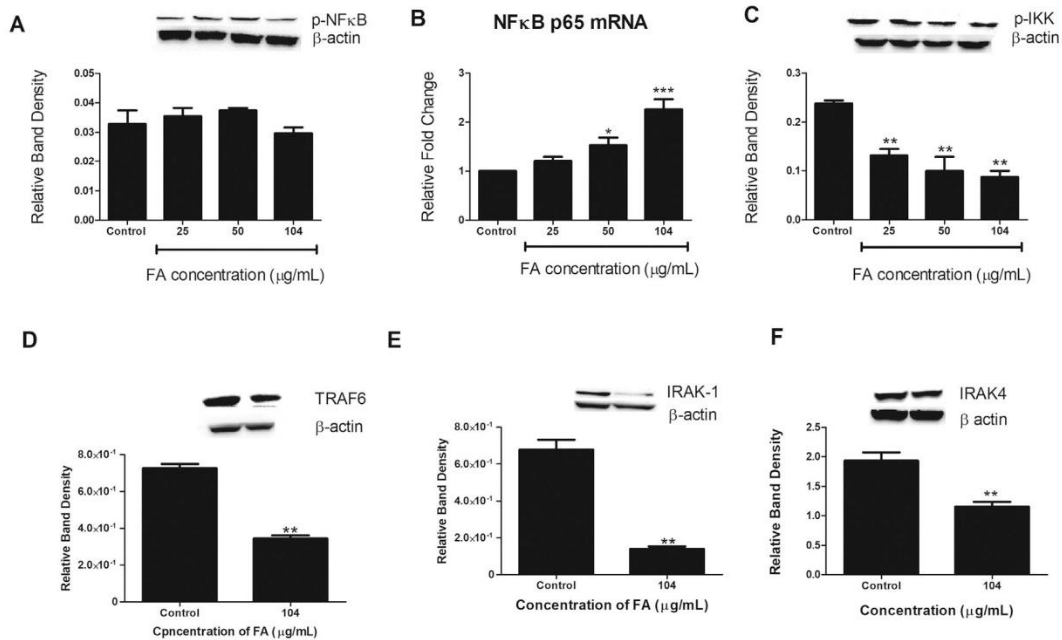

Fig. 2. Treatment with FA attenuates NF-κB activation. Assessment of NF-κB p65 subunit showed decreased activation (A) but elevated mRNA (B) in cells treated with FA. Analysis of upstream activators of this transcription factor showed down-regulation of activated IKKβ (C), TRAF6 (D), IRAK1 (E) and IRAK4 (F). Results are expressed as mean and SD (n = 3). * - control *vs* FA μg/mL p = .01–0.05, ** - control *vs* FA μg/mL p 0.001–0.05, *** - control *vs* FA μg/mL p < .001.

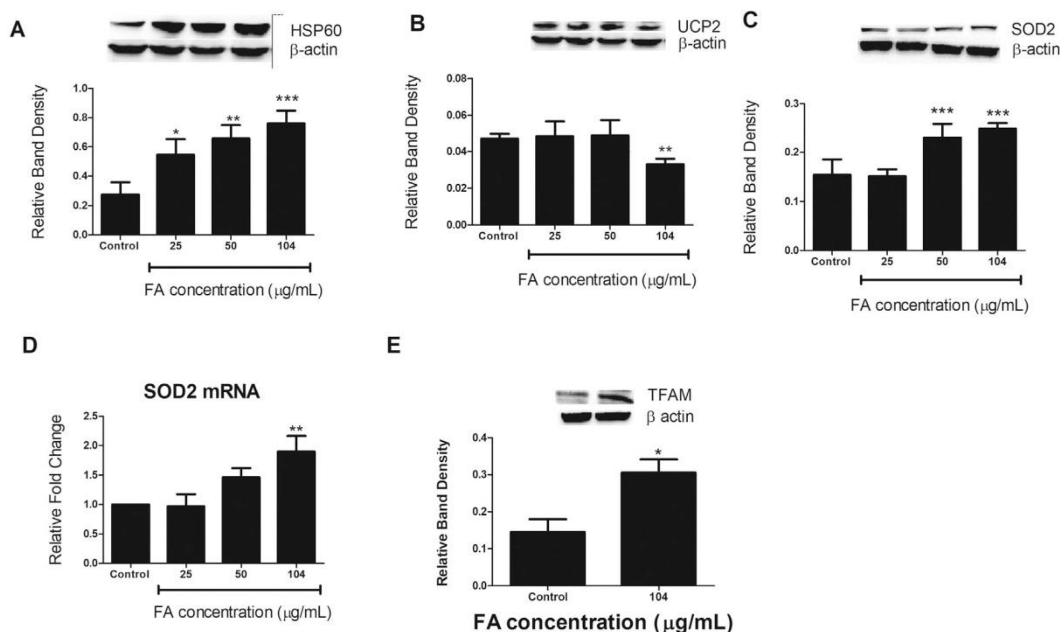

Fig. 3. FA elevated mitochondrial stress markers and enhanced defense mechanisms against mtROS damage. FA increased HSP60 (A) but decreased UCP2 (B) expression. Enhanced expression of the mitochondrial specific anti-oxidant enzyme, SOD2, was seen at protein (C) and transcript levels (D). TFAM expression was up-regulated (E). FA aberrantly affects mitochondrial integrity as indicated by enhanced membrane depolarization (F). Results are expressed as mean and SD ($n = 3$). *- control *vs* FA μg/mL $p = .01–0.05$, ** - control *vs* FA μg/mL p 0.001–0.05, *** - control *vs* FA μg/mL $p < .001$.

protein 60 (HSP60) and uncoupling protein 2 (UCP2). A dose dependent increase by FA in HSP60 expression was observed in cells (Fig. 3A). This suggests that FA is able to induce mitochondrial stress responses at lower concentrations.

The mitochondrial oxidative phosphorylation uncoupler, UCP2 protein expression was significantly decreased only at the IC_{50} concentration (Fig. 3B). These results validate FA as an inducer of mitochondrial stress, particularly at the IC_{50} concentration. Given that down-regulation of UCP2 is a marker for mitochondrial ROS generation and oxidative stress, we next determined if cells can up-regulate defenses against oxidative stress. FA significantly elevated SOD2 protein (Fig. 3C) and transcript levels (Fig. 3D) when compared to control. It is noteworthy that SOD2 levels (both protein and mRNA) peaked at the IC_{50} concentration. Further, mitochondrial transcription factor A (TFAM) expression, which supports mitochondrial biogenesis and mtDNA maintenance during oxidative stress, was significantly increased by the selected concentration (104 μg/mL) when compared to control (Fig. 3E). Mitochondrial membrane potential is an indicator of mitochondrial integrity. FA induced a dose dependent depolarisation of the mitochondrial membrane (Fig. 3F).

3.4. Fusaric acid induced NRF2 accumulation and activation

Mitochondrial dysfunction is linked to oxidative stress as these organelles are both generators and targets of ROS. Reduced glutathione (GSH) was assessed as a measure of oxidative challenge in FA treated HepG2 cells. FA depleted intracellular GSH levels at the highest selected concentrations indicating oxidative stress (Fig. 4A). The NRF2 transcription factor translocates to the nucleus under oxidative stress conditions and upregulates cytoprotective genes. Phosphorylation of NRF2 at serine 40 promotes its dissociation from Kelch-Like-ECH Associated Protein 1 (KEAP1). The increased phosphorylation of NRF2 (S40) expression was dose dependent (Fig. 4C). Further investigations revealed a similar trend in total NRF2 protein expression in FA stimulated cells compared to control (Fig. 4B).

3.5. Mitophagy contributes to the anti-inflammatory effect of FA

The p62 sequestosome and PINK1 are part of the cytoprotective protein battery induced by NRF2. FA induced a decrease in p62 expression which was observed to be dose dependent, however statistical analysis was only achieved at the IC_{50} (Fig. 5A). Accumulation of PINK1 recruits Parkin which results in enhanced clearance of damaged mitochondria. FA significantly increased PINK1 expression at the highest tested concentrations (104 μg/mL, Fig. 5B) when compared to the control. The impact of FA on autophagy was assessed by quantifying the transcript levels of autophagic markers. Both *ATG5* (Fig. 5C) and *ATG7* (Fig. 5D) which are involved in autophagosome expansion were significantly increased. The *LC3* receptor, which recruits mitochondria into autophagosomes, was also up-regulated (Fig. 5E). It is of interest that the increased transcription of all three tested markers correlated with an increase in FA concentrations, with peak mRNA levels at the IC_{50} concentration.

4. Discussion

The immune system is responsible for defense against pathogens. The liver is considered an immune competent organ owing to its blood supply and cell populations. The constant exposure of the liver to blood-borne pathogens makes it highly susceptible to inflammatory stimuli (Jenne and Kubes, 2013). Inflammasomes are multiprotein complexes that function as innate immune sensors with NLRP3 being the best characterized. Excessive inflammasome activation results in hepatocyte pyroptosis, inflammation and fibrosis (Wree et al., 2014) while loss of inflammasome activation results in decreased IL-1β processing, attenuating the inflammatory response. Further, decreased NLRP3 has been observed in hepatocellular carcinoma patients (Wei et al., 2014).

FA, a niacin related compound and known chelator of divalent

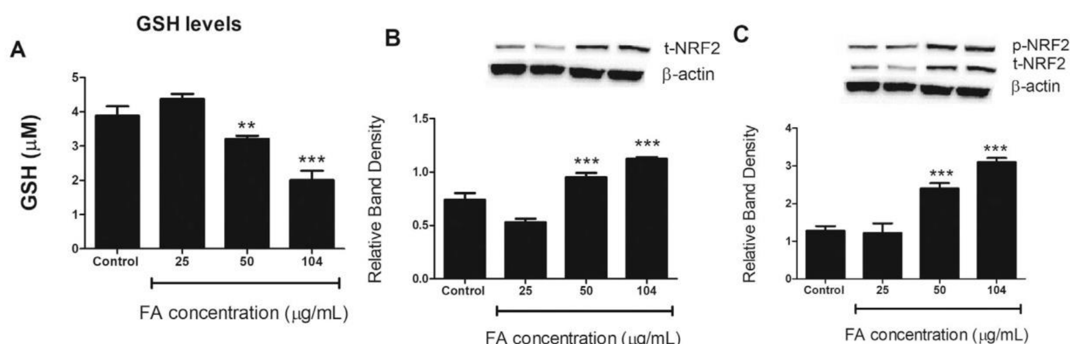

Fig. 4. Depletion of GSH triggers oxidative stress and the NRF2 pathway. FA was shown to diminish intracellular GSH (A) and induce accumulation (B) as well as transcriptional activation of NRF2 (C). **t-NRF2 – total NRF2, p-NRF2 – phosphorylated NRF2.** Results are expressed as mean and SD ($n = 3$). *- control *vs* FA μg/mL $p = .01–0.05$, ** - control *vs* FA μg/mL p 0.001–0.05, *** - control *vs* FA μg/mL $p < .001$.

cations (Stack Jr et al., 2004; Yin et al., 2015) is cytotoxic to several human cell lines (Abdul et al., 2016; Devnarain et al., 2017; Ogata et al., 2001). The canonical mechanism of action associated with FA is generation of ROS and disruption of mitochondrial function (Abdul et al., 2016; Telles-Pupulin et al., 1998). Recently, FA was shown to cause downregulation of the mitochondrial stress response proteins: SIRT3 and LON, leading to oxidative stress in human liver cells (Abdul et al., 2016).

Mitochondria are the metabolic hub of cells and function as regulators of the innate immune response. Damaged mitochondria produce excessive ROS and release oxidized mtDNA which act as DAMPs to initiate NLRP3 assembly and activation - eliciting a pro-inflammatory response (Nakahira et al., 2011; Zhou et al., 2011).

Our results showed FA significantly decreased expression of pro-IL-1β and mature IL-1β (Fig. 1D and E). A study on rat liver hepatocytes showed that IL-1β is the major inducer of nitric oxide (NO) (Kitade et al., 1996). Further, IL-1 promotes NF-κB activation by phosphorylation (Beg et al., 1993). Thus, FA down–regulates one of the critical pro-inflammatory cytokines of the innate immune system.

The synthesis and maturation of IL-1β is tightly regulated by a two-step mechanism. The transcription of IL-1β is controlled by NF-κB. In the inactive state, NF-κB is sequestered in the cytoplasm by its inhibitor, IκB. The activation and nuclear translocation of NF-κB requires the phosphorylation and degradation of IκB by IKKβ. FA up-regulated the mRNA expression of the transcriptionally active p65 subunit (Fig. 2B) but decreased the expression of activated p65 (Fig. 2A). Phosphorylation at S536 is required for p65 nuclear translocation and acetylation in the nucleus resulting in NF-kB activation (Beg et al., 1993; Cogswell et al., 1994).

To validate NF-κB p65 non-phosphorylation, we evaluated the

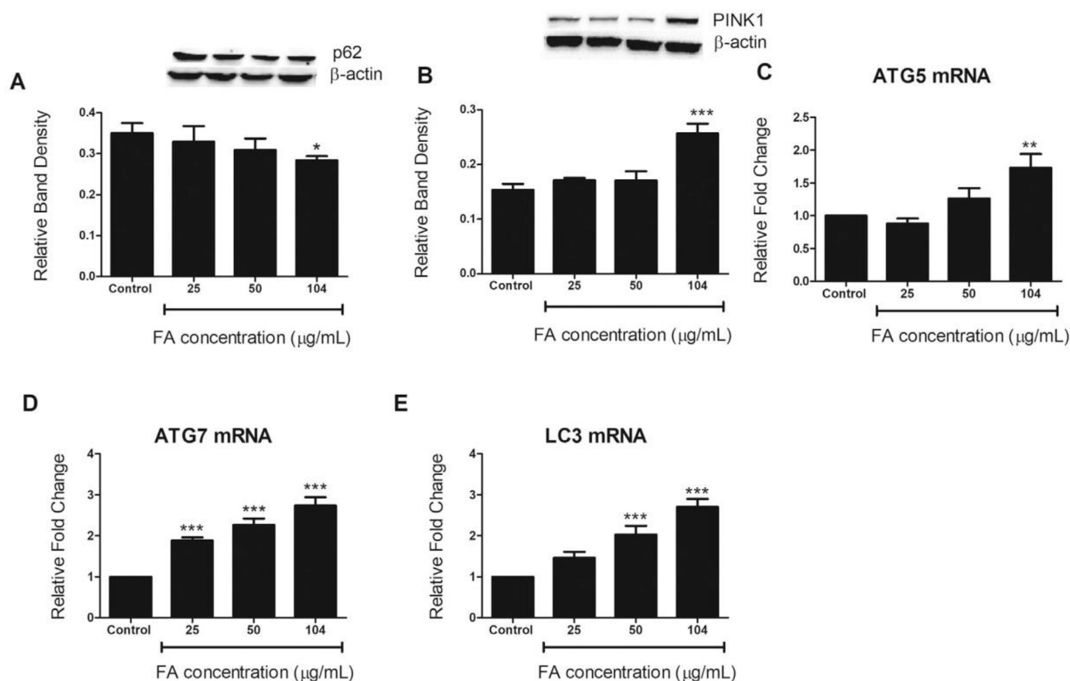

Fig. 5. Autophagy/Autophagosome and mitophagy markers were elevated by FA. The p62 sequestersome showed significant decreases (A) while the mitophagy marker PINK1 was significantly increased (B). FA increased expression of autophagy markers: *ATG5* (C), *ATG7* (D) and *LC3* (E). Results are expressed as mean and SD ($n = 3$). *- control *vs* FA μg/mL $p = .01–0.05$, ** - control *vs* FA μg/mL p 0.001–0.05, *** - control *vs* FA μg/mL $p < .001$.

effects of FA on IKKβ activation. Numerous up-stream adaptor molecules are recruited for IKKβ phosphorylation. IRAK-4 activates other IRAK family members including IRAK-1. The IRAKs then dissociate from myd88 and interact with TRAF6 to mediate IKKβ phosphorylation (Lawrence, 2009). FA inhibited IKKβ activation as evidenced by its decreased protein expression (Fig. 2C). Up-stream adaptor molecules and kinases recruited for IKKβ phosphorylation revealed significant decreases to TRAF6, IRAK1 and IRAK4 by FA (Fig. 2 D, E and F). TRAF6 is an essential adaptor molecule required in TLR signalling cascades. The really interesting new gene (RING) domain and first zinc finger of TRAF6, coordinate signalling by IL-1. FA is a known chelator of divalent ions, including zinc (Stack Jr et al., 2004), thus the chelation of zinc from TRAF6 may result in impaired signalling cascades. Taken together this data suggests FA exerts an inhibitory effect on IKKβ activation through down-regulation of up-stream kinases as well as aberrant TRAF6 signalling. Further, the NAD+ precursor, nicotinamide (NAM) was shown to down regulate the constitutive levels and translocation of NF-κB (Crowley et al., 2000; Chen et al., 2012). FA is a structural analogue of NAM. We previously reported that FA and NAM exert similar effects *in vitro* (Abdul et al., 2016). These results strongly suggest that FA abrogates NF-κB at the post-translational level.

The maturation of IL-1β is regulated by the NLRP3 inflammasome, a multiprotein platform that activates caspase 1. This inflammatory caspase cleaves the IL-1β zymogen into a biologically functional protein (Latz et al., 2013; He et al., 2016). NLRP3 inflammasomes require a priming step in which the components of this inflammasome are up-regulated. The transcription factor NF-κB is responsible for the expression of *NLRP3* and *caspase 1 (*Connat et al., 2018*;*Bauernfeind et al., 2009*)*. Analysis of gene transcripts for *NLRP3* and *caspase 1* showed FA to decrease these inflammasome components (Fig. 1B and C). These results can be attributed to a loss of transcriptionally active NF-κB (Fig. 2A). Furthermore, FA inhibited the activation of caspase 1 (Fig. 1A). Caspase 1 activation is an essential mediator of inflammasome function and its activity is considered a marker of inflammasome activation (O'Brien et al., 2017). This further lends support to NLRP3 dysfunction and aberrant IL-1β processing by FA.

An inflammatory response initiated by NLRP3 can be triggered by a variety of stimuli including host DAMPs, the majority of which are linked to mitochondrial damage and dysfunction with subsequent generation of ROS. Mitochondrial dysfunction is linked to ROS production due to the incomplete reduction of molecular oxygen by the electron transport chain (ETC) of the oxidative phosphorylation (OxPhos) complexes; mtROS damages macromolecules including mtDNA. The susceptibility of mitochondria to stressors have led to the evolution of a set of conserved machinery, known as the mitochondrial quality control (MQC) system, which sustains mitochondrial integrity and function (Bohovych et al., 2015). Mitochondrial protein homeostasis is maintained by a family of chaperone proteins (heat shock proteins, HSPs). The molecular stress protein HSP60 is up-regulated in response to mitochondrial impairment and plays a role in correct mitochondrial protein folding (Pellegrino et al., 2013). FA increased the expression of HSP60 (Fig. 3A), considered a good marker for mitochondrial stress. The proper folding of proteins is an ATP dependent process; FA interferes with the function of this protein since it depletes intracellular ATP levels. Further, FA was shown to down regulate the expression and acetylation of LON (Abdul et al., 2016), a key protease required for the degradation and removal of oxidatively modified or aggregated proteins in the mitochondria (Gibellini et al., 2014). These results validate the mitotoxic potential of FA.

Induction of mitochondrial dysfunction leads to oxidative stress and enhanced expression of UCP2, a negative regulator of ROS (Mao et al., 2017). FA decreased UCP2 expression (Fig. 3B), which compromises mitochondrial homeostatic response to ROS and exacerbates oxidative stress (Mao et al., 2017). FA impairs mitochondrial function as indicted by a higher percentage of depolarised mitochondria in treated cells (Fig. 3F). Such perturbations to the mitochondrial membrane can be a result of the acidic nature of FA which uncouples the ETC and can cause ROS production. Mitochondrial transcription factor A (TFAM) maintains mitochondrial homeostasis by regulating and maintaining mtDNA. The close proximity of mtDNA (that exists as circular DNA and not bound to histone proteins) to sites of ROS generation makes it highly susceptible to oxidative damage (Shokolenko et al., 2009). TFAM forms nucleoid-like structures when bound to mtDNA, protecting mtDNA from damage caused by ROS (Alam et al., 2003; Kang and Hamasaki, 2005). FA increased the protein expression of TFAM suggesting cellular defense against oxidative stress after toxic insult. The overexpression of TFAM may be due to downregulation of the ATP dependent LON protease (Abdul et al., 2016), which selectively degrades TFAM (Matsushima et al., 2010). Oxidized mtDNA directly binds to the NLRP3 inflammasome causing its activation. Our findings suggest a mechanism through which FA dampens inflammasome activation through accumulation of TFAM and confers resistance of mtDNA to ROS mediated damage.

