MODULATION OF MITOCHONDRIAL-MEDIATED APOPTOSIS IN THE HEART AND LIVER OF STREPTOZOTOCIN-INDUCED DIABETIC RATS BY QUERCETIN AND VITAMIN E

BY

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ABSTRACT

A major feature of Type 2 Diabetes Mellitus (T2DM) is tissue wastage, arising from excessive apoptosis. Excessive apoptosis could be a function of mitochondrial disorders in T2DM. Antioxidants, such as quercetin and Vitamin E, have been shown to be useful in the delay of progression of diabetes-induced complications. However, their effect on the apoptotic process in T2DM is unknown. This study was designed to investigate the effects of quercetin and vitamin E on mitochondrial Permeability Transition (mPT) pore, an event preceding apoptosis, in streptozotocin-induced diabetic rats.

Diabetes was induced in male Wistar rats with a single intraperitoneal administration of 40 mg/kg streptozotocin (STZ). Animals which had consistent 72 hours fasting blood glucose concentrations of ≥250 mg/dL were considered diabetic. Thirty-six diabetic rats (100-120 g) were equally divided into six groups, treated orally and daily for 28 days with water (diabetic control; DC), 10 mg/kg quercetin (Q_{10}), 30 mg/kg quercetin (Q_{30}), 10 mg/kg Vitamin E, 10 mg/kg each of quercetin and Vitamin E (Q_{10} &VitE) and 0.6 mg/kg glibenclamide separately. Additional 6 normal male rats were used as non-diabetic control. Animals were sacrificed, liver and heart mitochondria were isolated by differential centrifugation. Mitochondrial lipid peroxidation (mLPO), mPT and mitochondrial ATPase (mATPase) were determined by standard methods using a spectrophotometer. Levels of Cytochrome C Release (CCR), activities of caspase 9 (C9) and caspase 3 (C3) were determined immunohistochemically in heart, and liver. Insulin level was determined using ELISA. Formalin-fixed heart and liver tissues were examined microscopically after Haematoxylin and Eosin staining. Data were analysed using descriptive statistics and ANOVA at $\alpha_{0.05}$.

The percentage reduction in mLPO in the liver and heart were 11.2, 22.0 (Q_{10} & V); 13.4, 13.1(Q_{10}); 22.4, 27.2 (Q_{30}); 13.1, 38.0 (Vitamin E) and 15.4, 41.3 (glibenclamide) respectively relative to DC (liver 61.3; heart 78.0). Similarly, the percentage inhibition of mPT pore in the liver and heart were 89.2, 87.3, 64.0, 85.2, 70.1 and 50.3, 45.4, 82.0, 50.2 and 43.4, respectively in the Q_{10} & V, Q_{10}, Q_{30}, Vitamin E and glibenclamide compared with DC (liver 14.3; heart 20.0). Liver and heart mATPase were reduced when treated with Q_{10} & V, Q_{10}, Q_{30}, and Vitamin E relative to DC. Significant decreases in CCR were observed in the liver (41.0, 23.3 and 58.1%) and heart (33.2, 20.3 and 17.1%) after treatment with Q_{10} &V, Q_{30} and Vitamin E compared with DC (liver 90.4%; heart 98.0%). The STZ-induced CCR was associated with reduction in liver C9 by 12.0% in Q_{10} & V and in the heart by 28.2% in Vitamin E. Activation of C3 in T2DM rats were reduced in the liver by 10.2% in glibenclamide and in the heart by 16.1% in Vitamin E relative to DC (liver 73.4%; heart 65.2%). Insulin was increased significantly in all the treatment groups relative to DC. Histological examinations revealed congestion of vessels in the heart and liver.

Co-administration of quercetin and Vitamin E synergistically protected the heart and liver of rats with Type 2 Diabetes Mellitus via down regulation of mitochondrial-mediated apoptosis.

Keywords: Mitochondrial permeability transition, Type 2 diabetes mellitus, Cytochrome C release, Caspases 3 and 9

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CERTIFICATION

I certify that this work was carried out by Oluwatoyin Osinimega Daniel in the Department of Biochemistry, College of Medicine, University of Ibadan, Nigeria.

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DEDICATION

To God Almighty, the giver of life and strength, who kept me in all my days during the course, may your name be exalted.

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ABBREVIATIONS

T2DM	Type 2 diabetes mellitus
dATP	deoxy Adenosine Triphosphate
DNA	deoxyribonucleic acid
TNF	Tumor necrosis factor
Fas L	Fas ligand receptor
FADD	Fas-associated death domain
DISC	Death inducing signaling complex
mPT	mitochondrial Permeability Transition
STZ	Streptozotocin
F_0F_1ATPase	F_0F_1Adenosine trphosphatase
DM	Diabetes mellitus
CoA	Co-enzyme A
ATP	Adenosine triphosphatase
IL-6	Interleukin-6
CRP	C-reactive protein
TNF-α	Tumor necrosis factor- α
ETC	Electron transport chain
ROS	Reactive oxygen specie
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate

SDH	Sorbitol dehydrogenase
AGEP	Advanced glycated end-products
NF-κB	Nuclear factor-κB
PKC	Protein kinase C
VEGF	Vascular endothelial growth factor
eNOS	Endothelial nitric oxide synthase
TGF	Transforming growth factor
UDP-GluNAc	UDP-N-acetyl glucosamine
GluNAc	N-acetyl Glucosamine
GLUT 1,2,3,4	Glucose transporter 1,2,3,4
MSG	Monosodium glutamate
EMED	Encephalomyocarditis
VDAC	Voltage dependent anion channel
FADH	Flavin adenine dinucleotide
RNA	Ribonucleotide
Cyt C	Cytochrome C
AIF	Apoptosis Inducing Factor
ANT	Adenine Nucleotide Translocase
Cyp D	Cyclophilin D
PPIase	Peptidylpropylcis-transisomerase
CsA	Cyclosporin A

$\Delta\psi$	Membrane potential change
SFA	Sangliferin A
PiC	Phosphate carrier
ADK	Adenylate kinase
SMAC/DIABLO	Second mitochondrial-derived activator of caspase
TSPO	Translocator Protein
CRMT 1	Creatine kinase mitochondrial 1
HK I and II	Hexokinase 1 and 2
GSK 3β	Glycogen synthase kinase 3β
APAF-1	Apoptosis Inducing factor 1
TRADD	TNF receptor associated death domain
cAMP	cyclic Adenosine monophosphate
cGMP	cyclic Guanosine monophosphate
IPR	Inositol-3-phosphate receptor
PML	promyelocytic Leukemia Protein
PTEN	Phosphate and Tensin Homolog
$^{\cdot}OH$	Hydroxyl radical
$O2^{\cdot-}$	Singlet oxygen
H_2O_2	Hydrogen peroxide
LOOH	Lipid hydroperoxide
$RO^{\cdot}$	Alkoxyl radical

$^{\cdot}OOH$	Peroxyl radical
$SO_4^{\cdot}$	Sulfate radical
AST	Aspartate amino transferase
ALP	Alanine amino transferase
PIPES	Piperazine-N, N'-bis (2-ethanesulfonic acid)
ESPAS	N-ethyl-N-sulfopropyl-m-anizidine
ELISA	Enzyme-linked aminosorbent assay
TMB	3, 3', 5, 5'-tetramethylbenzidine
FSH	Follicle Stimulating Hormone
OD	Optical Difference
BSA	Bovine Serum Albumin
MDA	Malondialdehyde
TBARS	Thiobarbituric acid reactive specie
SDS	Sodium deodocyl sulfate
Pi	Inorganic phosphate
HMG-CoA	3-hydroxyl-3-methyl-glutaryl CoA
Q_{10}VitE	Steptozotocin-induced diabetic rats treated with 10 mg/kg each of Quercetin and Vitamin E
Q_{10}	Steptozotocin-induced diabetic rats treated with 10 mg/kg Quercetin
Q_{30}	Steptozotocin-induced diabetic rats treated with 30 mg/kg Quercetin

PQ	Rats pretreated with 30 mg/kg quercetin for three days before Steptozotocin-induction of diabetic and continuation of treatment for 28 days
VitE	Steptozotocin-induced diabetic rats treated with 10 mg/kg Vitamin E
Glib	Steptozotocin-induced diabetic rats treated with 0.6 mg/kg Glibenclamide
IMM	Inner mitochondrial membrane
OMM	Outer mitochondrial membrane
MSH suspension	210 mMMannitol, 70 mM Sucrose 5 mM HEPES-KOH
MST	220 mMMannitol, 70 mM Sucrose 5 mM (Tris (hydroxymethyl) aminomethane) Tris-HCl suspension
NTA	No Triggering agent
TA	Triggering agent
HEPES	N-2-hydroxyethylpiperazine-N-N'-2'-ethanesulfonic acid salt
LPO	Lipid peroxidation
Pi	Phosphate
PBS	Phosphate buffered saline
CARD	Caspase Activation and Recruitment Domain
PARP	Poly ADP-ribose polymerase
APES	Aminopropyltriethoxysilane

CHAPTER ONE

1.0 INTRODUCTION

Diabetes is an enigma that has taken its toll on the world population of 382 million, with an escalating prediction to affect about 592 million people globally in 2035 (IDF, 2013; Ojieabu *et al.,*2017). It has been predicted that by 2035, 23.9 million people would be affected in Africa with Nigeria having the highest prevalence (Ojieabu *et al.,*2017). Oputa and colleagues noted that Type 2 Diabetes (T2DM) contributes 95% to the overall cases of diabetes reported in Nigeria with prevalence more in females than males (Oputa *et al.,*2015). Discovery of new treatment molecules for the disease is on the increase since synthetic drugs have shown limited effects on complications of the disease including gastro intestinal discomfort, susceptibility to risk of cancer of the bladder, cardiovascular abnormality, edema and in post-menopausal women distal bone fracture (Vaeron, 2013).