The most common ROS produced by the mitochondria is superoxide (O^2). A key component of the mitochondrial anti-oxidant response is the enzyme SOD2, which catalyzes the conversion of O^2 to hydrogen peroxide (H_2O_2). Excess mitochondrial ROS is a potent inducer of oxidative damage within the organelle itself. FA increased the expression of SOD2 at both the protein (Fig. 3C) and transcript levels (Fig. 3D). SOD2 expression is regulated by the activation of NF-κB (Xu et al., 1999; Kiningham et al., 2008). These results are in contradiction with our findings as we showed elevated SOD2 expression despite a decrease in NF-κB (Fig. 2A and B). We therefore explored the possibility of NRF2 being an alternate transcriptional regulator of SOD2 in response to FA. Many studies have confirmed that the Keap1/NRF2/ARE redox-sensitive signalling system increases SOD2 expression (Gwarzo, 2009; Vnukov et al., 2015). The binding of NRF2 to the ARE, leads to transcriptional activation of anti-oxidant enzymes making it an important transcriptional regulator of oxidative stress responses.

The ROS scavenger, GSH, was assessed as a measure of cellular antioxidant status. FA drastically decreased GSH levels (Fig. 4A), leading to an oxidative environment that is in agreement with previous studies (Abdul et al., 2016; Devnarain et al., 2017). The increased levels of intracellular ROS, modify the cysteine residues on KEAP1 causing a conformational change that triggers the release of NRF2 (Dinkova-Kostova et al., 2005). FA induces the accumulation of NRF2 in cells (Fig. 4C) and is in agreement with our previous findings (Abdul et al., 2016). Furthermore there was an increase in the phosphorylation of NRF2 (Fig. 4B), a post-translational modification that results in transcriptional activation of NRF2 (Huang et al., 2002). A study by Dinkova-Kostova et al. (2005) showed that the use of metal chelators inhibit KEAP1 from binding optimally to NRF2 (Dinkova-Kostova et al., 2005). Thus, the up-regulation of NRF2 by FA could be through conventional ROS signalling as well as the potential removal of zinc from its repressor KEAP1. Taken together, these results show that FA prevents the release of DAMPs from mitochondria despite its evident and established mitotoxicity.

Recent studies have proposed enhanced mitophagy as a quality control process to negatively regulate NLRP3 inflammasome activation (Zhong et al., 2016; Nakahira et al., 2011; Zhou et al., 2011). Postulating that the anti-inflammatory effect of FA is mediated through removal of damaged mitochondria, we investigated the role of NRF2 gene products involved in autophagy and mitophagy. Sequence analysis have indicated the existence of ARE in the promoter regions of p62 (Jain et al., 2010) and PINK1 (Murata et al., 2015). Both these proteins which play a key role in tagging degrading damaged mitochondria to prevent release of DAMPs.

The p62 sequestersome is a multifunctional signalling scaffold and adaptor that binds polyubiquitinated proteins and damaged organelles, marking them for autophagosomal clearance (Zhou et al., 2011). Our data revealed a strong decrease in expression of p62 at the IC_{50} concentration of FA (Fig. 5A), and this is in agreement with the established

degradation of p62 after activation of autophagy.

Proteins belonging to the family of autophagy related genes (ATGs) co-ordinate the expansion of the phagophore to form the autophagosome (16). p62 has been proposed to mediate recruitment of damaged mitochondria to autophagosomes by binding to microtubule-associated protein 1A/1B-light chain 3 (LC3) (Ashrafi and Schwarz, 2013). We therefore examined the mRNA expression of *LC3* along with the expression of *ATG5* and *ATG7* (which are required for phagophore expansion and autophagosome formation). FA significantly increased all three markers of autophagy (Fig. 5). These results along with the decrease in p62 suggest FA up-regulates autophagy as a possible cell defense mechanism.

We then investigated the effects of FA on mitophagy. The serine/threonine kinase PINK acts as a molecular sensor of mitochondrial health. Under basal conditions PINK1 is degraded in the mitochondria. However, upon detection of mitochondrial dysfunction PINK1 selectively accumulates on damaged mitochondria where it signals for the recruitment and activation of Parkin. Parkin functions as an E3- ubiquitin ligase to recruit additional proteins necessary for removal of damaged mitochondria (Nguyen et al., 2016). FA significantly increased the expression of PINK1 (Fig. 5B) suggesting that PINK1/Parkin mediated clearance of damaged mitochondria is initiated and this prevents the accumulation of toxic mitochondrial products that would normally activate NLRP3.

Wang et al. (2012) showed that over expression of SOD2 prevents mitophagy (Wang et al., 2012). We show that FA enhanced the expression of mitophagy markers despite elevated SOD2 expression. At the posttranslational level, deacetylation of this enzyme by SIRT3 is essential for its activation (Chen et al., 2011). A recent study by Pi et al. (2015) showed that promotion of SOD2-SIRT3 interactions can ameliorate mtROS associated mitophagy (Pi et al., 2015). FA induced downregulation of SIRT3 at both the protein and transcript levels (Abdul et al., 2016). Furthermore, the manganese ion present in SOD2 is critical for its enzymatic activity, thus, chelation of this ion by FA would result in loss of enzyme activity. This will then promote mitochondrial ROS production leading to mitophagy.

The initial part of this study showed that the anti-inflammatory effect of FA was exerted through a decrease in NLRP3 inflammasome priming. The plausibility of the NRF2 mediated cytoprotective response in dampening of NLRP3 inflammasome activation was investigated in the latter part of the study. Loss of NLRP3 priming and activation provides evidence for the immunosuppressive effects of FA. The implications of this study extend beyond that of immunosuppression. Many studies highlight the protective effects of NRF2 (reviewed in (Ma, 2013)), however the excessive and prolonged expression of NRF2 has been shown to favour cancer progression (Jaramillo and Zhang, 2013). Furthermore, severe downregulation of NLRP3 was found in cases of hepatocellular carcinoma. NRF2 is responsible for the transcription of p62. A higher level of active NRF2 elevates the expression of p62. A dysfunctional autophagy and NRF2 regulatory mechanism has been identified in HCC. This occurs through the physical interaction of p62 with the NRF2 inhibitor Keap1, thus leading to increased stabilization and transcriptional activity of NRF2, an important reprogramming of metabolic and stress response pathways that can contribute to HCC (Bartolini et al., 2017). However, further experimentation is needed to confirm the carcinogenic potential of FA.

5. Conclusion

Despite the health hazards of fusariotoxins in food safety and high levels of mycotoxin exposure, FA remains neglected in terms of research and its molecular mechanisms of its toxicity is not well established. These results provide evidence that FA decreased NF-κB transcriptional activity, but more importantly upregulation of NRF2 is needed for the anti-inflammatory effects of FA by a mechanism that prevents inflammasome priming and activation in the liver derived HepG2 cells. These results reveal a novel mechanism of immune dysregulation by FA.

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Conflict of interest

None.

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CHAPTER 4

The neglected foodborne mycotoxin Fusaric acid induces bioenergetic adaptations by switching energy metabolism from mitochondrial processes to glycolysis in a human liver (HepG2) cell line

Mitochondrial toxicity and dysfunction is often associated with aberrant energy metabolism and can result in metabolic switching. This metabolic switch and subsequent metabolic inflexibility is a characteristic of the Warburg effect and cancer cells. In this chapter liver cells were supplemented with different carbon energy substrates to determine the effects of FA on mitochondrial ATP output. A shorter time period (6 hours) was selected for this study to determine mitochondrial toxicity in the absence of overt cytotoxicity. Galactose and palmitate were used as alternate fuel carbons to stimulate OxPhos and β–oxidation respectively. Fusaric acid rendered glucose as the preferred energy substrate and induced a phenotype reminiscent of the Warburg effect in HepG2 cells through constitutive up-regulation of HIF-1α and subsequent inhibition of PDH.

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The neglected foodborne mycotoxin Fusaric acid induces bioenergetic adaptations by switching energy metabolism from mitochondrial processes to glycolysis in a human liver (HepG2) cell line

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Abstract:

Metabolic flexibility defines the capacity of cells to respond to changes in nutrient status. Mitochondria are important mediators of metabolic flexibility and dysfunction is associated with metabolic inflexibility and pathology. Foodborne toxins are often overlooked as potential factors contributing to metabolic toxicity. Fusaric acid (FA), a neglected mycotoxin, is known to disrupt mitochondrial function. The aim of this study was to investigate the molecular mechanisms underlying a metabolic switch in response to FA. This study investigated the effects of FA on energy homeostasis in cultured human liver (HepG2) cells. HepG2 cells poised to undergo oxidative and glycolytic metabolism were exposed to a range of FA concentrations (4, 63 and 250 µg/mL) for 6 hours. We determined mitochondrial toxicity, acetyl-CoA levels and cell viability using luminometric, fluorometric and spectrophotometric methods. Expression of metabolic proteins (PDK1, PKM2, phosphorylated-PDH E1α and HIF-1α) and mRNAs (HIF-1α, PKM2, LDHa and PDK1) were determined using western blot and qPCR respectively. Our data connects a constitutive expression of HIF-1α in response to FA to the inhibition of pyruvate decarboxylation through up-regulation of PDK-1 and phosphorylation of Pyruvate Dehydrogenase E1α subunit. Moreover, we highlight the potential of FA to induce a glucose "addiction" and phenotype reminiscent of the Warburg effect. The findings provide novel insights into the impact of this neglected foodborne mycotoxin in the dysregulation of energy metabolism.

Keywords: Metabolic inflexibility

Mitochondria

Pyruvate dehydrogenase complex

Warburg effect

1. Introduction

The contamination of plants with mycotoxins is a growing problem throughout the world. Mycotoxins are a heterogeneous group of compounds that exert toxic effects in vertebrates. Of particular concern are the Fusarium toxins which are considered the most frequently occurring foodborne mycotoxins worldwide [1-3]. Fusaric acid (5-butylpicolinic acid, FA) is an often "neglected" polyketide derived secondary metabolite [4]. This mycotoxin is of interest because of its ubiquitous production by several *fusarium* species that commonly contaminate agriculturally important crops such as corn [5], with amounts as high as 643 µg/kg of sample being documented [6]. FA is a natural chelator of divalent cations [7,8] in addition to its associated plant pathogenicity [9,10] and reported "mild" toxicity in animals [11,12]. FA shows strong synergism with co-produced fusariotoxins [13-15], while FA alone results in teratogenicity [8,16] and overall cytotoxicity in recent studies on human derived cell lines [17-19]. Although exact mechanisms of FA toxicity are yet to be elucidated, human *in vitro* models have highlighted genomic, oxidative and mitochondrial toxicity [17,19,20]. Despite the established mitochondrial toxicity of FA in vertebrates [17,21] little is known about its effects on cellular energy metabolism.

Mitochondria regulate important energy processes in response to nutrient availability, thus making them key role players in metabolic flexibility (capacity to switch between energy substrates) [22]. Most cells exhibit a degree of metabolic flexibility to meet bioenergetic demands and cope with changes to the cellular environment [23]. The pathologic potential of such shifts is exemplified by the switch from mitochondrial oxidative phosphorylation (OxPhos) to aerobic glycolysis (Warburg effect) which is strongly associated with a cancer cell phenotype and aberrant proliferation [24,25].

The metabolic program is an active modulator of cellular phenotype [26] and changes to the metabolic program can influence multiple signalling cascades by providing or limiting substrates for cell protection or death [27]. Hypoxia inducible factor-1 (HIF-1) has been implicated as a key regulator of cancer cell glucose metabolism by upregulating expression of glycolytic enzymes and their activity. These enzymes result in the shunting of pyruvate away from the tricarboxylic acid cycle (TCA) and towards glycolysis via inhibition of the mitochondrial pyruvate dehydrogenase complex (PDC), leading to accumulation of lactate [28,29]. This multi-enzyme complex links glucose metabolism, fatty acid metabolism and the TCA cycle by acting as the branching point between substrate production and utilisation to maintain metabolic homeostasis. Dietary bioactive compounds are known to have

profound therapeutic effects by mitigating metabolic inflexibility [30], however very little is known regarding common foodborne toxins and their roles in metabolic disease initiation and progression.

Evidence suggests that mammalian cells redirect their energy metabolism in response to available carbon sources in culture media [31]. Cells cultured in galactose as the main fuel source are forced to rely on mitochondrial OxPhos to generate ATP since no net ATP is generated from galactose metabolism. Hence evaluation of mitochondrial dysfunction using comparative effects between glucose and galactose can demonstrate sensitization to mitochondrial toxins such as FA [32]. Consistent with this methodology we sought to determine the underlying molecular mechanisms of FA on energy metabolism in the liver derived HepG2 cell line.

2. Results

2.1. FA impairs mitochondrial function without overt necrosis

A compound is considered a potential mitochondrial toxin if it decreases the ATP measure by greater than 20% of the average of control cells with less than a 20% increase in cytotoxicity. To assess loss of mitochondrial function in response to FA, HepG2 cells were cultured under glycolytic and aglycolytic conditions. Immortalised cells grown in glucose rich media derive their ATP from glycolysis and by-pass OxPhos. By substituting glucose with galactose, cells are forced to use OxPhos to meet their ATP demands. These cells are aerobically poised and more susceptible to mitochondrial toxins. This is reflected in the severe decrease of ATP levels in the GAL supplemented media (Fig 1C), when compared to ATP levels in glucose supplemented cells which remained above 50% (Fig 1A and 1B) post FA exposure. To validate mitochondrial toxicity as a measure of intracellular ATP, HepG2 cells were incubated under the same metabolic conditions in the presence of the F_0-F_1 ATP synthase inhibitor, oligomycin A. Treatment with oligomycin A affirmed the mitotoxic potential of FA by having a more drastic effect on GAL using cells as compared to glucose supplemented cells (Fig 1A-1C). We further show that a relationship exists between the dose of FA and mitochondrial toxicity (ATP output via OxPhos). The mitotoxic dose of FA is much lower in aerobically poised cells (Fig 1C) than in cells primarily deriving ATP from glycolysis (Fig 1A and 1B).

The simultaneous assessment of intracellular ATP levels and cell membrane integrity (marker for cellular toxicity) is a good indicator of early mitochondrial dysfunction before overt necrosis. Cellular cytotoxicity was not observed under any of the tested culture conditions regardless of the

FA dose (Fig 1A-1C). Taken together these data reveal that FA induces considerable mitochondrial dysfunction without overt necrosis in all tested energy substrate models (Fig. 1A-1C).

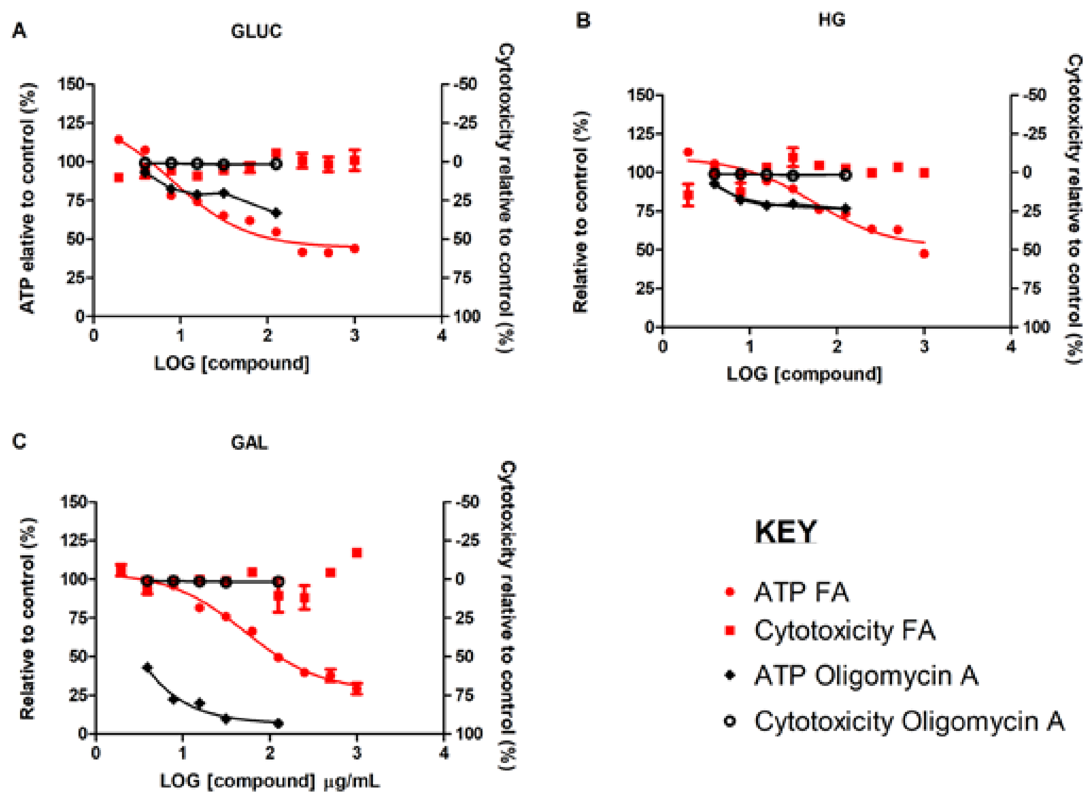


Fig. 1 The effect of FA on ATP levels and cytotoxicity in HepG2 cells. Serial concentrations of FA (µg/mL) and oligomycin A (µM) were tested in GLUC (A), HG (B) and GAL (C) supplemented media. FA inhibited ATP production in a dose dependent manner without causing overt cytotoxicity. The reduction in ATP with no loss to membrane integrity indicates that the compound is a mitochondrial toxin. The values are expressed as a percentage of the control ± SD (n=3).

2.2. FA influences cytoplasmic NAD(P)H

The WST-1 assay was used to quantify cytoplasmic NADPH generated under the selected substrate conditions. $NADP^+$ is a required co-factor for glucose-6-phosphate generation via glucose-6phosphate dehydrogenase (an early event of glycolysis) [33,34]. Cells cultured in glycaemic media (GLUC and HG) were stimulated to undergo glycolysis (as indicated by absorbance readings above

those seen in respective controls) regardless of FA dose. In contrast cells poised to undergo OxPhos showed increased NADPH levels only at mid to high FA concentrations (63 µg/mL - 250 µg/mL). These observed effects are considered biphasic with a low dose stimulatory effect and a high dose inhibitory effect (Fig 2A) that may be dependent on FA toxicodynamics (recovery equals injury) and toxicokinetics (elimination equals absorption). To validate this hypothesis and rule out mitochondrial reducing equivalent metabolism under tested substrate conditions, Rotenone, a mitochondrial complex 1 inhibitor was used. NADPH in glucose supplemented cells incubated with rotenone for 6 hrs was elevated when compared to OxPhos (GAL) stimulated cells (Fig 2B). This suggests enhanced glycolytic rates in response to mitochondrial toxins and maintenance of ATP levels. This provides evidence that FA shifts metabolism to a more glycolytic endpoint. Furthermore, the response to rotenone plateaus after the initial inhibitory concentration further alludes to a biphasic FA response.

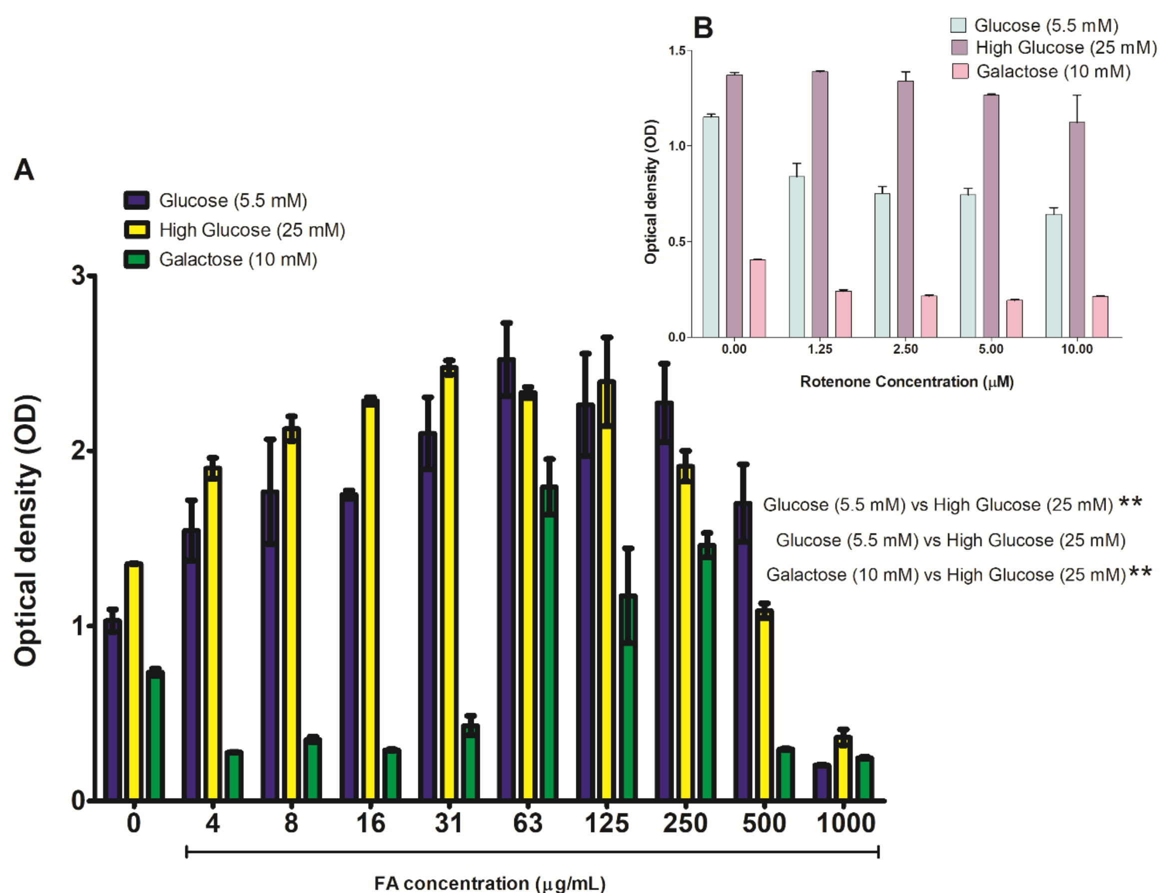


Fig. 2 Cytoplasmic levels of NADPH in HepG2 cells supplemented with GLUC, GAL and HG after treatment with varying FA concentrations. FA induces a biphasic response in which NADPH generation is characterised by low dose stimulation and high dose toxic effect. (A). Rotenone drastically depleted NADPH levels in cells treated with GAL (B). Values are expressed as mean OD ± SEM (n=3).

Key

**p<0.01

2.3. FA facilitates metabolic flux away from the TCA cycle

In glycolysis glucose is metabolised to pyruvate in the cytoplasm. Pyruvate can be metabolised either to lactate (augmenting glycolysis), or acetyl-CoA in the mitochondrion by PDH to serve as a substrate for the TCA cycle. Having established that FA is a mitotoxin, we investigated the fate of pyruvate by quantifying acetyl-CoA and lactate concentrations. FA treated cells showed a significant, dose dependent decrease in acetyl-CoA upon supplementation with GLUC and GAL (Fig 3A and 3C, $p < 0.0001$) when compared to control. However, acetyl-CoA in HG supplemented cells showed minimal changes (Fig 3B). It is noteworthy that the PDH activator, DCA significantly elevated acetyl-CoA levels in all tested models (Fig 3A-3C). Analysis of lactate levels revealed FA to induce an increase in glycolytically poised HepG2 liver cells (GLUC $p = 0.0042$ and HG $p = 0.0009$, Fig 3D and E). Moreover, stimulation of OxPhos did not prevent FA from elevating lactate production ($p < 0.0001$, Fig 3F).

We then determined whether the changes to acetyl-CoA and lactate levels were the result of a corresponding inhibition of PDH. Phosphorylation of PDH E1α (S293) is important for down regulation of enzyme activity. FA significantly increased phosphorylation of the PDH E1α subunit in a dose dependent manner compared to untreated cells (GLUC $p < 0.0001$, HG $p < 0.0001$, GAL $p < 0.0001$) while PDH phosphorylation was reduced in DCA treated cells in GLUC and GAL, thus strongly indicative of a glycolytic switch. In contrast, DCA induced a high degree of PDH phosphorylation in HG ($p < 0.0001$, Fig 3F-3I).

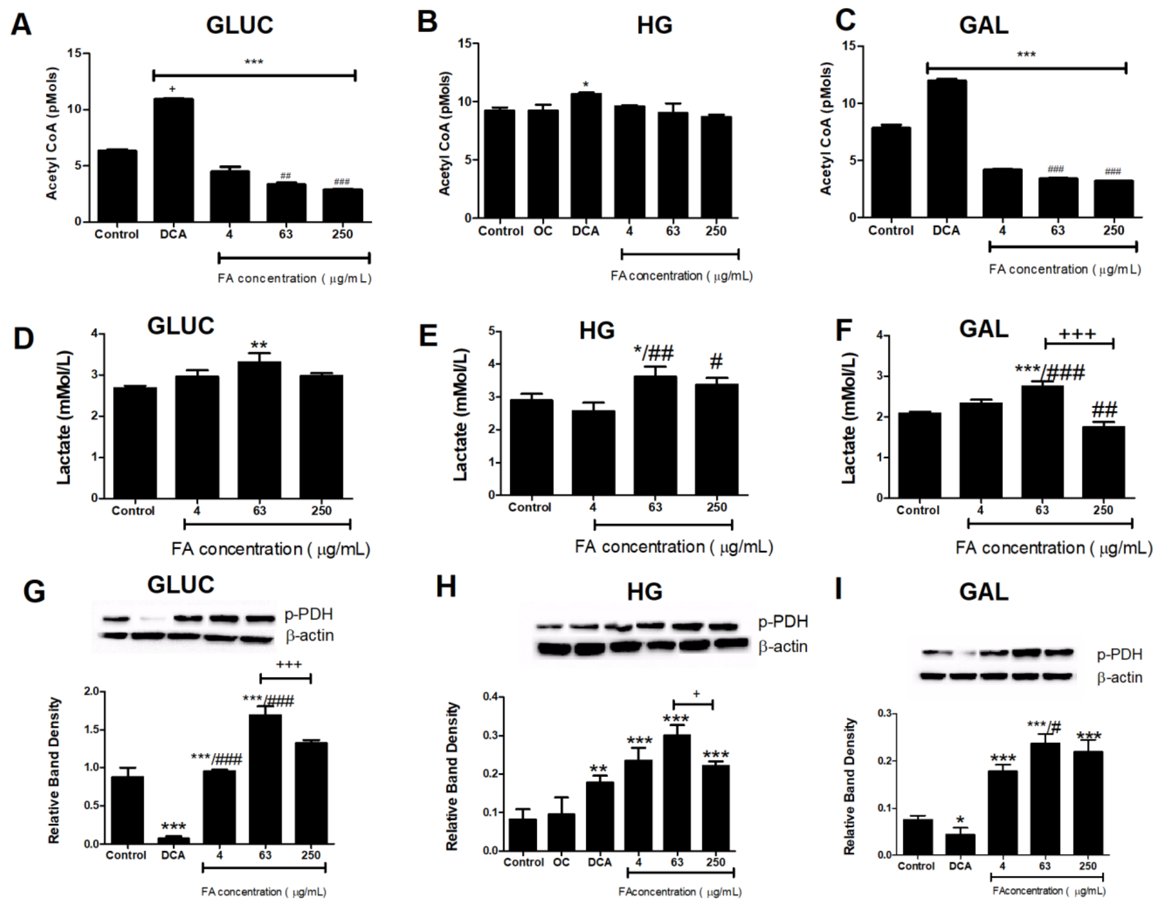


Fig. 3 FA attenuates pyruvate decarboxylation. A dose dependent decrease in acetyl-CoA levels was observed in FA treated cells (A-C) along with elevated lactate levels (D-F). Western blot detection of serine 293 phosphorylation showed FA increased expression of inactive PDH E1α (G-I). The results are expressed relative to the control ± SD (n=3).

Key

vs control ***p< 0.001, **p<0.01, *p<0.05

4 μg/mL *vs* 63 μg/mL, 4 μg/mL *vs* 250 μg/mL $^{+}$p< 0.001, $^{++}$p<0.01, $^{+}$p<0.05

63 μg/mL *vs* 250 μg/mL $^{###}$p< 0.001, $^{##}$p<0.01, $^{#}$p<0.05

2.4. FA mediated induction of HIF-1α

Given the role of HIF-1α in glucose metabolism and glycolytic switch we investigated the expression of this transcription factor at both mRNA and protein levels. A basal level of HIF-1α under normoxic conditions was observed in untreated cells and served as a control to which FA stimulated cells could be compared. qPCR analysis revealed mRNA levels of *HIF-1α* to be dose

dependently increased in response to FA. However, only at a concentration of 250 μg/mL was statistical significance observed across all tested energy substrates ($p < 0.0001$, Fig. 4A-4C).

Western blot analysis was performed to investigate the stability of HIF-1α in response to FA under normoxic conditions. In GLUC supplemented cells FA elevated the expression of HIF-1α at low concentrations but this was reversed at 250μg/mL of FA ($p < 0.0001$, Fig. 4D). Moreover, supplementation with GAL and HG effectively increased the expression of HIF-1α in a dose dependent manner ($p < 0.0001$, Fig. 4E and 4F). Taken together these data indicate FA promotes HIF-1α expression (mRNA) and stability (protein) under normoxia.

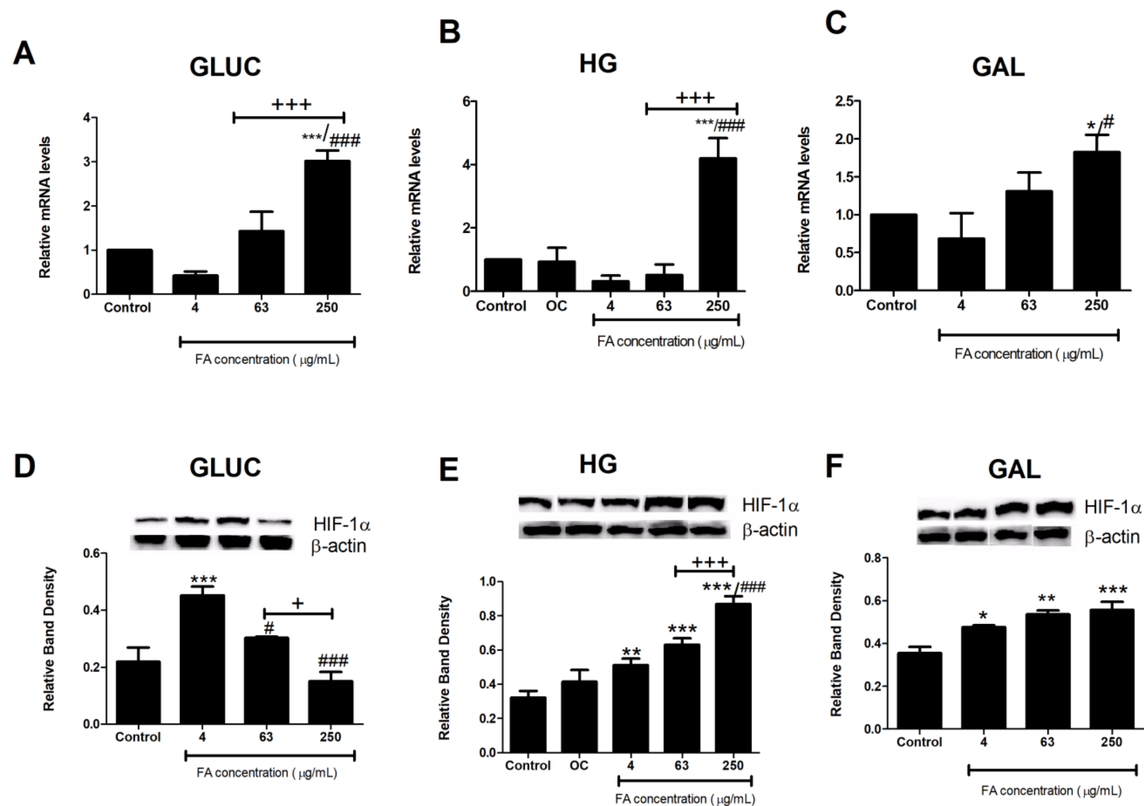


Fig. 4 Effects of FA on HIF-1α expression and stability under normoxia post FA exposure. *HIF-1α* mRNA (expression) was assessed with qPCR (A-C). HIF1-α protein expression (stability) was assessed by western blot (D-F). FA induces HIF-1α accumulation under the tested substrate conditions. The results are expressed relative to the control ± SD (n=3).

Key

vs control ***$p < 0.001$, **$p<0.01$, *$p<0.05$

4 μg/mL *vs* 63 μg/mL, 4 μg/mL *vs* 250 μg/mL $^{+}p < 0.001$, $^{++}p<0.01$, $^{+}p<0.05$

63 µg/mL *vs* 250 µg/mL $^{###}p< 0.001$, $^{##}p<0.01$, $^{#}p<0.05$

2.5. FA dampens kinase expression

The phosphorylation and activity of PDC is regulated by PDH kinase 1 (PDK1). To examine the effects of FA on PDC regulation qPCR and western blot was performed to determine the expression profiles of PDK1. At the transcript level FA increased *PDK1*at the two highest concentrations in GLUC model (p = 0.0003, Fig 5A). In HG, FA showed a significant increase only at 63µg/mL, while a highly significant decrease was observed at 250µg/mL relative to the control (< 0.0001, Fig 5B). In cells forced into OxPhos metabolism (GAL supplementation), PDK1 expression increased significantly at a FA concentration of 63 µg/mL (p < 0.0001, Fig 5C).

Under GLUC conditions FA increased the expression of PDK1 in a dose dependent manner, while HG supplemented cells showed steep increases at lower FA concentrations but a significant decrease at 250µg/mL (p < 0.0001, Fig 5D and 5E). In contrast, cells forced to undergo OxPhos (GAL supplemented cells) showed increased PDK1 expression only at 63 µg/mL when compared to control (< 0.0001, Fig 5F).

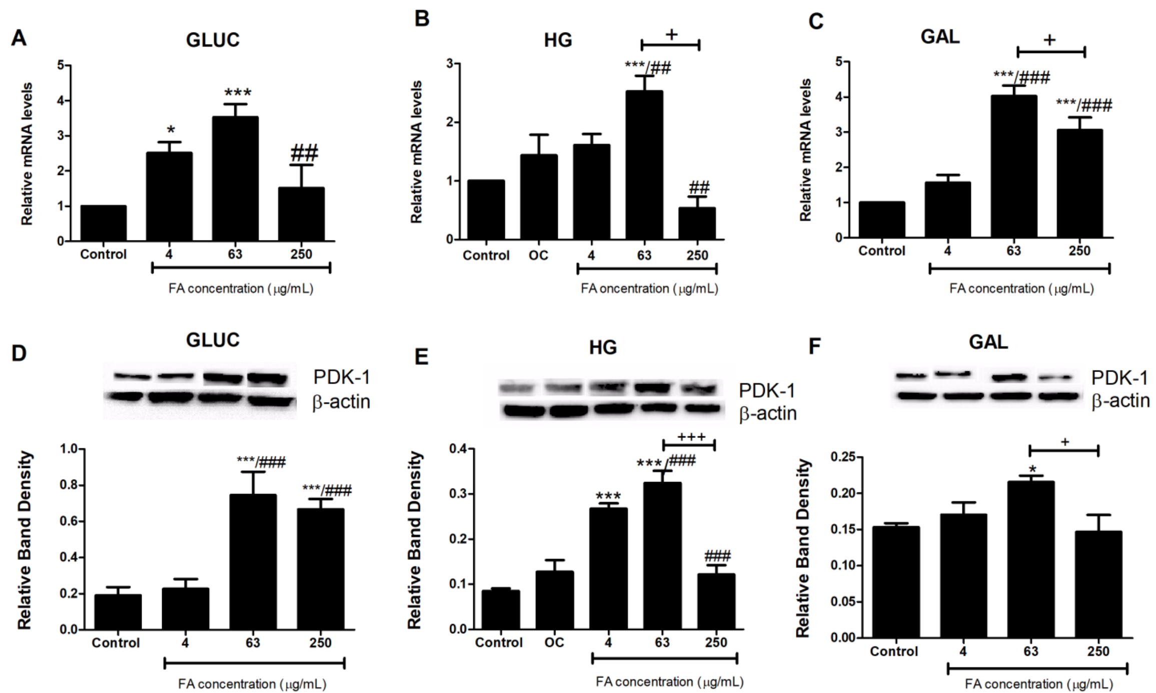


Fig. 5 Effects of FA on PDK1 protein and gene expression. FA induced a sharp increase to both mRNA (A-C) and protein levels (D-F) at 63µg/mL. In contrast strong decreases to mRNA (A-C)

and protein expression (D-F) were observed at highest FA concentration across all selected metabolic substrates. The results are expressed relative to the control ± SD (n=3).

Key

vs control ***p< 0.001, **p<0.01, *p<0.05

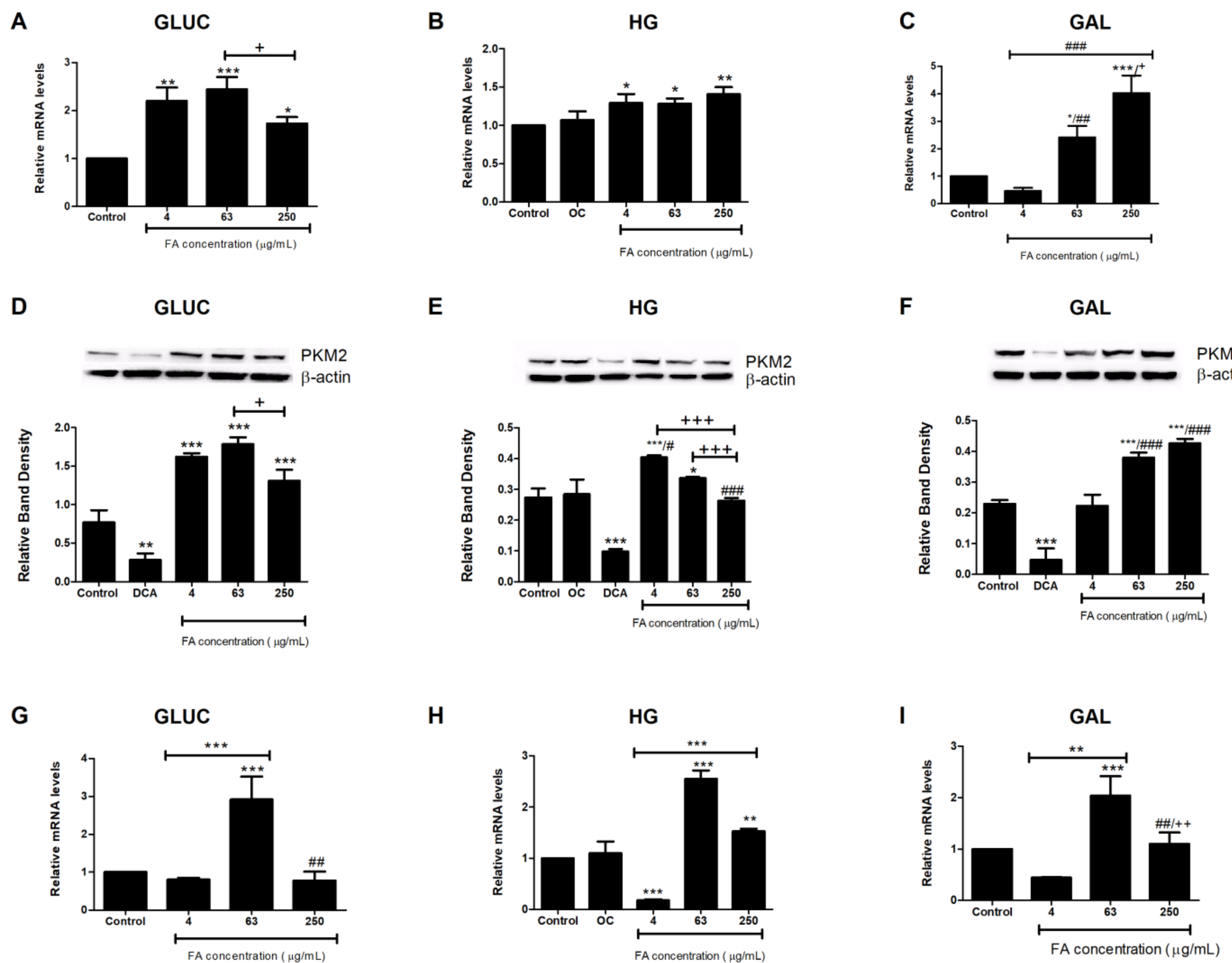
4 μg/mL *vs* 63 μg/mL, 4 μg/mL *vs* 250 μg/mL $^{+}$p< 0.001, $^{++}$p<0.01, $^{+}$p<0.05

63 μg/mL *vs* 250 μg/mL $^{###}$p< 0.001, $^{##}$p<0.01, $^{#}$p<0.05

2.6. FA shunts metabolism toward glycolytic endpoints

Given the established role of HIF-1α in circumventing pyruvate decarboxylation and OxPhos we examined targets of this transcription factor related to glycolysis induced by FA. Pyruvate kinase isoform M2 (PKM2) catalyses the final step of glycolysis. FA significantly increased gene expression of *PKM2* in all experiments relative to the control (GLUC p = 0.0002, HG p = 0.0012, GAL p < 0.0001, Fig. 6A-C). We further evaluated the effects of FA on PKM2 protein expression. In GLUC cultured cells there was increased PKM2 expression at all FA concentrations relative to the control (p < 0.0001, Fig. 6D). Cells cultured in HG showed a dose dependent decrease in PKM2 after FA stimulation; however protein expression was still higher in treated cells when compared to control (p < 0.0001, Fig. 6E). We also observed cells cultured in aglycaemic conditions (GAL) showed a dose dependent increase to PKM2 protein levels relative to the control (p < 0.0001, Fig. 6F). In contrast the PDH stimulator DCA decreased the expression of PKM2 across all culture conditions (Fig. 6D-F).