In a search of molecules that will be able to manage the complications more effectively, mitochondria have come into limelight, since excessive regulated cell death (apoptosis) is prominent in all forms of diabetes. Apoptosis or programmed cell death is a typical component of growth, development and well-being of multicellular organisms (Bernardi and Di Lisa*,* 2015). Upregulation of apoptosis has been implicated in numerous diseases including neurodegenerative, cardiovascular diseases and arthereosclerosis. In diabetes, damage of the pancreatic beta cells which are responsible for insulin production by

apoptosis causes elevation of blood glucose among other complications (Gong *et al.,*2011). It is now well established that apoptosis takes place through three major pathways, the mitochondrial-mediated pathway (intrinsic), receptor-mediated pathway (extrinsic) or granzyme morphologic pathway.

The intrinsic pathway is a cellular stress mediated occurrence in which antiapoptotic Bcl-xL is unable to engage proapoptotic Bax appropriately in an association that prevents the transmembrane movement of Bax to the outer mitochondria where oligomers are formed that enables the exit of cytochrome c (Cyt C). When Cyt C is released due to compromised mitochondrial membrane permeability or integrity, it associates with dATP which recruits caspase 9 in its inactive form, this arrange to produce apoptosome complex. As a result of the formed complex, the inactive caspase 9 (procaspase 9) becomes activated to caspase 9, which turns-on the activity of the executioner caspases 3, 6 and 7 which are mobilized to commission the fragmentation of DNA (Mcllwain *et al.,*2016). These events undoubtedly reveal the pivotal relevance of the mitochondrion in the highly conserved intracellular pathway of cell death (Mcllwain *et al.,*2016).

Extrinsic apoptosis on the other hand involves a cell death receptor which is found on the extracellular site of the cell that transmit signals that initiate cell death. They include tumor necrosis factor (TNF), Fas ligand receptor (Fas L) and others (Parrish *et al.,*2013). On receipt of signal by Fas L, the Fas L death domain (FADD) recuits initiator caspase 8 towards the death inducer called death inducing signaling complex, DISC, which enables activation of caspases 3, 6 and 7. This then results in DNA fragmentation and other apoptotic features (Mcllwain *et al.,*2016).

Some naturally occurring compounds found such as flavonoids, alkaloids, terpenes among others have been shown to possess antioxidant, antimicrobial, anti-inflammmatory roles in different types of diseases and they delay the progression of complications.

1.1 JUSTIFICATION OF THE STUDY

One major feature of diabetes mellitus is tissue wastage arising from increasing apoptosis. Apoptosis, a type of regulated cell death, has the unique physiological role of maintaining tissue homeostasis and regulation of embryonic development among other functions. The process is believed to take place via three (3) mechanisms including the death domain receptor or extrinsic apoptotic pathway, mitochondrial-mediated or intrinsic apoptotic pathway or the granzyme morphologic pathway. Interestingly, the mitochondrial-mediated pathway has been revealed to involve the release of Cyt C when the mitochondrial Membrane Permeability (mPT) pore opens thus making the pore a pharmacological target in diseases involving dysregulated apoptosis. Several researchers are looking for substances that may demonstrate pharmacological usefulness in the reversal of emaciation in complicated diabetic situation due to the inability of anti-diabetic drugs to completely alter the complications arising from the disease (Srinivasan and Indumathi, 2003).

1.2 GENERAL OBJECTIVE

This study was designed to determine the effectiveness of quercetin and vitamin E on the minimising of the complications in diabetes via the inhibition of mitochondrial-mediated apoptosis.