Since the intracellular fate of glucose seemed to be off set by FA we investigated the gene expression of *LDHa*, the enzyme catalysing the conversion of cytosolic pyruvate to lactate. We observed a peak in expression at 63μg/mL with strong represssion at the highest FA concentration in all culture conditions (Fig. 6G-I). These data point to a cytosolic fate for pyruvate and energy metabolism in FA treated cells.



<u>Fig. 6</u> FA enhances cytosolic energy metabolism. The effects of FA on mRNA expression (A-C) and protein expression (D-F) for PKM2 was assessed. Transcript levels of the cytosolic enzyme LDHa was also determined (G-I). The results are expressed relative to the control ± SD (n=3).

<u>Key</u>

vs control ***p< 0.001, **p<0.01, *p<0.05

4 µg/mL *vs* 63 µg/mL, 4 µg/mL *vs* 250 µg/mL $^{+}$p< 0.001, $^{++}$p<0.01, $^{+}$p<0.05

63 µg/mL *vs* 250 µg/mL $^{\#\#\#}$p< 0.001, $^{\#\#}$p<0.01, $^{\#}$p<0.05

<u>2.7 FA renders glucose as preferred metabolic substrate</u>

After confirming that FA upregulated glycolytic enzyme expression, we then investigated if cells could generate ATP through β-oxidation. For this we assessed ATP and biomass as measures of cell viability upon addition of glucose free-FFA. There was a significant dose dependent decrease in

ATP levels in palmitate supplemented cells when compared to glycaemic cultures ($p < 0.0001$, Fig 7A). Furthermore, treatment with FA under FFA and GAL conditions led to a significant and major decrease in biomass further highlighting preferential glucose utilisation in FA treated cells (GAL $p < 0.0001$, FFA $p < 0.0001$, Fig. 7B).

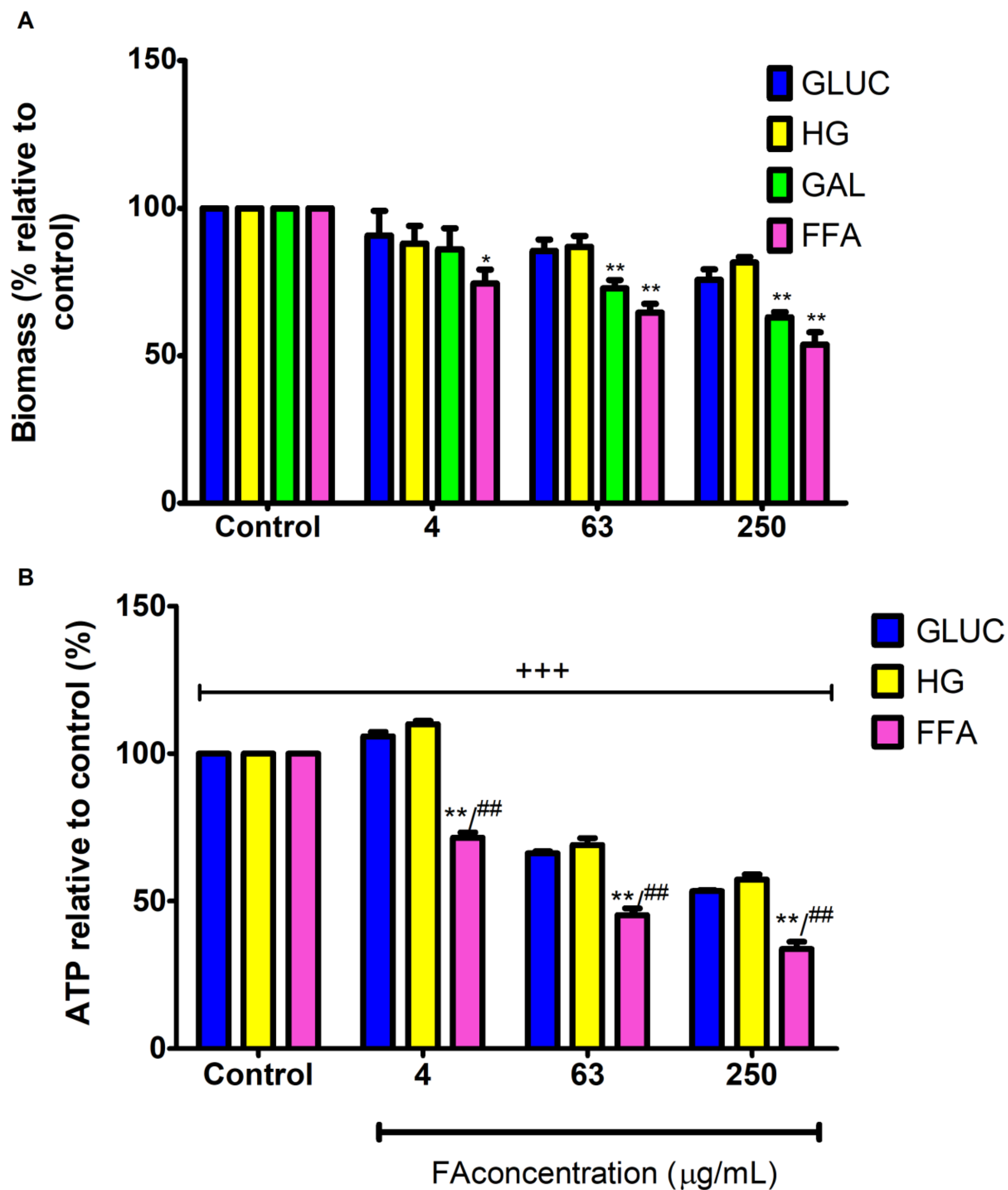


Fig. 7 Glucose dependence in the presence of FA: FA depleted ATP at higher concentrations across all energy substrates (A). Cell biomass was minimally affected under glycaemic conditions (GLUC

and HG) but severely reduced in aglycaemic media (GAL and FFA, B). The results are expressed relative to the control ± SD (n=6).

Key

vs control ***p< 0.001, **p<0.01, *p<0.05

4 µg/mL *vs* 63 µg/mL, 4 µg/mL *vs* 250 µg/mL $^{+}$p< 0.001, $^{++}$p<0.01, $^{+}$p<0.05

63 µg/mL *vs* 250 µg/mL $^{###}$p< 0.001, $^{##}$p<0.01, $^{#}$p<0.05

Discussion

We report on the impact of the *Fusarium* mycotoxin FA on mitochondrial toxicity and its outcome on cell energy metabolism in human liver (HepG2) cells. Determining mitochondrial toxicity *in vivo* is a challenge given that typical rodent models possess large respiratory capacity and that these efficiently functioning mitochondria can tolerate toxic insults [35]. Culturing of HepG2 cells in media substituted with GAL leads to circumvention of the Crabtree effect and stimulates OxPhos [32]; this model is considered relevant in determining mitochondrial toxicity and subsequent metabolic implications [32,35,36]. Using this model we investigated FA mediated glycolysis and glucose shunt at the molecular level in HepG2 cells. We show, for the first time, that FA impedes pyruvate decarboxylation while elevating expression of glycolytic enzymes and renders cells reliant on glucose to meet energy demands.

Recent *in vitro* studies showed that FA decreased intracellular ATP levels and initiated cell death after 24h [17-19]. By sequentially assessing cell membrane integrity and ATP content in OxPhos primed and glycolytic HepG2 cells after FA stimulation (6h), we demonstrate mitochondrial dysfunction in the absence of overt cytotoxicity (Fig 1). Substantial decrease in ATP concentration was observed in GAL supplemented cells when compared to glycolytic cells where ATP synthesis was maintained by glycolysis (Warburg effect). This is affirmed by the inclusion of the classic mitochondrial toxin oligomycin A. These findings are in accord with studies that highlight the primarily mitochondrial effect of FA [17,21]. Telles-Pupulin (1998) found that FA repressed mitochondrial metabolism by inhibiting key enzymes involved in the TCA cycle and OxPhos [21]. A recent study by our group showed FA to down regulate the expression of the mitochondrial deacetylase sirtuin 3, and inhibit mitochondrial biogenesis [17]. Thus FA can directly and indirectly interfere with reducing equivalent metabolism and mitochondrial ATP synthesis in OxPhos reliant cells.

Consequently, we hypothesised that the maintenance of ATP in glucose supplemented cells relative to OxPhos primed cells represents a compensatory shift toward glycolysis. WST-1 assayed cytosolic NADPH as an indirect measurement of glucose-6-phosphate dehydrogenase activity and marker of early glycolytic switch [33,34]. The glucose free GAL model revealed drastic suppression of NADPH synthesis suggesting enhanced glycolytic processing in glucose supplemented cells (Fig 2). The combined down regulation of mitochondrial ATP synthesis and up-regulation of cytoplasmic ATP synthesis further highlight the mitotoxic potential of FA.

We then investigated the intracellular fate of glucose offset by FA. Pyruvate is the end product of glycolysis and is at the branch point of cellular glucose metabolism. Cytosolic processing of glucose results in lactate production while PDH routes pyruvate to acetyl-CoA synthesis for use as a TCA cycle substrate [37]. Our results show that FA reduced acetyl-CoA levels in GLUC and GAL supplemented cells but not in those cultured under HG conditions (Fig. 3A-C). FA was shown to undergo chain elongation to form fusaryl CoA derivatives using acetyl-CoA as a C_2 donor [38] leading us to speculate that this mycotoxin can directly deplete acetyl-CoA pools. Further investigation into the effects of FA on acetyl-CoA synthesis revealed enhanced phosphorylation of PDH-E1α (Ser293) demonstrating inactivation of this important mitochondrial enzyme (Fig. 3 F-I) [39,40]. In support of our observations, the pharmacological activation of PDH by DCA showed elevated acetyl-CoA levels across all tested energy substrates [41]. Moreover, elevated lactate levels (Fig 3D-F) highlight the shunt of glucose toward cytosolic energy generation in FA stimulated cells. From these data it seems that FA facilitates glucose flux away from the TCA cycle and toward glycolysis.

Increased PDH phosphorylation under hyperglycaemic is mediated by enhanced mitochondrial deacetylation [42]. Furthermore, DCA was shown to increase both acetyl-CoA levels and protein acetylation in a mouse model [41]. These results are in line with our hypothesis that FA induces metabolic inflexibility since FA has previously been shown to downregulate sirtuin 3 (primary mitochondrial deacetylase) at protein and gene level [17].

The post translational modification of PDH was investigated by examining the expression profile of the serine kinase PDK1 by FA in cells. PDK1 dependent inhibition of PDH prevents pyruvate decarboxylation to acetyl-CoA, this results in reduced flow of glucose derived pyruvate into the TCA cycle [43]. FA elevated the transcription and translation of PDK1 in glycolytic and aglycolytic cells (Fig 5). PDK1 is considered as the most potent inactivator of PDH. PDK1 phosphorylates

PDH at three primary sites where it brings about strong phosphorylation and poor dephosphosphorylation of PDH blocking substrate (pyruvate) binding [39,44]. Furthermore, it has recently been established that PDK1 is specifically required for adaptation to metabolic stressors [43]. Based on these findings it seems FA facilitates glucose flux away from the TCA cycle and OxPhos.

Accumulating evidence shows that transcriptional activation and stabilisation of HIF-1α plays a crucial role in glycolytic metabolism and metabolic reprograming [45]. Hydroxylation of HIF-1α by iron dependent prolyl hydroxylases (PHDs) under normoxia allows the von Hippel-Lindau protein (VHL) to ubiquitinate HIF-1α for proteasomal degradation. During hypoxia, HIF-1α is saved from degradation allowing for accumulation and import into the nucleus [46,47]; FA induced HIF-1α transcription and stabilisation under normoxic conditions (Fig 4). However, transcription of HIF-1α lags behind its accumulating protein expression. These results suggest an important role for FA as a post-translational regulator given that such modifications reserved for proteins involved in rapid responses for cell adaptation to stress. In addition to oxygen dependent accumulation, loss of PHD and VHL function have been shown to induce HIF-1α stability. Iron chelators inhibit PHD and VHL by depleting Fe^{2+} allowing constitutive accumulation of HIF-1α, mimicking hypoxia [46,48]. The chelation of iron by FA is well established *in vitro* [7,49]. The nitrogen atom in the pyridine ring and the oxygen atom in the carboxyl group, may act as a chelator and form FA-iron complexes [49]. The mechanism by which FA exerts its effects *in vitro* is similar to that of established iron chelators ciclopirox olamine and deferoxamine mesylate [48].

To validate this we examined targets of the HIF-1α transcription factor. HIF-1α promotes transcription of several glycolytic enzymes including PDK1, PKM2 and LDHa [43,50,51]. FA elevated the expression of *PDK1* mRNA most likely through HIF-1α activated transcription. Aberrant expression of PDK1 is often associated with disease progression due to its pivotal role in regulating metabolic flexibility by altering glucose flux away from pyruvate decarboxylation and OxPhos [52]. Our study alludes to a signalling mechanism in which HIF-1α is the metabolic regulatory system in FA stimulated cells, resulting in a cell phenotype reminiscent of the Warburg effect.

The Warburg effect is defined as a metabolic switch favouring pro-oncogenic glycolytic processes over OxPhos even in the presence of ample oxygen [24,37,45]. Pyruvate kinase catalyses the final and rate limiting step of aerobic glycolysis in which phosphoenol pyruvate is converted to pyruvate producing ATP. The PKM gene is alternatively spliced to produce PKM1 and PKM2. The PKM2

variant is highly expressed in multiple cancer types including human hepatocellular carcinoma (HCC) [53,54]. Previous studies have demonstrated that *PKM2* gene transcription is regulated by HIF-1α and that PKM2 directly interacts with HIF-1α to promote transcriptional activation of genes possessing the hypoxia response element suggesting that PKM2 and HIF-1α form part of a positive feedback loop [55,56]. We demonstrated FA to be efficient at inducing the HIF-1α pathway activation and identified PKM2 expression for further study. Enhanced PKM2 expression was observed in response to FA. Interestingly pharmacological stimulation of PDH by DCA caused a significant decrease in PKM2 expression. Studies suggested that acetyl-CoA may be a regulator of PKM2 [37,57]. It therefore seems that FA promotes metabolic flux away from pyruvate decarboxylation.

The cytosolic conversion of pyruvate to lactate by lactate dehydrogenase and its expression is often associated with the Warburg effect and tumorigenesis [58]. We show that FA up-regulates transcription of LDH*a* across all tested energy substrates particularly at 63μg/mL (Fig. 6 G-I). Furthermore, OxPhos priming of HepG2 cells did not limit lactate production. (Fig 3F). Our data suggests that FA enhances the production of pyruvate and shunts this metabolite to lactate. This further supports FA mediated stabilisation of HIF-1α since pyruvate and lactate are known to elevate HIF-1α expression [59].

In most mammalian cells, oxidative metabolism by mitochondria generates substantial amounts of ATP needed for cell survival. Agents causing mitochondrial dysfunction often lead to loss of cell viability [32]. Telles-Pupulin et al (1998) postulated that FA inhibited cellular processes related to mitochondrial energy metabolism and ATP generation [21]. Free fatty acids such as palmitate offer an alternate source of ATP through β-oxidation in the mitochondria. The metabolic switch to glycolysis is highlighted by the major decrease in ATP levels in cells cultured in glucose free-FFA media (Fig. 7B). In support of this data, we observed strong decreases to cell biomass in aglycaemic media when compared to glucose supplemented cells (Fig. 7A). These data underline glucose as the preferred metabolic substrate in cells treated with FA.

The findings of this study highlight a transition between adaptation and toxicity in response to FA at tested concentrations, particularly when molecular aspects of this work are scrutinised. *In vitro* studies offer the advantage of being highly homogenous, comprised of a population of cells with similar susceptibility to a particular toxin. Under these conditions it is reasonable to assume that the response of the liver derived HepG2 cell line to FA is a hormetic (biphasic) response characterised

by low dose stimulation and high dose inhibition [60] to several of the tested metabolic enzymes regardless of energy substrate. The exact mechanisms of hormesis induced by FA are unknown. Existing studies on FA used a variety of cell lines, doses and incubation periods [17-21], highlighting the need for more systematic experiments to elucidate the extent of such a biphasic dose response. Moreover, in view of the established mitochondrial toxicity of FA [17,21] it is necessary to also investigate whether stress responses mediate hormetic adaptation.

Conclusion

Given the current worldwide epidemic related to metabolic diseases it is becoming increasingly essential to understand how foodborne toxins such as FA might affect energy metabolism and flexibility. We demonstrate that FA oxygen-independently promotes HIF-1α expression and stabilisation which is associated with the metabolic shift away from pyruvate decarboxylation and toward a glycolytic phenotype reminiscent of the Warburg effect. Our findings add a novel aspect to the importance of neglected mycotoxin research particularly in the fields of metabolism and toxicology.

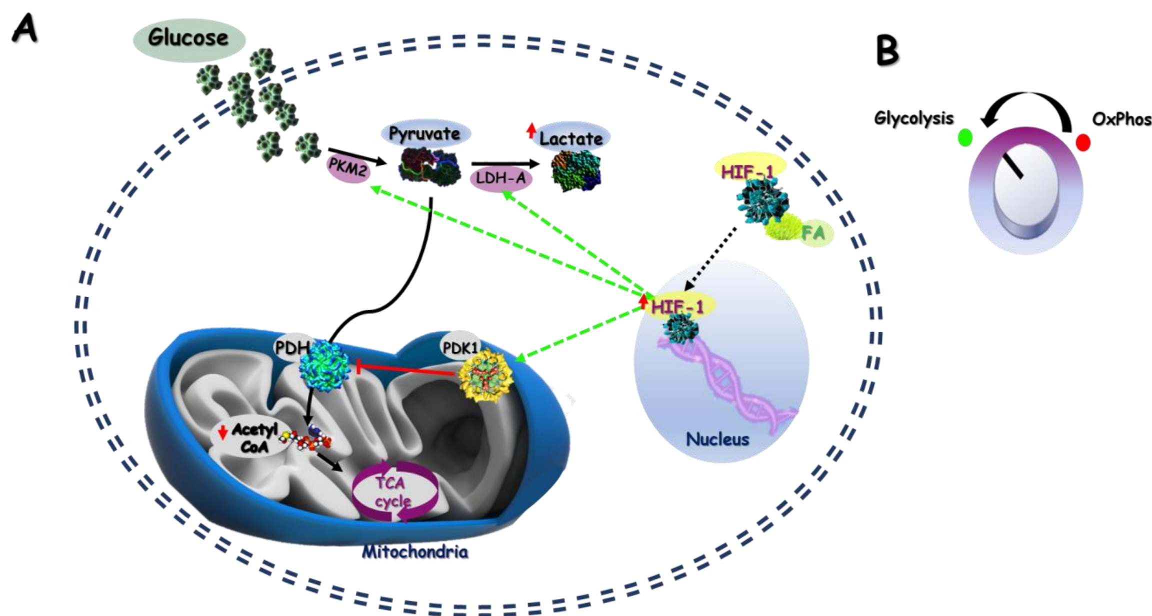


Fig. 8: Schematic diagram of the metabolic switch induced by FA. FA constitutively increases the expression of HIF-1α with subsequent transcriptional activation of PDK-1 that results in the inhibition of pyruvate decarboxylation. Furthermore, FA shunts pyruvate toward lactate production

by enhancing PKM2 and LDHa expression (A). Taken together, FA switches metabolism from OxPhos to glycolytic endpoint and induces a cell phenotype reminiscent of the Warburg effect (B).

3. Methods and materials

3.1. Reagents

The HepG2 human liver derived cell line was acquired from Highveld Biologicals (Johannesburg, SA). Western blot reagents were purchased from Bio-Rad (Hercules, CA, USA). FA isolated from *Gibberella fujikuroi* (F6513), Glucose (G8270-100G) and Galactose (G5388-100G) was acquired from Sigma-Aldrich (St Louis, MO, USA). All other reagents and consumables were purchased from Merck (Darmstadt, Germany), unless otherwise stated.

3.2. Cell culture and treatment protocol

HepG2 cells were cultured in 25cm^3 flasks in 5% CO_2 at 37°C as described by Marriquin *et al* [32]. Glucose free DMEM (Thermo-Fischer, A1443001) was supplemented as follows:

Low glucose (GLUC): 5.5mM glucose, 5mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 10% fetal calf serum (FCS), 1mM sodium pyruvate, and 1% penstrepfungizone. High glucose (HG): 25mM glucose, 2 mM HEPES, 10% FCS, 1mM sodium pyruvate and 1% penstrepfungizone. Mannitol (19mM) was used as an osmotic control (OC). Galactose (GAL): 10 mM galactose, 2mM glutamine, 5 mM HEPES, 10% FCS, 1mM sodium pyruvate, 1% penstrepfungizone. Free fatty acid (FFA): Sodium palmitate (Sigma, # P9767) was heat conjugated to fatty acid free bovine serum albumin (BSA) (1mM Sodium Palmitate/0.17mM BSA Solution) according to Schulz et al [61]. Cells were treated with a final concentration of 0.25mM palmitate and a 0.17mM BSA vehicle control (VC). These carbon sources were chosen to cover a broad range of metabolic backgrounds.

FA (2 mg/mL) stock solution was prepared using sterile phosphate buffered saline (PBS, 0.1 M). This was then diluted in DMEM supplemented with the above mentioned energy substrates to final concentrations of 4, 63 and 250 µg/mL (0 µg/mL FA in control). These concentrations were selected by correlating the number of metabolically active cells to energy outputs using the WST-1 (glycolysis) and Mitochondrial ToxGlo™ Assay (oxidative phosphorylation) data. Moreover, selecting doses capable of eliciting a biphasic response were hypothesised to provide a more complete characterization of FA toxicity.

A concentrated stock solution of oligomycin A and rotenone (10 mM) was prepared in Dimethyl sulphoxide (DMSO). This was diluted in media (0.1% v/v – benchmark DMSO concentration for *in vitro* experiments) to obtain final concentrations used in subsequent experiments. Sodium dichloroacetate (DCA, 1M) was dissolved in sterile PBS. This was then diluted in DMEM supplemented with the energy substrates of interest to a final concentration of 5mM. Cells were incubated for 6 hours (hrs) with test compounds to allow sufficient time for mitochondrial dysfunction/toxicity without overt cytotoxicity and cell death. All experiments were repeated at least three times (independently) in triplicate to validate results.

3.3. Assessment of mitochondrial toxicity and cytotoxicity

HepG2 cells were plated into a 96-well (15 000 cells, 200 µL/well) sterile white opaque luminometry plate. Following incubation with FA (0 – 1000 µg/mL, 37 °C, 5% CO_2, 6 hrs) in GLUC, HG and GAL supplemented media the Mitochondrial ToxGlo Assay (Promega, Madison, WI, USA, G8000) was performed as per the manufacturers' instructions. Briefly, cytotoxicity reagent was prepared by diluting bis-AAF-R110 substrate in assay buffer (5 µL/mL). The cytotoxicity reagent (20 µl/mL) was added to cells (100 µl media/well) and plate was shaken (30 secs) and subsequently incubated at 37°C (30 min). The fluorescent signal indicating dead cell protease activity was measured at 485 nm/520–530 nm (Excitation/Emission) using a Modulus™ microplate luminometer (Turner Biosystems, Sunnyvale, USA). The plate was then left to equilibrate (room temperature, 10 min). ATPase reagent (100 µl) was added to each well, the plate was shaken (1 min) before the luminescence signal was measured using a Modulus™ microplate luminometer.

3.4. Spectrophotometric quantitation of NAD(P)H using WST-1

Cells were seeded (20 000 cells/well) in a 96 well microtitre plate. Cells were treated with varying concentrations of FA (0 – 1000 µg/mL, 37 °C, 5% CO_2, 6 hrs) in triplicate (200 µL/well). The supernatant was then aspirated and 110 µl/well of a DMEM/WST-1 reagent solution (10:1) was added and incubated for 3 hrs (37 °C, 5% CO_2). The absorbance of the colorimetric reaction was measured at a wavelength of 450 nm with a reference wavelength of 620 nm using a spectrophotometer (Bio-Tek uQuant, Winooski, VT, USA).

3.5. Acetyl-CoA quantification

The PicoProbe Acetyl-CoA assay kit (Abcam 875460) was used to determine the levels of acetyl-CoA. Briefly, cells (2 x 10^6) were harvested and resuspended in assay buffer (500 µL) on ice. The homogenate was spun down (10,000xg, 10 min, 4°C) and the supernatant was collected and kept on ice. Acetyl-CoA standards (pMols), PicoProbe reagents and substrate mix were prepared and assayed as per manufacturer's guidelines. Fluorescence was measured at 535/589 nm (Excitation/Emission) using the Modulus™ microplate luminometer (Turner Biosystems, Sunnyvale, USA). The data is expressed as relative fluorescent units (RFU).

3.6. Measurement of lactate

Supernatants from cultured HepG2 cells were collected and lactate levels were assessed at a pathology laboratory (Global clinical and viral pathology, Amanzimtoti, Durban, South Africa).

3.7. Protein quantification

Protein expression was determined using western blots. Cytobuster™ Reagent (250 µL) (Novagen, San Diego, CA, USA, catalogue no. 71009) was used for protein extraction. Protein samples were quantified (bicinchoninic acid (BCA) assay)) and standardised to 1 mg/mL. The samples were then boiled (5 min) in Laemmli Sample buffer [dH_20, 0.5 M Tris-HCl (pH 6.8), glycerol, 10% SDS, β-mercaptoethanol, 1% bromophenol blue]. Prepared protein samples were electrophoresed on sodium dodecyl sulphate polyacrylamide (SDS-PAGE) gels (4% stacking gel, 7.5% resolving gel) at 150V (Bio-Rad Mini PROTEAN Tetra-Cell System). The separated proteins transferred onto nitrocellulose membranes using the Trans-Blot Turbo Transfer system (standard mixed protein program, Bio-Rad). The membranes were blocked with 5% BSA in Tris Buffer Saline with tween 20 (TTBS- NaCl, KCl, Tris, dH_2O, 0.5% tween 20, pH 7.5). Membranes were incubated with primary antibody (1:1000 dilutions Table 1) overnight (4°C). The membranes were incubated (RT) with HRP-conjugated secondary antibody for 2 hrs (1:5,000 dilution). Protein bands were detected using the Clarity western ECL chemiluminescent substrate and images were captured with the ChemiDoc™ XRS+ Molecular Imaging System (Bio-Rad). Protein expression was normalised against house-keeping protein, HRP-conjugated β-actin (Sigma, A3854). Protein expression was analysed using Image Lab Software version 5.0 (Bio-Rad) and the results were expressed as relative band density (RBD).

Table 1: List of antibodies used to perform western blot experiments:

Antibody	**Catalogue #**
Anti-Mitochondrial Pyruvate Dehydrogenase kinase 1 antibody (PDK1)	Abcam (Ab207450)
Anti-PKM2 antibody	Abcam (Ab150377)
Anti-Pyruvate Dehydrogenase E1α subunit (phospho S293)	Abcam (Ab177461)
Anti-HIF-1α antibody	Sigma (H6536)

3.8. RNA and qPCR

RNA was isolated from control and FA treated HepG2 cells using an in house protocol [62]. Total RNA was quantified (Nanodrop 2000, ThermoFischer), standardised (1000 ng/mL) and converted to complementary DNA (cDNA) using a commercially available kit (iScript™ cDNA Synthesis kit, Bio-Rad; catalogue no 107-8890). A 20 µL reaction volume containing 1µL RNA template, 4 µL 5X iScript™ reaction mix, 1 µL iScript reverse transcriptase and nuclease free water was prepared. Thermocycler conditions were 25 °C for 5 min, 42 °C for 30 mins, 85 °C for 5 min and a final hold at 4 °C.

mRNA expression levels of genes involved in glycolysis and PDH inhibition was assessed (Table 2) using the Sso Advanced™ Universal SYBR® Green Supermix (Bio-Rad, catalogue no. 172-5271) and the CFX Touch™ Real Time PCR Detection System (Bio-Rad, Hercules, CA, USA). Conditions for PCR reactions: initial denaturation (95°C, 8 min), denaturation (95°C, 30 sec), annealing (variable: Table 2, 40 sec) and extension (72°C, 30 sec). Each measurement was done in triplicate and normalised against GAPDH which was run under the same conditions. Data was analysed using the Livak and Schmittgen (2001) method and represented as fold change relative to the housekeeping gene ($2^{-\Delta\Delta Ct}$) [63].

Table 2: Primer sequences and conditions used for gene expression studies:

Sequence (5'-3')	Annealing temperature (°C)
HIF-1α	
Forward: GAAC GTCG AAAAGTCT CG	55
Reverse: CCTT ATCA AGAT GCGA ACTC ACA	
PKM2	
Forward: GTAC CATG CGGA GACC ATCA	55
Reverse: GTAG GCGT TATC CAGC GTGA	
LDHa	
Forward: GGTT GGTG CTGT TGCA TGG	60
Reverse: TGCC CCAG CCGT GATA ATGA	
PDK1	
Forward: CTGT GATA CGGA TCAG AAAC CG	57
Reverse: TCCA CCAA ACAA TAAA GAGT GCT	
GAPDH	
Forward: TCCA CCAC CCTG TTGC TGTA	Variable
Reverse: ACCA CAGT CCAT GCCA TCAC	

3.9. Intracellular ATP

Intracellular ATP levels were measured using the ATP CellTitre Glo reagent (Promega, Madison, USA). Following treatment cells (20,000 cells/well in GLUC, HG and FFA supplemented DMEM) were seeded into a 96-well opaque polystyrene microtitre plate in triplicate. The ATP CellTitre Glo reagent (25 µL/well) was added to each sample and incubated in the dark for 30 min (RT). Thereafter, the luminescence was measured on a Modulus^{TM} microplate luminometer (Turner Biosystems, Sunnyvale, USA). The data is expressed as relative light units (RLU).

3.10. Assessment of potential cytotoxicity (Biomass)

HepG2 cells were cultured in 96-well plates (15,000 cells/well). After treatment with FA (described in 2.2) biomass was stained by decanting media and incubating attached cells with crystal violet solution (0.5% w/v) crystal violet/20% (v/v) Methanol) for 10 mins. After thorough washing with sterile deionised water to remove excess dye the plate was left to dry overnight. The dye was then solubilised with ethanol (100 µL/well) and the absorbance was quantified at 595 nm using a spectrophotometer (Bio-Tek uQuant, Winooski, VT, USA).

3.11. Statistical analysis

Statistical analysis was performed using one way ANOVA followed by Bonferroni test for multiple group comparison. Results are expressed as mean ± standard deviation (SD) unless otherwise stated. Differences with $p<0.05$ were considered statistically significant.

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Conflict of Interest:

The authors declare that they have no conflict of interest.

Availability of data

The datasets generated and/or analysed during the current study are available from the corresponding author on reasonable request.

Author Contributions

NSA, SN and AC conceived and planned the experiments. NSA carried out the experiments. NSA, SN and AC contributed to the interpretation of the results. NSA prepared first draft of manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

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CHAPTER 5

Interactomic and molecular mechanisms of Fusaric acid induced mitochondrial sirtuin aberrations in glycolytically and oxidatively poised HepG2 liver cells

Chapter 4 underlined the induction of metabolic inflexibility by FA in HepG2 cells. Mitochondrial SIRTs are regarded as energy sensors and at the forefront of metabolic regulation and stress responses. In this chapter the inhibitory effects of FA on SIRTs 3 and 5 activities were determined using *in silico* methods. Furthermore, the expression profiles of these mitochondrial SIRTs were analysed along with their possible regulatory mechanisms at the transcriptional, post-transcriptional and translational levels. Fusaric acid strongly associates with SIRTs and downregulates their expression profiles in both glycolytic and OxPhos primed cells.

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Interatomic and molecular mechanisms of Fusaric acid induced mitochondrial sirtuin aberrations in glycolytically and oxidatively poised liver (HepG2) cells

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Key words

Micro RNA

Molecular docking

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PGC-1α

Post translational modifications

Abstract

Fusaric acid (FA) is a ubiquitous yet neglected mycotoxin. The toxicity of FA is associated with mitochondrial dysfunction and oxidative stress. Sirtuins (SIRTs) are key mediators of cell stress responses through deacetylation of antioxidant, mitochondrial maintenance and energy metabolism proteins. Dietary bioactive compounds have profound effects on SIRT activity, however little is known regarding common foodborne toxins and SIRTs. In this study the interaction of FA with mitochondrial SIRTs – SIRT3 and SIRT5, were firstly studied by molecular docking. Thereafter we substantiated the in silico findings by investigating the effect of FA on expression profiles of SIRT3 and SIRT5, and transcriptional and post-transcriptional regulators, PGC-1α and miRNA-30c using western blots and qPCR *in vitro*. FA was predicted to bind to the active site of SIRT3 and SIRT5 having implications for biological activity. Furthermore, protein expression of SIRT3 and SIRT5 was down-regulated despite elevated mRNA levels. Further experimentation revealed post-transcriptional regulation of both SIRTs as evidenced by elevated miRNA-30c despite induction of PGC-1α. This study highlights the potential of a diet contaminated with FA to dysregulate mitochondrial specific proteins that can lead to initiation and progression of disease.

1. Introduction

Mitochondria are essential organelles that regulate diverse aspects of cell biology and homeostasis, and are central to cellular energy metabolism and fuel utilisation. [1-3]. Mitochondrial dysfunction is associated with metabolic inflexibility and is implicated in the development of several metabolic diseases including cancer [4] and insulin resistance [5]. To maintain mitochondrial integrity, these organelles are equipped with innate protective mechanisms that are systematically and biochemically linked to metabolic processes and energy substrate availability [6-9].

The mammalian family of sirtuins (SIRTs) has attracted major interest given their links to protein acetylation and diseases relating to metabolism and aging [10]. The SIRTs are integral post-translational regulators of protein activity in response to nutrient availability and stressors through deacetylation of lysine residues [11-13]. Sirtuin 3 has been shown to directly deacetylate and activate numerous mitochondrial proteins involved in the tricarboxylic acid (TCA) cycle, oxidative phosphorylation (OxPhos), fat metabolism and stress responses [12, 14-17]. Knockout models of SIRT3 exhibit hyperacetylation of mitochondrial proteins highlighting its strong deacetylase activity [18]. SIRT5 was recently described to more efficiently demalonylate or desuccinylate its substrates due to the presence of an Arg105 residue and Tyr102 residue in its pocket [14], this primary mitochondrial dessucinylase is reported to regulate the urea cycle by targeting carbamoyl phosphate synthase 1 (the rate limiting enzyme of the urea cycle) [19]. Sirtuin 5 has also been shown to influence mitochondrial metabolism through succinylation of the pyruvate dehydrogenase complex and succinate dehydrogenase [20]. Pathway analyses of targets of SIRT5 show hypersuccinylation in knockout mice and subsequent perturbations to fatty acid oxidation and ketone body production [21].

Peroxisome proliferator-activated receptor γ (PPARγ) coactivator 1α (PGC-1α) is a transcriptional co-activator vital in maintenance of mitochondrial function, protection and biogenesis [12, 22-24]. The elucidation of the transcriptional regulation of diverse stress and metabolic pathways have led to the discovery of mitochondrial SIRTs 3 and 5 as putative downstream target genes of PGC-1α [13]. These mitochondrial SIRTs are also regulated at the post-transcriptional level by microRNAs (miRNA), these short non-coding RNAs regulate gene expression by degrading or inhibiting translation of targeted mRNA [25].

The biochemical activation of SIRT activity is dependent on NAD^+ and release of nicotinamide (NAM) and O-acetyl ADP ribose as by-products, and NAM itself can inhibit SIRT activity [26, 27]. Thus, SIRT activity is linked to cellular energy and redox status [28]. Dietary bioactive compounds

are known to have profound effects on sirtuin activity, however very little is known regarding common foodborne toxins and their roles in SIRT regulation.

Mycotoxins are fungal secondary metabolites that adversely affect humans, animals and plants and their contamination of food and feed presents a significant problem to food security [29, 30]. Mycotoxins are a structurally diverse group of compounds that display structural similarities to biologically relevant molecules, and this contributes significantly to their toxic mechanisms of action [31]. The *Fusarium* mycotoxin, fusaric acid (FA) is a common contaminant of maize with a reported contamination rate of up to 13,593 µg/kg sample [32, 33]. Despite its ubiquitous presence this mycotoxin is neglected in terms of research. Several studies have highlighted the synergistic and additive effects of FA and co-produced mycotoxins in animal models [34-36]. More recent single toxin analyses in human cell lines indicate genotoxicity, cytotoxicity, deregulation of cell signalling and activation of cell death machinery [37-40].

Canonical FA toxicity is ascribed to oxidative stress, cell death and mitochondrial dysfunction [39-41]. FA is regarded as a putative mitochondrial toxin that inhibits enzymes of the TCA cycle and OxPhos [42]. Furthermore FA was shown to induce mitochondrial stress and impair mitochondrial biogenesis as well as inhibiting ATP synthesis [39, 42]. However, the full effects of mitochondrial perturbations by this niacin related mycotoxin has yet to be fully elucidated.

Many highly proliferative cells almost exclusively utilise glycolysis as a means to generate ATP despite the presence of functional mitochondria. Such anaerobically poised cells are resistant to mitochondrial toxins, elevated rates of glycolysis act as a compensatory measure to mitochondrial ATP generation impairment. In contrast, cells grown in galactose are forced to rely on mitochondrial OxPhos to meet their energy demands [43]. Consistent with this methodology we identified alterations to NAD^+ levels in glycolytic and aerobic poised HepG2 liver cells and elucidate the impact of FA on mitochondrial SIRT 3 and 5 expression, at the transcription and translation levels.

2. Methods and materials

2.1. Reagents

The HepG2 liver cell line was acquired from Highveld Biologicals (Johannesburg, SA). Fusaric acid (isolated from *Gibberella fujikuroi*- F6513), Glucose (G8270-100G) and Galactose (G5388-

100G) was acquired from Sigma-Aldrich (St Louis, MO, USA). Western blot reagents were purchased from Bio-Rad (Hercules, CA, USA). All other reagents and consumables were purchased from Merck (Darmstadt, Germany), unless otherwise stated.

2.2. Molecular docking (*in silico*)

Molecular docking was performed to visualise the orientation of ligand in the active site pocket of AIRT3 and SIRT5 to understand the mechanism of substrate/inhibitor selectivity [44]. Three-dimensional structures of SIRT3 and SIRT5 were taken from the Protein Data Bank (PDB codes 4v1c and 5xhs respectively) [45]. The chemical structure of FA was drawn using sketch molecules module of BIOVIA Discovery Studio (Dassault Systèmes, San Diego*)*. The hydrogen was initially added to optimise the geometry of ligands through molecular mechanics using universal force fields and steepest descent algorithm present in Avogadro tools [46]. The docking confirmed the binding affinity of FA with SIRT3 and SIRT5. Fusaric acid was docked by defining the grid box with spacing of 1 Å and size of 30 × 30 × 30 pointing in x, y and z directions around the SIRT3 and SIRT5 active sites following the standard docking protocol [47, 48] using AutoDockTools and AutoDockVina [49] with default docking parameters. The Lamarckian Genetic Algorithm was used as the search algorithm with default parameter values. The most suitable docked conformation was taken for further analysis. Details of docking parameters are described in our previous communications [50, 51]. PyMol [52], Discovery Studio Visualizer [53] and LigPlot^{+} [54] were used for the visualisation and structure analysis of docked complex.