1.3 SPECIFIC OBJECTIVES

1. To assess the effect of quercetin (*in vivo*) on the opening of mPT pore of normal rat heart and liver.
2. To assess the mPT status in STZ-induced diabetic rat heart and liver and investigate the inhibitory status of vitamin E and quercetin on mPT pore in STZ-induced diabetic rat heart and liver.
3. To detemine the effects of STZ-induced diabetes and treatment with quercetin and vitamin E on mitochondrial F_0F_1 ATPase activities of the heart and liver cells.
4. To determine the ameliorative role of vitamin E and quercetin on mitochondrial lipid peroxidation in STZ-induced diabetic rats.
5. To determine the inhibitory role of vitamin E and quercetin on the release of mitochondrial Cyt C in the liver and heart of STZ-induced diabetic rats.
6. To assess the extent of activation extent of caspases 3 and 9 in certain visceral organs in STZ-induced diabetic rats and probable intervention by vitamin E and quercetin.
7. To investigate the pathophysiology of some tissues in STZ-induced diabetic rats and probable intervention with vitamin E and quercetin.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 DIABETES

A chronic endocrine malady branded by elevation of blood glucose (hyperglycemia) in the body owing to disrupted insulin secretion or utilization or both is Diabetes Mellitus (DM). The pancreatic β-cells of the Langerhans is the domain for the synthesis of insulin hormone, which is a key player in the systematic maturation of tissues and critical in the regulation of glucose homeostasis. This hormone is responsible for glucose mobilization into the muscle and adipose tissues. It reduces hepatic glucose concentration by gluconeogenesis, glycogenolysis and stimulates glycolysis and glycogen synthesis (Giacco and Brownlee, 2010; Riaz, 2015). It is required in lipid metabolism by increasing lipid synthesis while inhibiting lipolysis. It also increases amino acid absorption and body protein synthesis. Insulin action is triggered by the activation of its tyrosine kinase receptor which occurs by binding of the hormone to the α-subunit of the receptor causing autophosphorylation. When the hormone is diminished or completely absent, antagonistic hormones, epinephrine and glucagon dominate metabolic regulation. This increases gluconeogenesis and inhibits glycolysis.

It has been well known that diabetes-induced hyperglycemia is responsible for morbidity and mortality caused by micro and macro vascular complications (Bastaky 2005; Rains and Jain, 2010; Ullah *et al.,*2015). Diabetes is a worldwide prevalent metabolic disorder that has affected about 23.6 million American population, 12.1 million African population (Whiting *et al.,*2011) and it affected 210 million of the world population of people in 2010, with the number predicted to increase to about 300 million in 2025 (Ullah, 2015) and thereafter upsurge to about 592 million by 2035 (Guariguata *et al.,*2014).The higher majority of the victims are those that live in countries with low and middle per capital income confirming the large burden of the disease in developing world. Studies by Abdulganiyu and Fola (2014) established that the annual national cost of illness for diabetes in Nigeria may be about N225 billion presenting 88% of annual per capital income. The incidence of the disease has increased from 2.2% in 1997 to 5% by 2013 with one-third in the rural regions and two-third in the urban dwellers. Also about 2 million of the population with diabetes is undiagnosed. Statistics have shown that Nigeria is tasked with the highest prevalence of diabetes in the continent with South Africa following closely behind with 2.6 million affected by the disease. About 1.9 and 1.7 million people have been shown to be living with diabetes in Ethiopia and Tanzania respectively (Oputa and Chineye, 2015). Since this have huge consequences on the world's failing socio-economy burden and health care system, decrypting the mechanism of the disease causality would probably be a path to proffer a better model of treating the disorder.

There are two classical types of the disease namely, the type I and 2 while others include idiopathic and gestational diabetes (Ullah *et al.,*2015).

2.2 TYPE I DIABETES MELLITUS

Type I diabetes mellitus (TIDM) has been generally referred to as the insulin dependent DM or a diabetes that occur at juvenile stage, which is approximately 5-10% of all diabetes cases. This type of diabetes is linked with the DQA and DOB genes that are influenced by DRB genes. The rate of destruction of the β-cells varies, for infants, rapid destruction is observed as compared with slower destruction in adults indicating that the severity might be more in children while the adults may retain residual insulin which probably may prevent ketoacidosis. (ADA, 2008). Patients with T1DM may also present with graves disease, hashimoto thyroiditis, addison disease, vitiligo among others (ADA, 2008). Patients with T1DM consequently require insulin for treatment and withdrawal of this results in ketoacidosis (Sadeghi *et al.,*2016).

There are two types of this class of disease namely type IA and IB. The type IA diabetes is said to be mediated by the immune system because these cells destroy the β-cells, while type IB is caused by insufficient insulin production by the β-cells (Arora *et al.,*2013).

Symptoms of Type 1 Diabetes Mellitus

a. Increased blood glucose which exceeds normal concentration range of 5-9 mM.
b. The increased formation of ketone bodies (ketoacidosis) leads to elevation of the blood pH
c. Increased level of lipoproteins referred to as blood fats.
d. Increased plasma and urine levels of urea occurs due to the accelerated protein breakdown in muscle, since the muscle couldn't assimilate glucose again, it switches role to protein breakdown for its essential requirements of ATP. Since

nitrogen is disposed from degraded amino acids it generates alanine and glutamine, which are translocated to the liver to supply substrates to the urea cycle and gluconeogenesis.

e. Increased thirst due to loss of fluid as a result of osmotic activity of glucose in urine and increased urine flow.
f. Acetone smell (acetone is one of the ketone bodies).
g. Observed weight loss due to the degradation of protein and fat in the body.
h. In severe cases, the patients may be unconscious (comatose).