2.3. Cell culture and treatment protocol

HepG2 cells were cultured in 25cm^3 flasks in 5% CO_2 at 37°C as described by Marriquin *et al* (2007) [43]. Glucose free DMEM (Thermo-Fischer, A1443001) was supplemented as follows:

Low glucose (GLUC): 5.5mM glucose, 5mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 10% foetal calf serum (FCS), 1mM sodium pyruvate, and 1% penstrepfungizone. High glucose (HG): 25mM glucose, 2 mM HEPES, 10% FCS, 1mM sodium pyruvate and 1% penstrepfungizone. Mannitol (19mM) was used as an osmotic control (OC). Galactose (GAL): 10 mM galactose, 2mM glutamine, 5 mM HEPES, 10% FCS, 1mM sodium pyruvate, 1% penstrepfungizone.

FA (2 mg/mL) stock solution was prepared using sterile phosphate buffered saline (PBS, 0.1M). This was then diluted in DMEM supplemented with the above mentioned carbon sources to final concentrations of 4, 63 and 250 µg/mL (0 µg/mL FA in control) for 6 hours. These concentrations were selected by correlating the number of metabolically active cells to energy outputs using the WST-1 (glycolysis) and Mitochondrial ToxGlo™ Assay (oxidative phosphorylation) data (Sheik Abdul 2018, submitted to Scientific reports, SREP-18-28513, unpublished data). All experiments were conducted three times (independently) in triplicate.

2.4. NAD^+ quantification

The NAD^+/NADH Assay Kit (ab 65348, Abcam) was used to quantify intracellular NAD^+ levels, according to the manufacturer's recommendations with no amendments. A standard curve was generated and used to extrapolate total NAD^+ (pmol) in glycaemic and aglycaemic culture conditions.

2.5. Protein quantification

Protein expression was determined using western blots. Cytobuster™ Reagent (250 µL) (Novagen, San Diego, CA, USA, catalogue no. 71009) was used for protein extraction. Protein samples were quantified (bicinchoninic acid (BCA) assay)) and standardised to 1 mg/mL. The samples were then boiled (5 min) in Laemmli Sample buffer [dH_20, 0.5 M Tris-HCl (pH 6.8), glycerol, 10% SDS, β-mercaptoethanol, 1% bromophenol blue]. Prepared protein samples were electrophoresed on sodium dodecyl sulphate polyacrylamide (SDS-PAGE) gels (4% stacking gel, 7.5% resolving gel) at 150V (Bio-Rad Mini PROTEAN Tetra-Cell System). The separated proteins transferred onto nitrocellulose membranes using the Trans-Blot Turbo Transfer system (standard mixed protein program, Bio-Rad). The membranes were blocked with 5% BSA in Tris Buffer Saline with tween 20 (TTBS- NaCl, KCl, Tris, dH_2O, 0.5% tween 20, pH 7.5). Membranes were incubated with primary antibody (1:1000 dilutions Table 1) overnight (4°C). The membranes were incubated (RT) with HRP-conjugated secondary antibody for 2 hr (1:5,000 dilution). Protein bands were detected using the Clarity western ECL chemiluminescent substrate and images were captured with the ChemiDoc™ XRS+ Molecular Imaging System (Bio-Rad). Protein expression was normalised against house-keeping protein, HRP-conjugated β-actin (Sigma, A3854). Protein expression was analysed using Image Lab Software version 5.0 (Bio-Rad) and the results were expressed as relative band density (RBD).

Table 1: List of antibodies used to perform western blot experiments:

Antibody	Catalogue #
PGC-1α	Cell Signalling Technology (#2178)
SIRT3	Abcam (#86671)
SIRT5	Abcam (#13697)
Phospho-eiF2α (Ser51)	Cell Signalling Technology (#3597)

2.6. RNA and qPCR

RNA was isolated from control and FA treated HepG2 cells using an in house protocol [55]. Total RNA was quantified (Nanodrop 2000, ThermoFischer), standardised (1000 ng/mL) and converted to complementary DNA (cDNA) using a commercially available kit (iScript™ cDNA Synthesis kit, Bio-Rad; catalogue no 107-8890). A 20µL reaction volume containing 1µL RNA template, 4µL 5X iScript™ reaction mix, 1µL iScript reverse transcriptase and nuclease free water was prepared. Thermocycler conditions were 25°C for 5 min, 42°C for 30 min, 85°C for 5 min and a final hold at 4 °C. Transcript expression levels of mitochondrial specific *SIRT* genes (Table 2) were quantified using the SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad, catalogue no. 172-5271) and the CFX Touch™ Real Time PCR Detection System (Bio-Rad, Hercules, CA, USA). Conditions for PCR reactions: initial denaturation (95°C, 8 min), denaturation (95°C, 30 sec), annealing (Table 2, 40 sec) and extension (72°C, 30 sec). Each measurement was done in triplicate and normalised against GAPDH which was run under the same conditions. Data was analysed using the Livak and Schmittgen (2001) method and represented as fold change relative to the housekeeping gene ($2^{-\Delta\Delta Ct}$) [56].

Table 2: Primer sequences and conditions used for gene expression studies:

Sequence (5’-3’)	Annealing temperature (°C)
SIRT3	
Forward: 5’-CGGC TCTA CACG AGAA CATC-3’	57
Reverse: 5’-CAGC GGCT CCCC AAAG GAAC AC-3’	
SIRT5	
Forward: 5’-CGAA ACCA GCCT GAA-3’	57
Reverse: 5’-TTCT CCAA ACCA CACG ACGT-3’	
GAPDH	
Forward: 5’-TCCA CCAC CCTG TTGC TGTA-3’	Variable
Reverse: 5’-ACCA CAGT CCAT GCCA TCAC-3’	

2.7. MicroRNA 30c analysis

A standard miRNA target prediction tool (TargetScan) was used to search for potential candidate miRNAs that may regulate the mitochondrial SIRTs. MiRNA 30c (miR-30c) was predicted to regulate both SIRT3 and SIRT5. The cDNA for miRNA analysis was prepared by using a 10µl reaction mix of the miScript II RT kit [cat. No. 240 MS00003528 (5× miScript HiSpec Buffer, miScript Reverse Transcriptase Mix 10× miScript 241 Nucleics Mix; Qiagen)] as per manufacturer’s instructions. MiR-30c levels were then quantified using the miScript Primer Assay (Qiagen). Data was normalised against human RNA U6 small nuclear 2 (RNU6-2) and analysed using the Livak and Schmittgen (2001) method and represented as fold change relative to the housekeeping gene ($2^{-\Delta\Delta Ct}$) [56].

2.8. Statistical analysis

Statistical analysis was performed using one way ANOVA followed by Bonferroni test for multiple group comparison. Results are expressed as mean ± standard deviation (SD) unless otherwise stated. Differences with $p<0.05$ were considered statistically significant.

3. Results

3.1. Docked complex interactions of ligand FA and target enzymes SIRT3 and SIRT5

Computational methods such as molecular docking are reliable for the prediction of binding modes and affinities of ligands and enzymes. We first determined if FA could act as a ligand for SIRT3. An analysis of the docked complex of SIRT3 revealed significant interactions of FA with the enzyme. The total free energy of binding was estimated to be -6.4 kcal/mol. FA readily interacted with the target protein through a variety of interactions including hydrogen bonding, p-sigma bonding, alky bonding and metal chelation of the zinc atom (Figure 1A). Similarly, FA formed close interactions to residues of SIRT5 as shown by conventional hydrogen bonding, pi cation bonding and alkyl bonding. The binding efficiency of FA to SIRT5 was relatively strong with an estimated affinity of – 5.1 kcal/mol (Figure 1B).

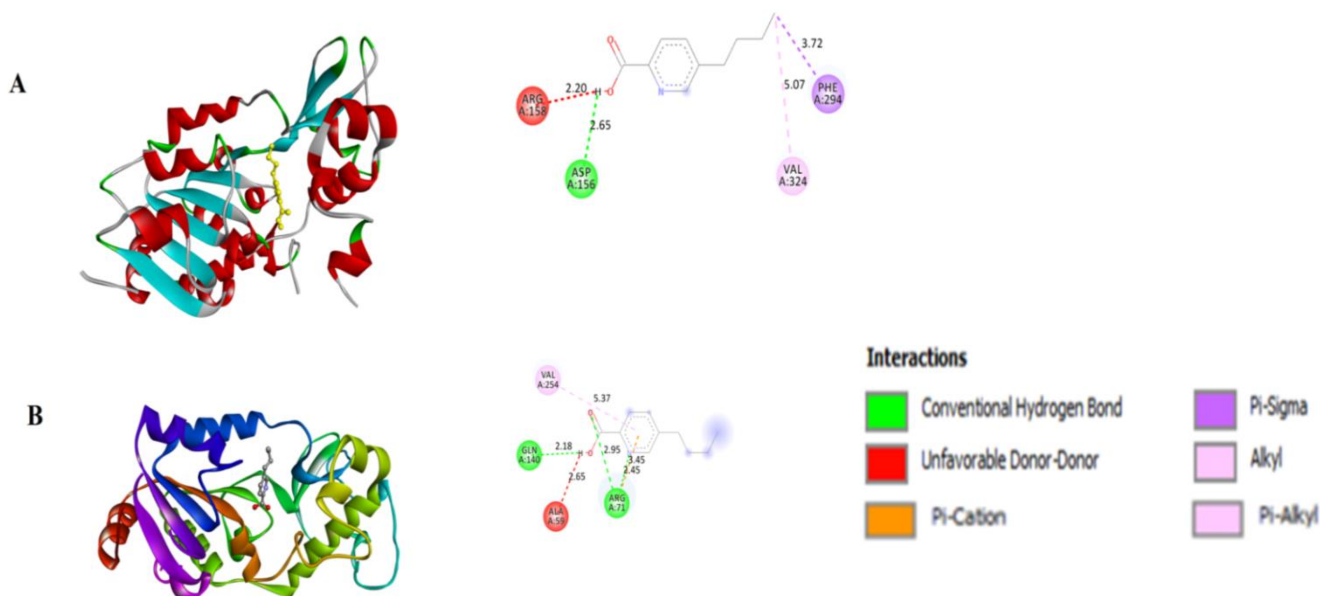

Figure 1: Structural rendering of the docked FA in complex with SIRT3 (A) and SIRT 5 (B).

3.2. NAD$^+$ is differentially regulated by FA in a concentration dependent manner

NAD$^+$ is a necessary co-substrate for the biochemical activity of SIRTs. The involvement of FA in NAD$^+$ perturbations was assessed using a colorimetric assay. Under low glucose (GLUC) and OxPhos (GAL) primed conditions FA significantly elevated NAD$^+$ levels at 4 and 63 µg/mL (Figure 2A and C). NAD$^+$ levels in HG cells showed a remarkable increase only at 63 µg/mL followed by a steep decline at 250 µg/mL (Figure 2B).

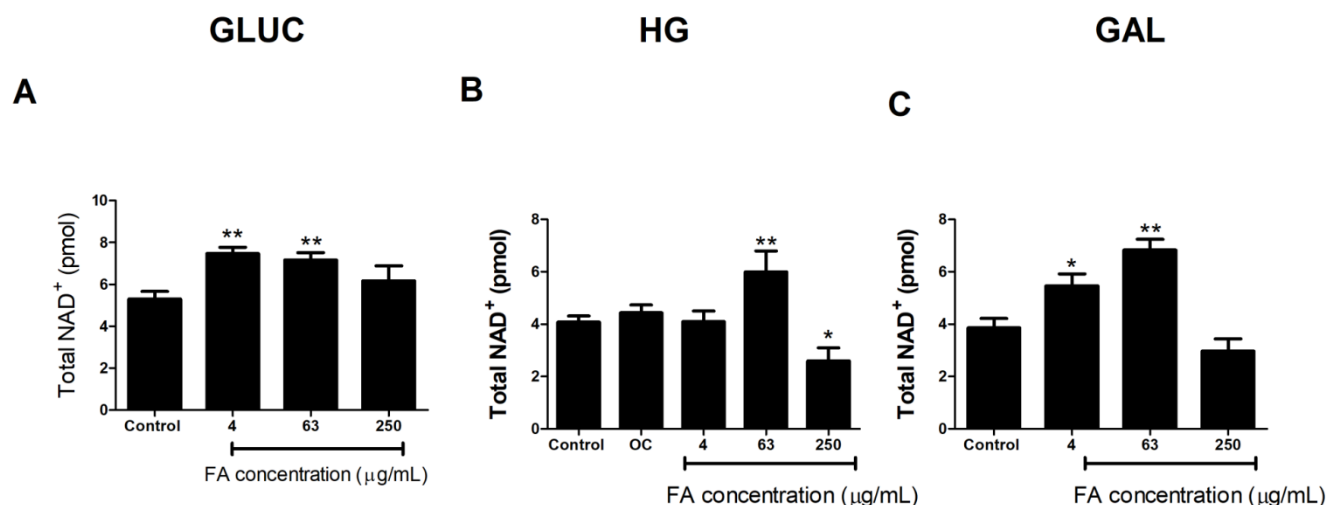

Figure 2: FA alters NAD$^+$ concentrations in a dose and energy substrate dependent manner. The results are expressed relative to the control ± SD (n=3).
**p<0.01, *p<0.05 *vs* control

3.3 FA suppresses SIRT3 protein expression despite elevated transcription

FA induces oxidative stress as its canonical mechanism of toxicity. SIRT3 is a stress response deacetylase that activates anti-oxidant defences. We therefore examined the influence of FA on SIRT3 expression profiles.

FA, at all tested concentrations, significantly decreased SIRT3 protein levels. This decrease was dose dependent with the 250 µg/mL FA exerting the greatest effect across all conditions tested (Fig 3A-C). In contrast FA significantly elevated *SIRT3* mRNA expression except at the highest dose of

FA (Fig 3D-F). These results were independent of the energy substrate present in the media indicating that SIRT3 is a putative target of FA.

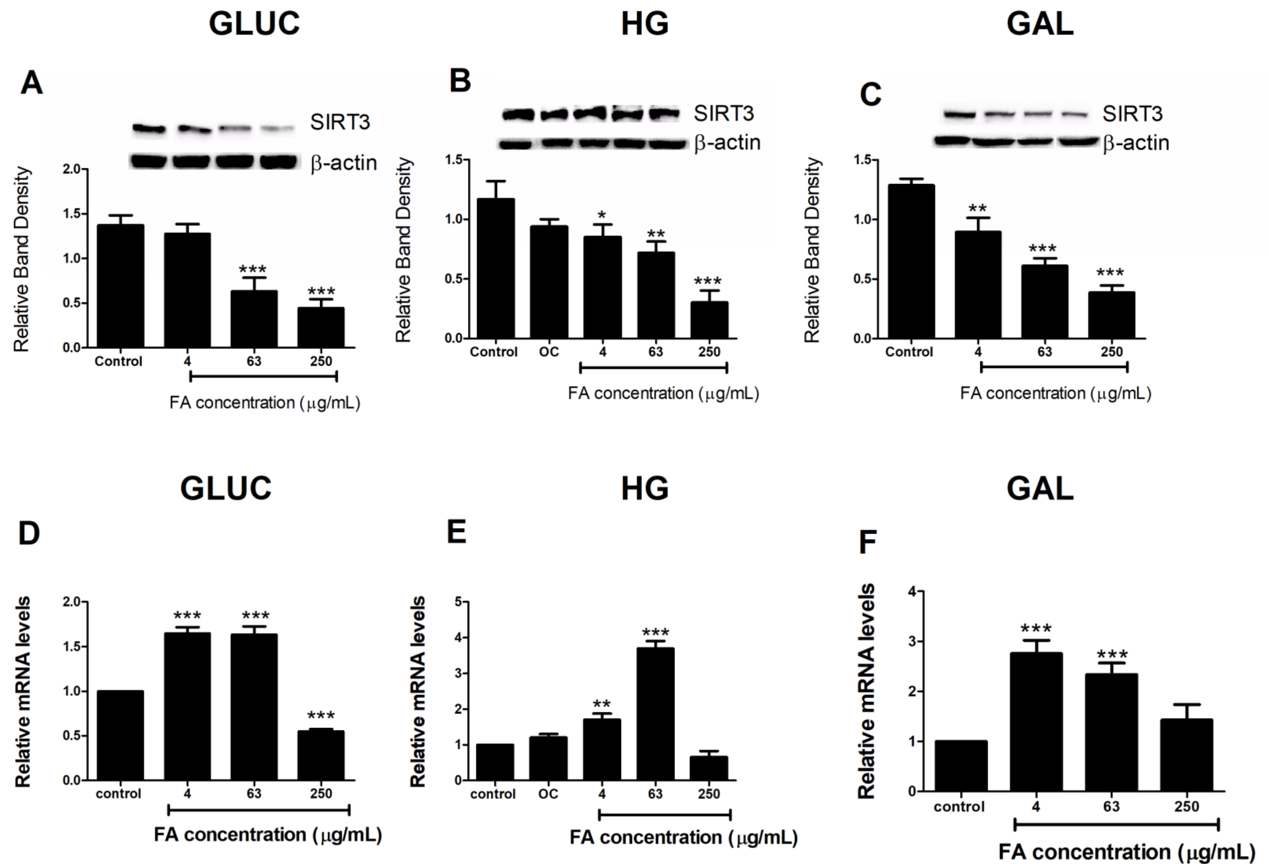

Figure 3: Western blot analysis of SIRT3 revealed significant dose dependent decreases after stimulation with FA (A-C). In contrast *SIRT3* mRNA levels were significantly elevated (D-F). The results are expressed relative to the control ± SD (n=3).

***p< 0.001, **p<0.01, *p<0.05 *vs* control

3.4. SIRT5 protein expression is downregulated in spite of higher transcript levels in response to FA stimulation.

We then investigated the expression profile of the mitochondrial desuccinylase, SIRT5. FA significantly decreased the protein levels of SIRT5 under glycaemic conditions (Fig 3A and B). In OxPhos primed cells the lowest concentration of FA elevated SIRT5 expression levels while at 250 µg/mL a significant decline in expression was noted (Fig 4C). This observation is in sharp contrast

with transcript levels as we noted an increased expression of SIRT5 mRNA across all energy substrates except at 250 µg/mL (Fig 4D-4E).