2.3 TYPE 2 DIABETES MELLITUS

This class is the most common type of diabetes in the globe, showing a data of about 90-95% of all diabetic cases with most of the patients being obese and having no observable ketoacidosis (ADA, 2008). There is defect of the secreted insulin and or resistance probably because of increase in age and obesity. It is known that T2DM also occurs in people who subsist on poor diet; poor exercise habit and those genetically predisposed (Lee *et al.,*2017). Peripheral tissues show less sensitivity to insulin, and so increased levels of insulin are essential to achieve the necessary response in the peripheral cells. Findings of Chartzigeorgiou *et al.* (2009), have shown that high percentage of patients with T2DM do not have large body mass but they show low secretion of insulin with reduced resistance than the obese phenotype.

The T2DM is found in patients who may have their insulin level elevated, showing that their β-cell function may still be active. It occurs more in patients with hypertension and

dyslipidemia. Robust data from Lee and colleagues (2017) showed that deletion of the kcne 2 gene potassium regulating subunit causes diabetes by down regulating insulin β receptor and substrate 1 (Lee *et al.,*2017). The intracellular metabolic dysregulation in T2DM is similar to that in T1DM, but typically less acute, since the residual insulin retains at least partial efficacy. T2DM is linked to other factors like obesity, lack of sleep, genetic factors among others (Arora *et al.,*2013). The causative factors of diabetes are many but findings of Fei and Zao (2013) have linked microbial infection to obesity and diabetes (Fei and Zao, 2013). Biochemical examinations revealed that diabetic obese subjects showed normal blood glucose levels when treated for microbial infection that caused weight loss. This showed that *Enterobacter* specie may be the source of the cause of diabetes (Arora *et al.,*2013, Fei and Zao, 2013). Studies have also shown the relationship between T1DM and the auto-reactive $CD8^+$ T cells (Devendra *et al.,*2004). Studies have shown that there is involvement of the immune response with T2DM and gestational diabetes with hypothesis that non-specific response immune system might cause the association of interleukin-6 (IL-6) with obesity and C-reactive protein (CRP) (Arora *et al.,*2013). Nutrition has also been shown to influence the level of IL-6 and CRP which directly increase the threat of susceptibility to diabetes because increase in the fat content of the meal increases their levels, which in turn increases the lipid content and then triggers oxidative damage and crowding the macrophages (Arora *et al.,*2013). The consequence of this is increased TNF-α, and insulin dependent uptake of glucose (Arora *et al.,*2013, Weisberg *et al.,*2003).

T2DM is modeled in moderately sized dosage of streptozotocin injection which had been shown to induce a gradually progressive DM with preserved β-cell mass in the pancreas (Lee *et al.,*2017). It is also studied in non-overweight and overweight animal models, both

having different degree of resistance to insulin and destruction of the β-cells (Tripathi *et al.,*2014). The disease is so common that it affects the young and old and only drugs for management are available. However, numerous side effects of these drugs have prompted researchers to look for alternative therapies that may be able to manage the disease more effectively.

Obesity has been shown to be a contributing factor in T2DM, it induces the release of leptin, TNF-α, resistin, adiponectin that modulate insulin secretion and contributes to its resistance (Riaz, 2015). This ultimately causes β-cell failure that results into release of inflammatory protein markers like IL-6 and CRP (Riaz, 2015).

2.4 GESTATIONAL DIABETES

This is also called Type 3 diabetes (T3DM) and it occurs in ~4% of all pregnancies in America and particularly in the third trimester of pregnancy. Studies have shown prevalence of about up to ~10% in the UK and black South Africans with an unknown data in Nigeria (Macaulay *et al.,* 2014; Macaulay *et al.,* 2018). It has its first occurrence at the onset of pregnancy and resolves after childbirth, although it increases the risk for T2DM in future (Ullah, 2015).

2.5 IDIOPATHIC DIABETES

Idiopathic diabetes has neither known etiologies nor evidence of autoimmunity but probably genetically inherited (ADA, 2008). Diabetes is one of the diseases where

excessive apoptosis is observed. It occurs when there is an increase in mitochondrial reactive species production which culminates into excessive death of cells. There is a prevailing hypothesis by Brownlee that proposes that hyperglycemia induced mitochondrial ROS generation has exacerbating effect on pathology of cells (Brownlee, 2005). This hypothesis explained that due to increased availability of NADH in the ETC, the tempo of electron flow upsurges, causing a mitochondrial membrane potential above the threshold level responsible for increased ROS generation (Brownlee 2005; Mapanga and Essop, 2016). Studies have also shown that this increase in mitochondrial potential can result into alteration of movement of electrons in complexes I and III causing accumulation in Coenzyme Q (Brownlee 2001; Du *et al.,*2002; 2003; Mapanga and Essop, 2016). This effect reduces molecular oxygen to mitochondrial superoxide which can cause destruction of the respiratory chain components and increase the ROS production (Paradies *et al.,*2004; Mapanga and Essop, 2016).

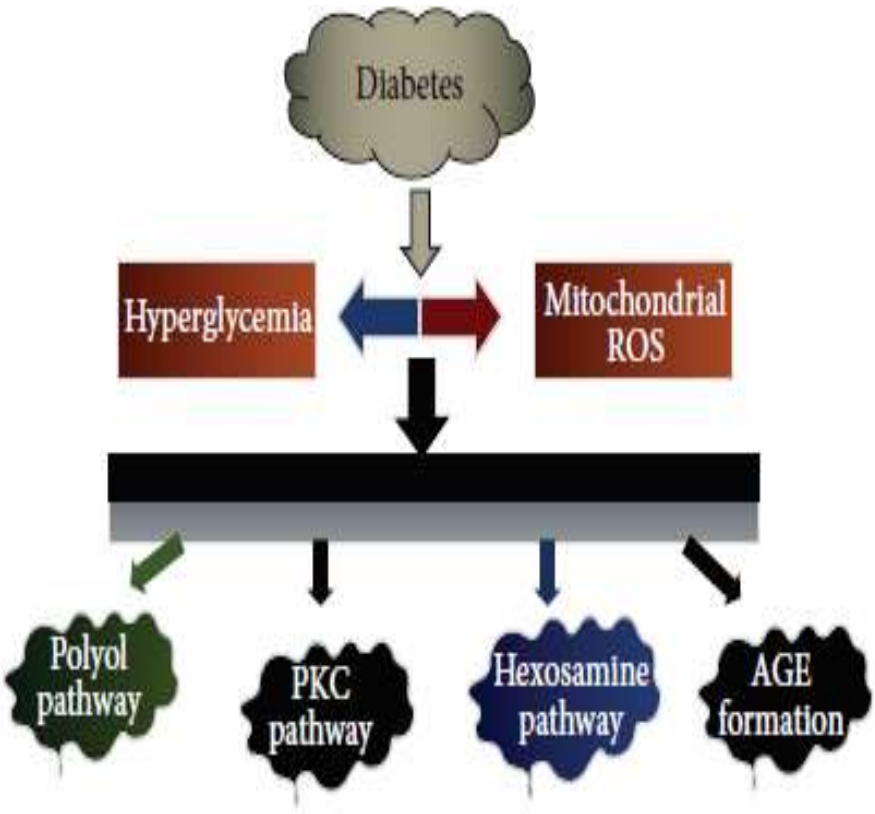


Figure 1: The mechanism of generation of mitochondrial ROS in Diabetes (Safi *et al.*, 2014).

It is now well known that there are four (4) mechanisms by which mitochondrial ROS are generated in complications arising from diabetes, they include:

The advanced glycated end products (AGEP), protein kinase C, polyol pathway flux, and hexosamine pathway flux.

1. Increased Polyol Pathway Flux

In normal physiological condition, glucose is oxidized by the glycolytic pathway and only ~3% goes into the polyol pathway but in diabetes due to hyperglycemia, the polyol pathway metabolizes ~30% glucose. This pathway becomes active in diabetic condition, when excess glucose is diverted into it due to the inability of the glycolytic pathway to manage the mass-imbalance. Aldo-keto reductase reduces the carbonyl compounds to their sugar alcohols (sorbitol) and this process utilizes NAD^+ as a cofactor and sorbitol dehydrogenase (SDH). The SDH oxidizes the sorbitol to fructose that can be funneled back into the glycolytic pathway. Glucose permeate cells readily through the aid of insulin hormone, but in certain cells that are insulin-independent like retina lens, glomerulus of the kidney, vascular cells and nervous tissue, glucose moves in an uncontrolled manner into them and the excess is diverted into polyol compound pathways. There is increased affinity of aldo reductase for glucose in hyperglycemia making sorbitol to accumulate, NADPH to be consumed as well as SDH. Since NADPH is required as a co-factor in glutathione (GSH) and nitric oxide generation, its consumption will result into depletion, hence an uprise in hemolysis, oxidative stress and eventually cell death (Brownlee 2001; Giacco and Brownlee, 2010; Ramasamy and Goldberg 2010; Behl *et al.,*2015; Javed *et al.,*2015). Activation of the polyol pathway alone do not result in diabetes complication but a

synergism with other dysfunctions trigger damaging outcomes in diabetes (van Dam *et al.,*2013).