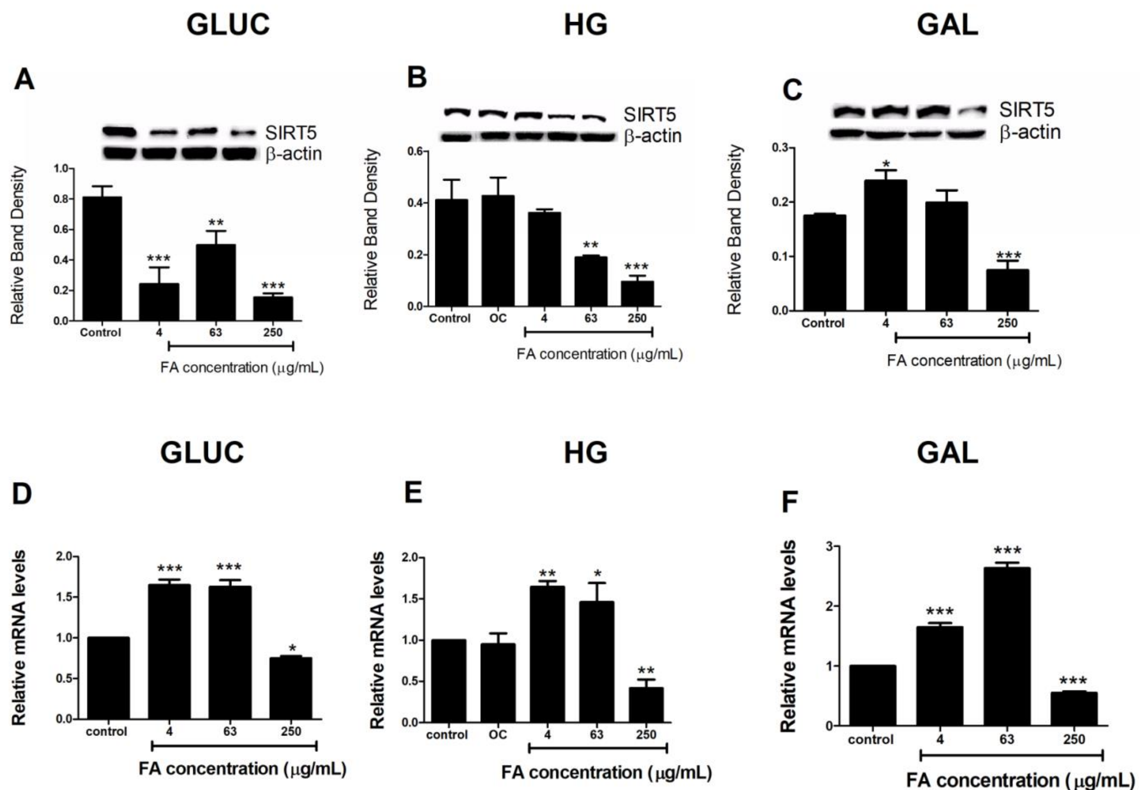

Figure 4: SIRT5 protein expression was consistently and significantly decreased under glycaemic conditions (A-C). This was in contrast to the mRNA levels which showed significant elevation across all tested energy substrates. The results are expressed relative to the control ± SD (n=3). ***p< 0.001, **p<0.01, *p<0.05 *vs* control

3.5. SIRT3 and SIRT5 transcriptional and translational machinery is dysregulated by FA.

Both SIRT3 and SIRT5 are downstream targets of PGC-1α. Stimulation of PGC-1α has been shown to elevate SIRT3 and SIRT5 protein expression and mRNA.

Cells cultured under low glucose (GLUC) showed a significant decrease to in PGC-1α expression at the lowest and highest FA concentrations whilst 63 µg/mL stimulated expression significantly (Fig 5A). Cells supplemented with high glucose (HG) showed significantly decreased PGC-1α expression regardless of FA concentration when compared to the control (Fig 5B). In contrast, FA dose dependently increased the expression of PGC-1α in OxPhos primed cells (GAL, Fig 5C).

Phosphorylation of the eukaryotic initiation factor 2 (eIF2) α subunit is a well-documented mechanism to decrease protein synthesis. We explored the potential of FA to dysregulate the phosphorylation status of eIF2α as a marker of defective translation. We found that FA enhanced phosphorylation of eIF2α in cells poised to undergo glycolysis (Fig 5D and E) and OxPhos (Fig 5F). Collectively these results show that FA impairs translation but not transcription of SIRT3 and SIRT5.

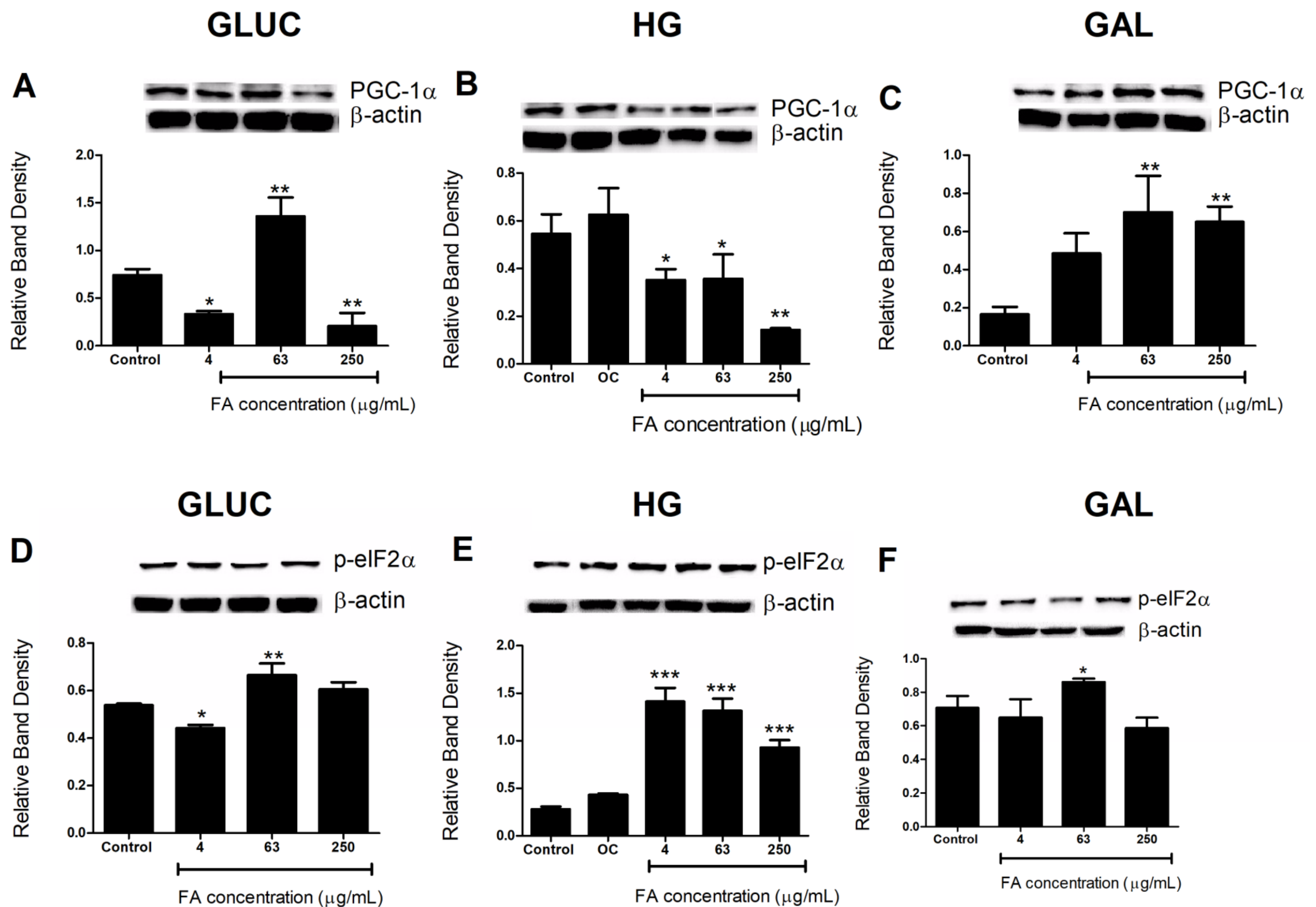

Figure 5: FA dysregulates transcriptional (PGC-1α; A-C) and translational (p-eIF2α; D-E) machinery of SIRT3 and SIRT5. The results are expressed relative to the control ± SD (n=3). ***p< 0.001, **p<0.01, *p<0.05 *vs* control

3.6. FA elevates MiRNA 30c in glycolytic and OxPhos primed cells

Using standard predicts miRNA target tools (TargetScan) we identified MiRNA 30c as a common post-transcriptional regulator of SIRT3 and SIRT5 (Fig 6A). Profiling of MiRNA 30c revealed FA to up-regulate its expression particularly at higher concentrations and across all tested energy substrates (Fig 6B-D).

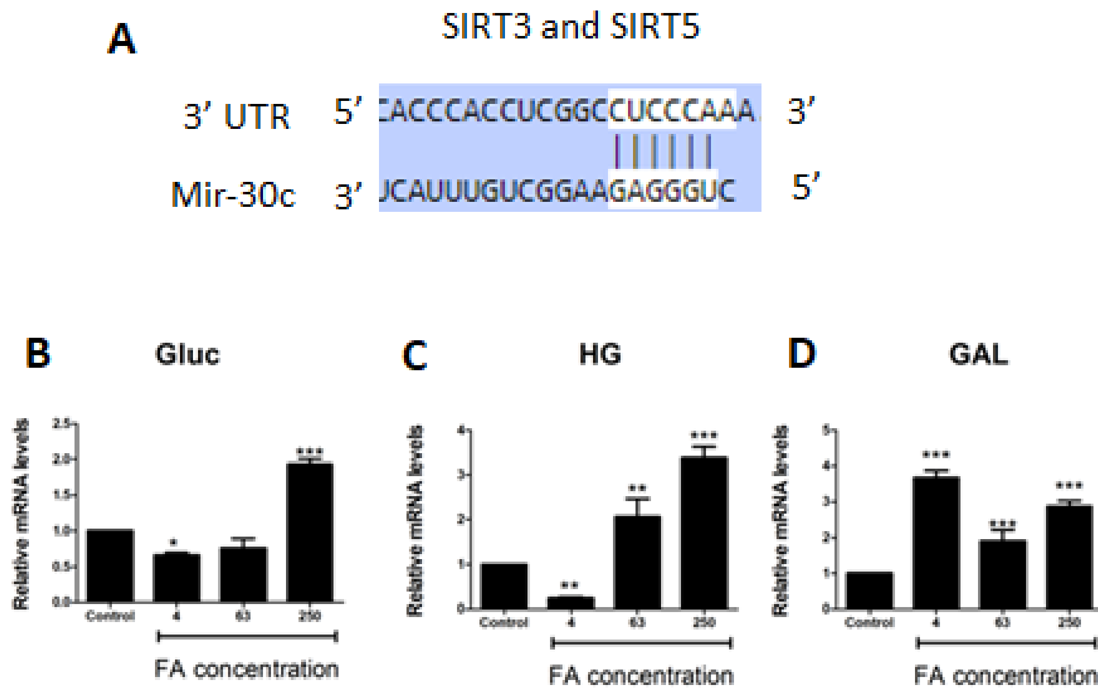

Figure 6: Sequence homology alignment between *SIRT3* and *SIRT5* and predicted target sequence of miR-30c (A). FA up-regulates miR-30c at higher concentrations (B-D). The results are expressed relative to the control ± SD (n=3).
***p< 0.001, **p<0.01, *p<0.05 *vs* control

Discussion

The canonical mechanism of FA toxicity is associated with oxidative stress and mitochondrial dysfunction [39, 41, 42, 57]; however the notion that FA is a putative mitochondrial toxin is yet to be clarified. Mitochondrial SIRTs are NAD^+ dependent enzymes implicated in several physiological

and disease states [58] mainly by their involvement in preventing mitochondrial stress and maintaining metabolic flexibility [12, 59, 60].

Sirtuins, a family of seven deacetylases, share a highly conserved catalytic core and differ only in their N- and C-terminal domains. The catalytic core is made up of two domains, a large Rossman fold and a smaller zinc binding domain [61]. The acetylated protein and the NAD^+ co-factor bind in a cleft between the two domains. The enzymatic reaction yields a deacetylated protein product and NAM, a competitive inhibitor of SIRT activity [62]. In this study, using *in silico* molecular docking, we show that FA directly interacts with SIRT3 and SIRT5 through several chemical bond interactions at the active pocket (Fig 1A and 1B) resulting in ligand-enzyme docked conformations that decrease biological activity. Acylation of lysine is an important protein modification with SIRT3 and SIRT5 displaying distinct acyl substrate preferences which have evolved to operate in unique physiological processes [63]. The diverse deacylation activities for SIRTs are related to differences in the amino acids present in the acyl binding pocket. While SIRT3 is a robust deacetylase, SIRT5 is recognised as a desuccinylase/demalonylase enzyme. The preference for succinyl and malonyl groups by SIRT5 is attributed to the presence of an arginine residue (Arg105) and tyrosine residue (Tyr102) in the acyl pocket, thus making it a weaker deacetylase than SIRT3 [64].

The synthesis and catabolism of NAD plays a vital role in the regulation of NAD-consuming enzymes, such as SIRTs. Several dehydrogenase enzymes of the TCA cycle reduce NAD^+ to NADH; that is needed to drive ATP production via the electron transport chain and OxPhos. Mitochondrial function and acetylation status is highly dependent on the limiting levels of NAD^+ [65]. Fusaric acid is known to inhibit α-ketoglutarate dehydrogenase [42], a key TCA cycle enzyme that reduces NAD^+ to NADH during the conversion of α-ketoglutarate to succinyl-CoA. Our observations of elevated NAD^+ (Fig 2), especially in cells poised to undergo OxPhos, could be a direct result of this inhibitory effect as NAD^+ can no longer be reduced to NADH. These results are in agreement with our previous study, which showed FA to dysregulate reducing equivalent metabolism in favour of a higher NAD: NADH ratio as evidenced by the MTT assay [39].

The universal SIRT regulators, NAD^+ and NAM, are essential for mitochondrial acetylome maintenance [66, 67]. Both SIRT3 and SIRT5 share a similar affinity for NAD^+ as a co-substrate. Structural and activity data, however, suggest differential sensitivities of SIRTs to NAM inhibition,

with SIRT3 being more sensitive than SIRT5 [68]. Nicotinamide is niacin related, pyridine derivative and is structurally similar to FA. A recent study revealed that novel bioisosteric analogues of NAM can inhibit SIRT3 activity [69]. We speculate that FA acts as a competitive inhibitor and, once bound to the co-substrate site, decreases SIRT activity by preventing NAD^+ catalysis. We have previously shown that FA inhibits SIRT3 activity in a similar manner to NAM [39]. Taken together, FA may be more selective for SIRT3 over SIRT5 as evidenced by a higher binding affinity (S1) and differential sensitivities to NAM inhibition.

It is important to underpin the difference between enzyme expression and activity. The expression levels of SIRTs are regulated at several levels. Analysis of SIRT3 and SIRT5 protein expression revealed down-regulation at the translated level (Fig 3A-C and 4A-C) but elevated transcript levels (Fig 3D-F and 4D-F). The net balances of forces often determine whether a protein will be its native folded conformation or denatured [70]. The protein stability of SIRT3 and SIRT5 is dependent on zinc ions co-ordinated by four cysteine residues [61, 71], FA being an established chelator of divalent cations [41, 72] can interact with zinc causing loss of protein stability and structural integrity.

At the transcriptional level, both SIRT3 and SIRT5 are regulated by PGC-1α. This co-transcription factor up-regulates the mRNA levels of *SIRT3* [12] and *SIRT5* [13]. Moreover, SIRT3 regulates PGC-1α activity, forming a positive feedback loop linking together oxidative stress, metabolism and mitochondrial function [12]. It is well established that PGC-1α is up-regulated during mitochondrial stress conditions as mechanism of cell defence [73, 74]. We show that FA dysregulates the expression of PGC-1α in a substrate dependent manner (Fig 5 A-C). Fusaric acid induced a biphasic response in cells cultured under low glucose conditions (Fig5 A) but elevated the expression of PGC-1α at all tested concentrations in OxPhos primed cells (Fig 5C). Cells supplemented with GAL as the energy substrate or limited glucose supply is more sensitive to mitotoxins and induce a greater response to overcome mitochondrial stress and its implications such as oxidative stress. A recent study by Gounden et al (2014) linked high glucose levels to elevated expression of PGC-1α expression [75]. However, FA significantly decreased the expression of PGC-1α. These results show FA initiates cytoprotective mechanism to the putative mitochondrial toxin FA through activation of the SIRT transcription pathway and concomitant increase to *SIRT3* and *SIRT5* mRNA levels, suggesting translational inhibition rather than transcriptional inhibition.

Protein synthesis is often inhibited in cells undergoing stress by enhanced phosphorylation of eIF2α. Translational control fine tunes rapid responses and flexibly to external signals and various forms of cell stress [76]. The phosphorylation of eIF2α occurs under situations often associated with mitochondrial dysfunction such as the unfolded protein response, decreased ATP levels and induction of oxidative stress [77, 78]. Fusaric acid significantly elevated the expression of p- eIF2α across all tested energy substrates (Fig 5D-F). There are several ways in which FA can contribute to inhibited protein translation including down regulation of protein chaperones [39], loss of ATP production and production of reactive oxygen species [39, 40, 42].

Extensive post-transcriptional, translational and stability regulation along with accumulation and degradation of corresponding mRNAs are essential for protein abundance [79]. MicroRNAs are negative regulators of protein expression and have become increasingly recognised as key role players in cell metabolism and stress responses. MicroRNAs associate with their mRNA targets through base pair complementarity. Imperfect binding of miRNA to target mRNA causes translational inhibition [80], this results in loss of protein expression. *SIRT3* and *SIRT5* share a common seed sequence with miR-30c with partial complementarity. FA was able to significantly upregulate the expression of miR- 30c at all tested concentrations (Fig 5A-C). This suggests FA can regulate SIRT expression at the epigenetic level by affecting post-transcriptional regulatory processes. Taken together the data validates that FA dysregulates translation of mitochondrial SIRTs 3 and 5 rather than transcription.

SIRT3 is the primary mitochondrial deacetylase [18] involved in the regulation of OxPhos through reversible acetylation of succinate dehydrogenase and links the TCA cycle to OxPhos [81]. SIRT3 also plays a key role in anti-oxidant defences through activation of SOD2 [82]. SIRT5 is involved in the urea cycle as well as mitochondrial function through regulation of PDH [64, 83]. Mitochondrial sirtuins SIRT3 and 5 functions as sensors and integrators of several distinct stress and metabolic inputs to coordinate responses for maintaining cellular homeostasis and aberrations to their expression and activity can be detrimental [84].

Conclusion

Post-translational modifications of proteins such as changes to the acylation status are strongly interconnected to sirtuins and metabolic phenotypes in response to nutrient availability and metabolites. Loss of expression and activity of sirtuins have been shown to reduce metabolic

flexibility and has been implicated in insulin resistance, obesity and cancer. Ingestion of foodborne toxins such as the neglected mycotoxin FA and their roles in metabolic decline is under estimated especially when coupled to changes in acetylation and redox status of cells. Loss of function or loss of expression of these important mitochondrial regulators can lead to metabolic inflexibility as well as decreased anti-oxidant capacity of the cell. FA targets translational processes of SIRT 3 and 5 which may contribute to disease initiation and progression.

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Abbreviations:

Sirtuin - SIRTs

MicroRNAs - miRNA

Peroxisome proliferator-activated receptor γ (PPARγ) coactivator 1α - PGC-1α

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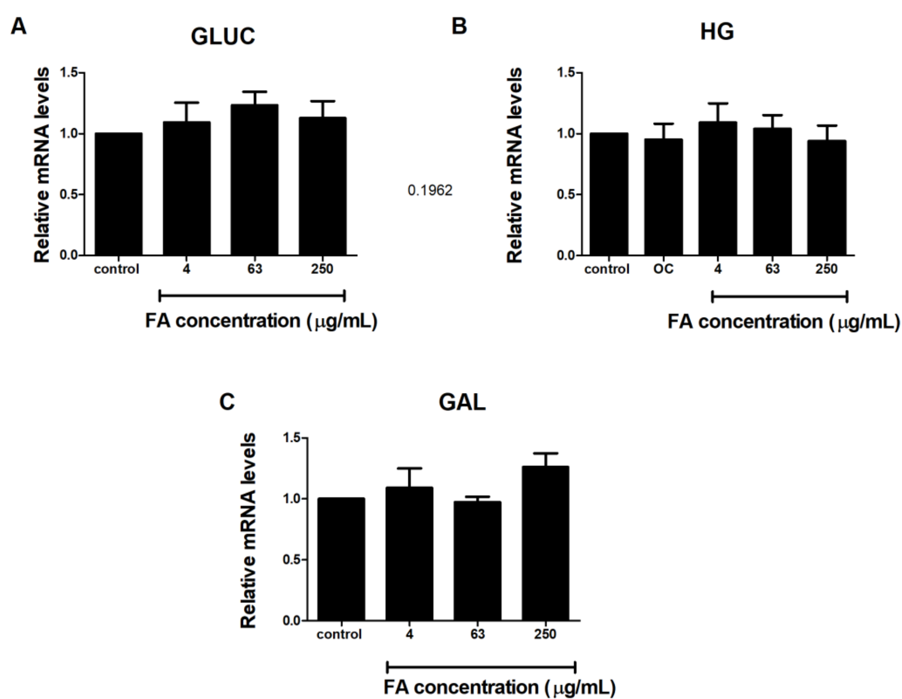
Supplementary data for manuscript:

Table 1: The binding affinity and list of residues participating in interactions with Fusaric Acid (FA) and SIRTs.

S. No.	Complex	Binding Affinity (kcal/mol)	Residual interactions
1.	Sirtuin 3 - Fusaric Acid	-6.4	Phe_{294}, Val_{324}, Arg_{158}, and Asp_{156}
2.	Sirtuin 5 - Fusaric Acid	-5.1	Val_{254}, Gln_{140}, Arg_{71}, and Ala_{59}

Supplementary figure 1:



S1: FA had no effect on PGC1α transcript levels.