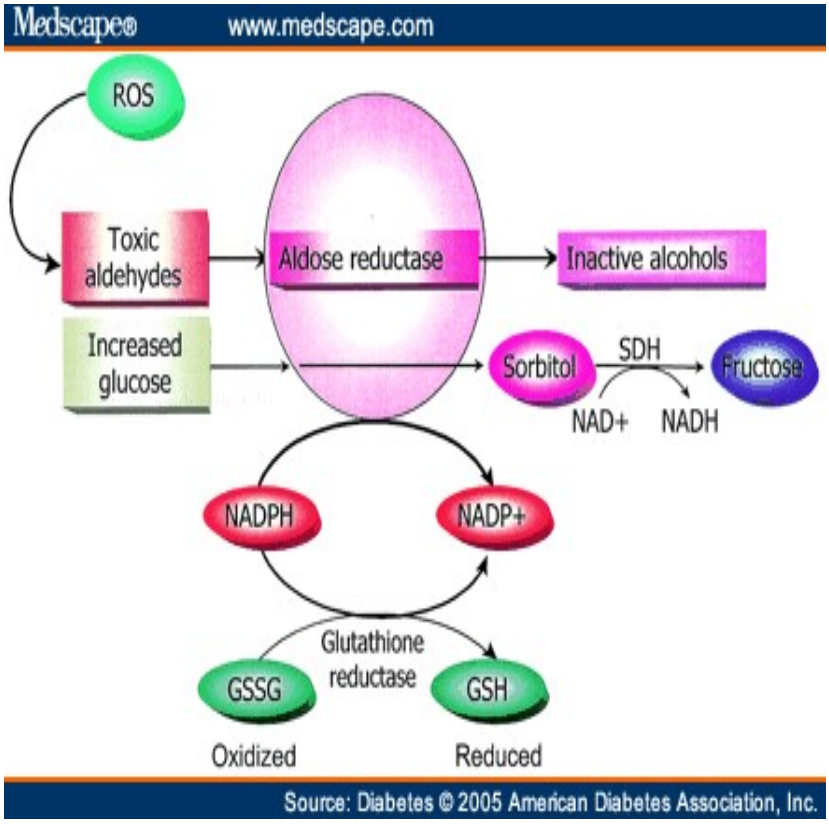


Figure 2: The Increase glucose flux into the polyol pathway due to hyperglycemia (Brownlee, 2005).

In the presence of aldose reductase, more sorbitol is obtained from glucose, this process utilizes NADPH, consequently making it unavailable for the generation of glutathione which subsequently lowers nitric oxide generation therefore reducing the antioxidant available in the body to combat available ROS. Whereas when this reaction is catalyzed by sorbitol dehydrogenase, it produces NADH (Mapanga and Essop, 2016).

From the figure above, it is shown that aldo-reductase toxify the system and damage DNA by reducing aldehydes generated from lipid peroxide to inactive alcohols. Generation of fructose from sorbitol increases the level of NADH which is a substrate of NADH oxidase required for superoxide production. Fructose-3-phosphate along with 3-deoxyglucosone are obtained from fructose, the end product of the polyol pathway, these compounds are also nonenzymatic glycation agents that increases the availability of advanced glycated end-products (AGEP) in hyperglycemia. It has been shown that methylglyoxal (an AGEP) increases aldo-reductase expression and this has effect on the p38 kinase pathway showing that the p38 kinase pathway increases AGEP formation subsequently elevating the generation of ROS (Srivastava *et al.,*2005; Mapanga and Essop 2016).

2. Increased Intracellular Advanced Glycated End Products Formation

The AGEP are predominant in diabetes due to increased glucose and carbonyl compounds obtained from fatty acids oxidation in arterial endothelial cells like heart. These products are non-enzymatically glycated and they can damage cells by three mechanisms including:

a) Alteration of vital proteins in the body by AGEP leading to their altered function.
b) Modification of the extracellular matrix components lead to altered functions and abnormal interaction with other matrix components expressed on the cell surface.

c) Modification of plasma proteins which subsequently bind to the AGEP receptors. Since these proteins are strange components on the receptors, they cause generation of reactive species which may activate the nuclear factor (NF)-κB. This would result in aberrant gene expression, ultimately causing excessive death of cells in diabetes (Giacco and Brownlee, 2010).

The protein glycation occurs owing to the reaction of the carbonyl atom of the reducing sugar with the amine group of the proteins to generate a Schiff base that forms an amadori product in a rearrangement reaction. The Schiff base has the unique characteristics of producing methylglyoxal and oxoaldehydes glyoxal in the Namiki pathway of the Maillard reaction that ensues during glycation formation (Mapsanga and Effsop, 2016). Due to the toxicity of methylglyoxal, there is immediate metabolism facilitated by the intracellular system, glyoxalase I and II acts in the presence of GSH to obtain D-lactate.. In diabetes, there is dysregulation of glyoxalase I, hence maintaining the high intracellular level of methylgloxal and its analogs would increase AGEP formation (Paradies *et al.,*2004; Mapanga and Essop, 2016).