CHAPTER 6

6.1. Synthesis and conclusion

The *Fusarium* fungi are known to have pathogenic potential to animals and plants. *Fusarium* metabolites are of economic importance due to their significant impact on public health and animal productivity across several countries. These toxins have been implicated in disease progression and initiation in both animals and humans. In the African context, where maize is the staple diet for the majority of the population, the emergence of highly prevalent but neglected fusariotoxins is of particular concern and will worsen an already precarious situation. The threat of human and animal exposure to these mycotoxins has led to ongoing elucidation of chemical structures, but the limited existence of mechanistic toxicological data has contributed to the difficulty in determining their toxic potential (Chilaka et al., 2017).

Fusaric acid is a highly prevalent and ubiquitous fusariotoxin (Streit et al., 2013, Bacon et al., 1996) that acts with co-produced mycotoxins which results in synergistic toxic manifestations (Bacon et al., 1995, Porter et al., 1995). However, the toxicity of FA is grossly underestimated as only a few studies have determined its effects on organisms as an individual agent. In both plants and animals, FA is considered a putative mitochondrial toxin. Fusaric acid has multiple sites of action within mitochondria including the disruption of ATP synthesis through inhibition of the TCA cycle, OxPhos (Telles-Pupulin et al., 1996, Telles-Pupulin et al., 1998) and induction of mitochondrial depolarization (Singh and Upadhyay, 2014). However, there is a dearth of knowledge surrounding the molecular mechanisms of FA induced aberrant energy metabolism and inflammatory responses associated with mitochondrial toxicity.

Enormous strides have been made in understanding mitochondrial dysfunction and associated pathobiology. This study provided key evidence to support the role of FA in perturbing mitochondrial homeostasis. Fusaric acid (24 hours, IC_{50} = 104 μg/mL) was found to down regulate the expression and activity of SIRT3, a critical deacetylase involved in the regulation of the mitochondrial stress responses. In addition, FA negatively impacts on mitochondrial biogenesis by significantly decreasing the expression of PGC-1α, NRF-1 and p-CREB, which are required for the transcription of nuclear encoded mitochondrial proteins. Loss of healthy mitochondria was shown to elevate ROS within FA exposed cells and as a consequence activate the NRF2 mediated cytoprotective response.

Classically, mitochondrial dysfunction has been linked to excessive ROS production and subsequent oxidative stress. In recent years, an intimate link between the release of mitochondrial DAMPs and innate inflammatory responses has been established. The NLRP3 inflammasome is a signalling platform that mediates the activation of caspase 1 and maturation of IL-1β in response to mitochondrial aberrations and stress. Inflammasomes are tightly regulated by a priming and activation step to ensure potent inflammatory responses are initiated only when needed (He et al., 2016). Quantitative and qualitative data show that FA inhibits inflammasome priming, an observation supported by decreased expression of the transcriptionally active p65 subunit of NF-κB. In this study FA disrupted the canonical NF-κB activation through down regulation of up-stream kinases. The ability of cells to defend against oxidative stress is critical for the dampening of inflammasome activation (He et al., 2016). Mitochondrial dysfunction is known to elevate ROS and subsequently induce the release and translocation of NRF2 to the nucleus where it induces the expression of a cytoprotective gene battery, including PINK-1 (Murata et al., 2015). Fusaric acid elevated the expression of NRF2 and may therefore selectively up-regulate the autophagic clearance of damaged mitochondria via increased expression of PINK-1. Moreover, FA decreased the expression of p62 but increased autophagosome components (ATG5, ATG7 and LC3). Loss of NLRP3 priming and activation provides evidence for the immunosuppressive effects of FA. The implications of this study extend beyond that of immunosuppression. Severe downregulation of NLRP3 was found in cases of hepatocellular carcinoma. Furthermore, many studies highlight the protective effects of NRF2 (reviewed in (Marin et al., 2013)), however the excessive and prolonged expression of NRF2 has been shown to favour cancer progression (Jaramillo and Zhang, 2013).

The ability of cells to defend against FA induced mitochondrial and oxidative stress may selectively manipulate cell phenotype leading to resistance against mitochondrial toxicity, thus allowing altered metabolic states. The roles of mycotoxins are often overlooked as potential agents in metabolic toxicity. Metabolic switching and subsequent metabolic inflexibility is associated with several pathologies including insulin resistance and cancer (Galgani et al., 2008). The metabolic shift in cancer cells is referred to as the Warburg effect and is characterised by the preferential use of glucose and shunting of pyruvate towards lactate production rather than OxPhos to generate ATP (Vander Heiden et al., 2009). Fusaric acid induces a cell phenotype reminiscent of the Warburg effect through constitutive stabilisation and expression of HIF-1α. This transcription factor transcribes for a set of genes (PDK1, LDH-A, PKM2) that inhibit the decarboxylation of pyruvate in the mitochondria and elevate lactate production. FA was shown to phosphorylate PDH E1α in a

PDK1 dependent manner and concomitantly increase the expression of both PKM2 and LDH-A suggesting a switch in metabolism away from OxPhos and toward glycolysis (shunt to lactate production). The capacity of FA to reduce viability and biomass of cells cultured in GAL and palmitate but not glucose suggest that FA renders these cells "addicted" to glucose. This is the first evidence to suggest that FA mitochondrial toxicity induces an adaptive cellular response to mitigate loss of mitochondrial function and compensate ATP production by enhancing glycolysis.

Fusaric acid results in dysregulated mitochondrial stress responses and metabolic flexibility. Mitochondrial SIRTs are at the forefront of facilitating metabolism and mitochondrial homeostasis. In silico analysis showed FA to strongly bind to mitochondrial SIRTs (3 and 5) and impede their catalytic activity despite the presence of ample NAD^+ levels. Analysis of protein expression of SIRT 3 and 5 revealed significant decreases despite elevated transcript levels. The transcriptional co-activator of SIRT 3 and 5, PGC-1α, was elevated and is in agreement with the observed mRNA levels. However, upon further analysis of the transcriptional activation of SIRT 3 and 5 it was apparent that FA downregulates translation of these proteins. This was validated by elevated phosphorylation of EIF-2α and increased expression of miRNA 30c. Taken together these data suggest that FA targets translational processing of SIRTs.

An accumulating body of evidence indicates that mitochondria are targeted by environmental toxins. This study highlights the importance of mitochondrial function and maintenance and the toxicities resulting from a neglected foodborne toxin. The data clearly indicates that FA is mitotoxic and induces cellular adaptive responses that lead to several toxic outcomes including suppression of pro-inflammatory cytokine maturation, enhanced glycolysis and lactate shunt to compensate for decreased mitochondrial outputs and aberrant SIRT regulation through manipulation of miRNA milieu. The mechanisms of FA toxicity can have radical alterations on mitochondrial function, thus reducing the availability of rate limiting mitochondrial-produced molecules (e.g., methyl groups for DNA methylation or cofactors such as NAD+, acetyl-CoA, and α-ketoglutarate) required for epigenetic histone and protein modifications.

Vast strides have recently been made in understanding the biology and pathobiology of mitochondria, with mitochondrial dysfunction being implicated in many disease states including obesity, insulin resistance and cancer. This work contributes significantly to the role of dietary toxins in modulating a cross talk between cell stress responses and toxicological outcomes. These

findings add a novel aspect to the importance of mycotoxin toxicity particularly in the fields of mitochondrial and environmental toxicology.

Mycotoxin producing fungal species are contaminants of agricultural commodities under a wide range of environmental conditions, making them common foodborne toxins. The economic burdens of mycotoxin contamination are of great consequences. Crops with large amounts of mycotoxins are often destroyed. Despite several national and international organizations that set recommended guidelines and maintain up-to-date information on regulatory statutes of principal mycotoxins, FA is often neglected. This thesis supports the classification of FA as an "emerging mycotoxin" and highlights the acute toxicity of FA. Studies on acute exposure to mycotoxins are highly relevant in the South African context. In developing countries, access to safe food is often limited, leading to the consumption of contaminated food. Because of this and together with the recently available information on frequent occurrence and *in vitro* mechanisms of FA toxicity, more data on the exposure of humans and animals as well as *in vivo* studies clarifying possible toxic effects are warranted.

6.2. References

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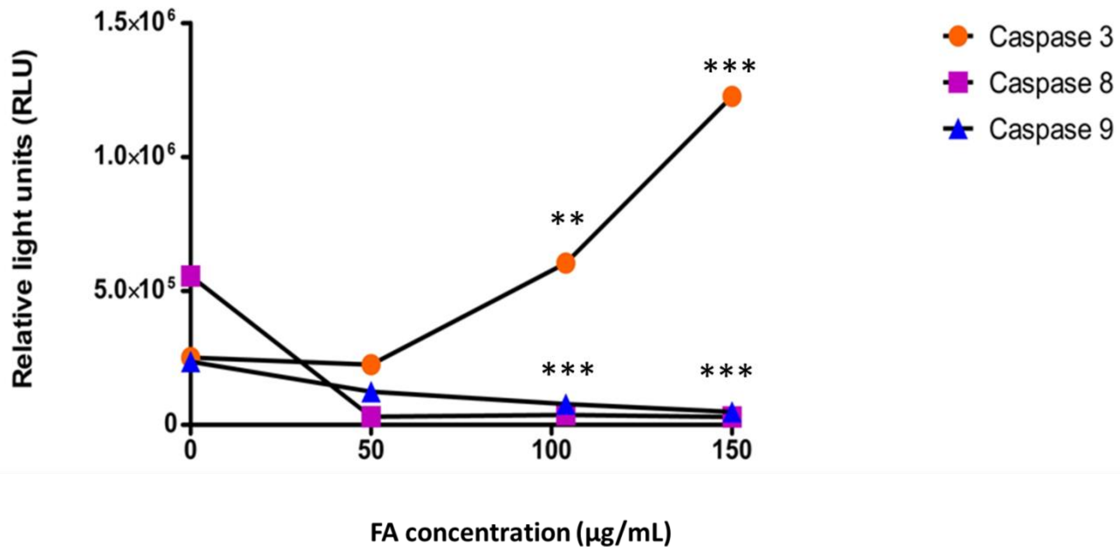
ADDENDUM A

Apoptosis Assessment (Dose dependent caspase activation)



Addendum A: Figure 1: Initiator caspases decreased at higher concentrations of FA while executioner caspases increased at higher concentrations of FA. Results are expressed as mean and SD (n=3). ** - control vs FA µg/mL p 0. 001-0.05, *** - control vs FA µg/mL p < 0.001

Assessment of apoptotic proteins involved in the intrinsic pathway.

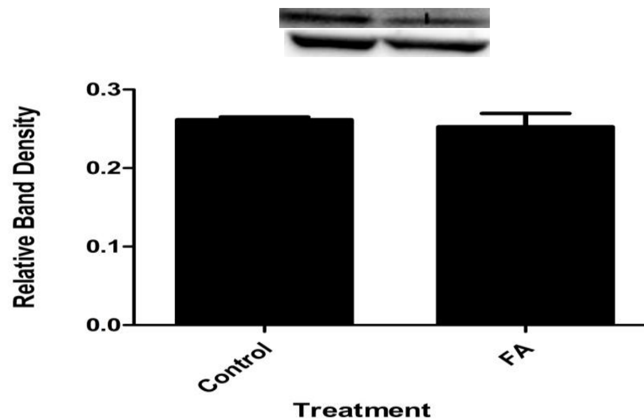


Figure 2: BAX showed no significant difference between control and IC_{50} (104 µg/mL). Results are expressed as mean and SD (n=3).

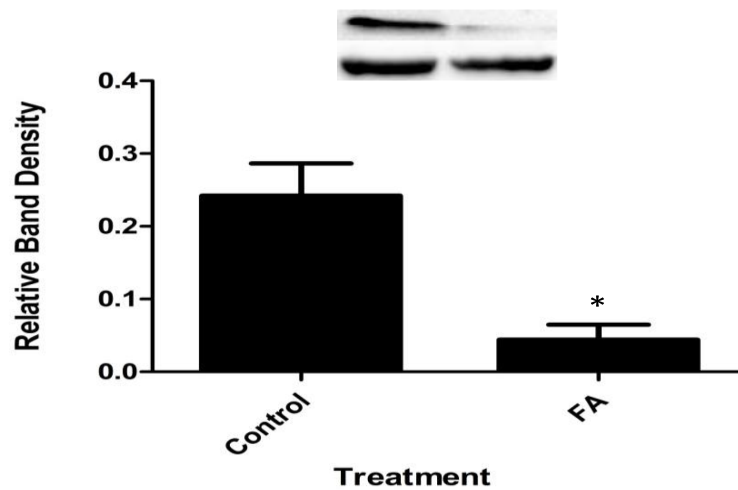


Figure 3: SMAC was significantly decreased (p=0.0199) between control and IC_{50} (104 µg/mL). Results are expressed as mean and SD (n=3). * - control vs FA µg/mL $p < 0.05$

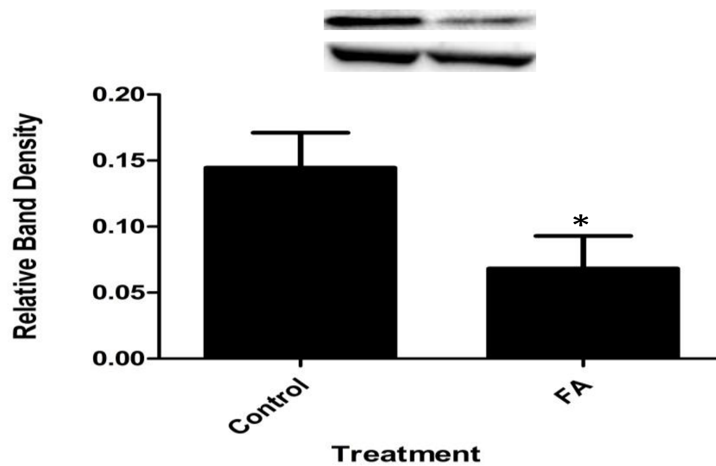


Figure 4: ILP2 was significantly decreased (p=0.0352) between control and IC_{50} (104 µg/mL). Results are expressed as mean and SD (n=3). * - control vs FA µg/mL $p < 0.05$

ADDENDUM B

Effects of FA on inflammatory mediators

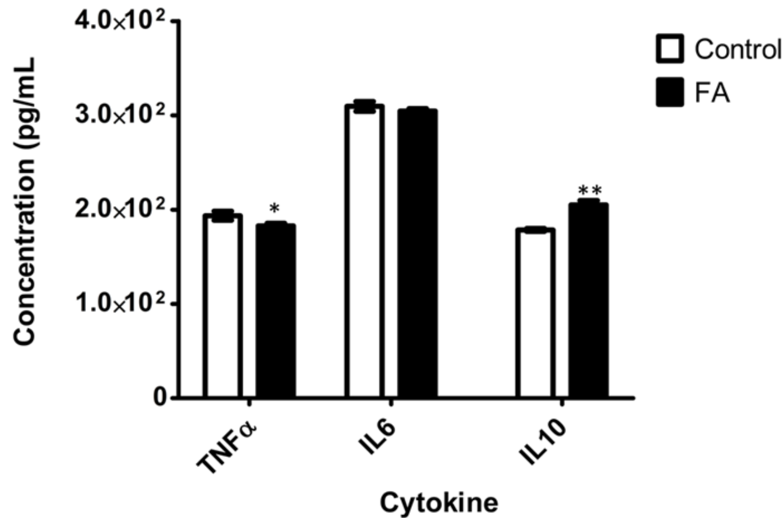


Figure 1: TNFα was significantly decreased (p=0.00435) and IL10 was significantly increased (p=0.0017) between control and IC_{50} (104 µg/mL). Results are expressed as mean and SD (n=3). * - control vs FA µg/mL $p < 0.05$, ** - control vs FA µg/mL p 0. 001-0.05

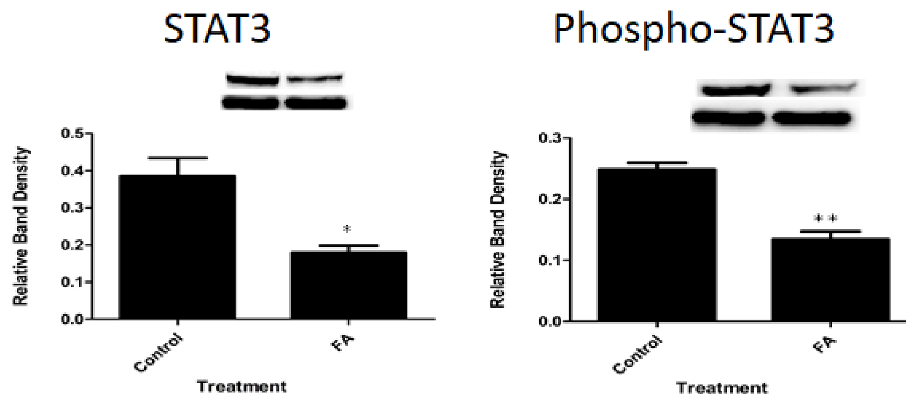


Figure 2: Both total and phospho stat was significantly downregulated between control and IC_{50} (104 µg/mL)._Results are expressed as mean and SD (n=3). * - control vs FA µg/mL $p < 0.05$, ** - control vs FA µg/mL p 0. 001-0.05.

ADDENDUM C

Mitochondrial dysfunction induced by FA as a function of time

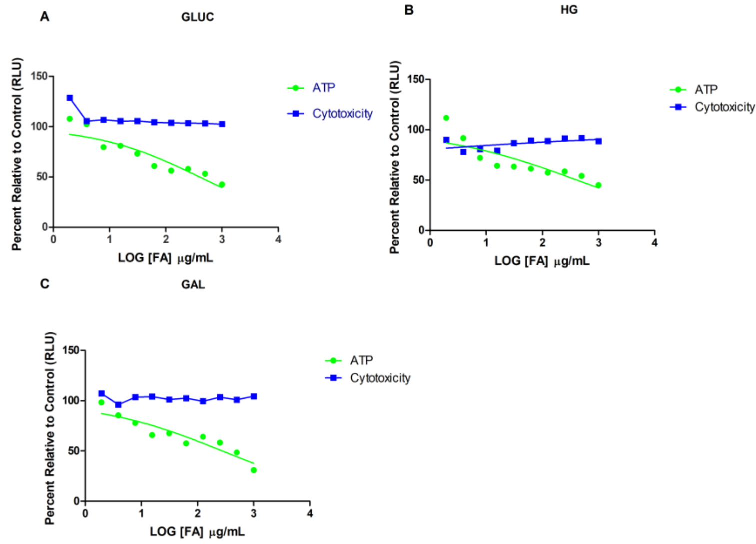


Figure 1: After 2 hours exposure to FA, the ATP output did not decrease below 50% for any of the chosen substrates (A – Glucose, B – High Glucose and C- Galactose).

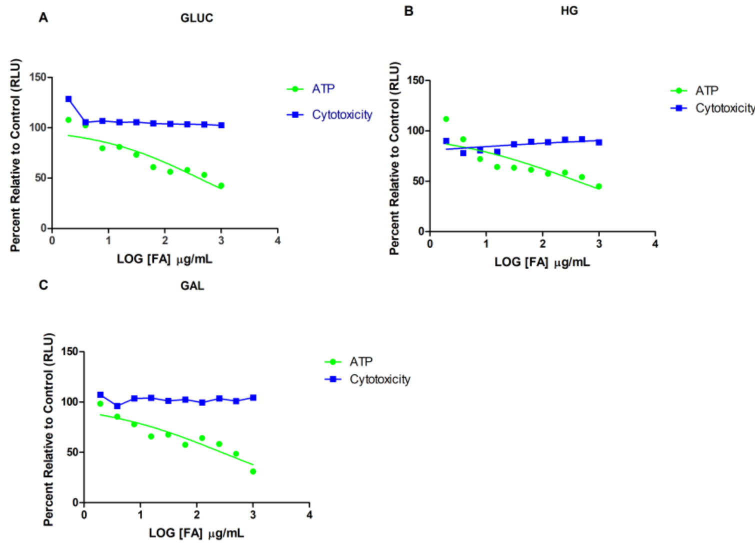


Figure 2: After 4 hours exposure to FA, the ATP output did not decrease below 50% for any of the chosen substrates except GAL at the highest tested FA concentration (A – Glucose, B – High Glucose and C- Galactose).