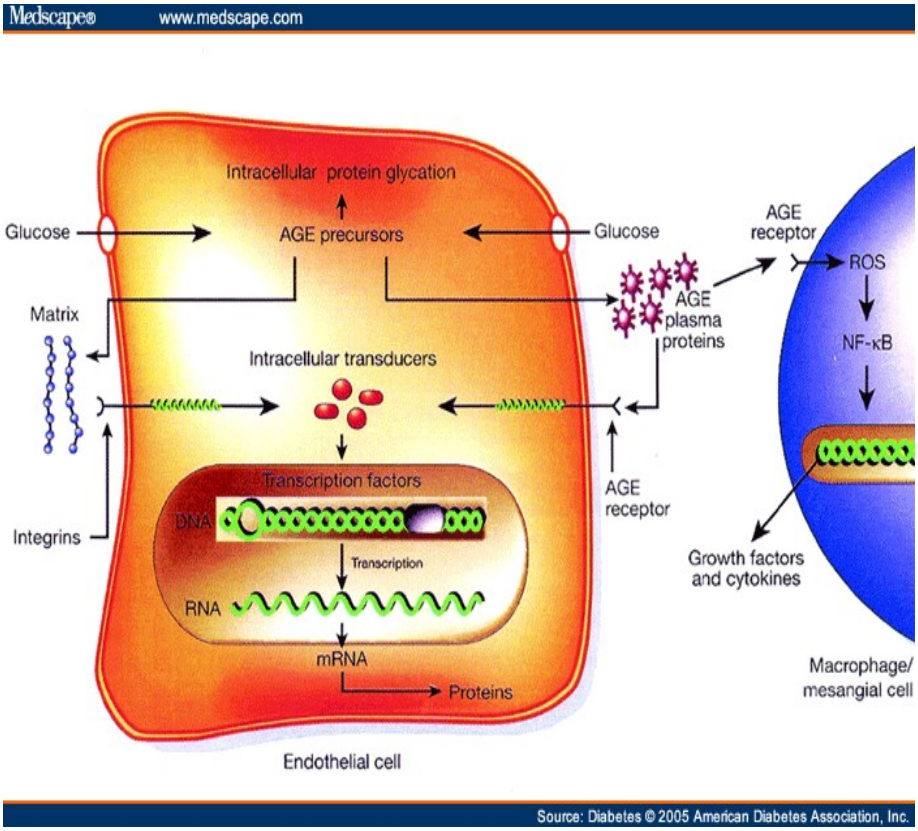


Figure 3: Formation of advanced glycated end-products (Brownlee, 2005).

3) Increased Activation of Protein Kinase C

The Protein kinase C (PKC) belongs to a complex class of enzyme which phosphorylates specific target proteins that carry out signal transduction in the intracellular system. Different isoforms of this protein exist with twelve (12) of them recorded in literature (Mapanga and Essop, 2016). These isoforms are activated by intracellular Ca^{2+}, phosphatidylserine and diacylglycerol (Giacco and Brownlee, 2010). In diabetes, intermediates of the glycolytic pathway can be converted to dihydroxyacetone phosphate which eventually can be converted to diacylglycerol therefore activating PKC isoforms and hence generating complications in diabetes (Giacco and Brownlee, 2010). Studies in vascular culture cells have validated the influence of diabetes on certain isoforms of PKC (β and δ), while in the retina cells, an unidentified target of the enzyme's signaling is increased by p38α, mitogen-activated protein kinase (MAPK), and PKC (Giacco and Brownlee, 2010).

More studies have revealed that in hyperglycemic conditions, PKC isoforms (α, β and δ) show damaging effects on the myocardium leading to abnormal contraction (Song *et al.,*2015; Mapanga and Essop, 2016). The protein also regulates functions in the vascular-like growth factor signaling endothelial activation, release of vasodilator, and enhancement of macromolecules permeability across the endothelial by phosphorylation of cytoskeletal protein. This indirectly control growth modulators expression. The PKC also induce the expression of smooth muscle cell tumor angiogenesis factor and vascular permeability factor, simply referred to as vascular endothelial growth factor (VEGF) (Rains and Jain, 2010; Paneni *et al.,*2013; Mapanga and Essop 2016). Increased activity of this protein also

decreased the smooth muscle cell's nitric oxide thereby downregulating the expression of eNOS stimulated by insulin (Mapanga and Essop, 2016).

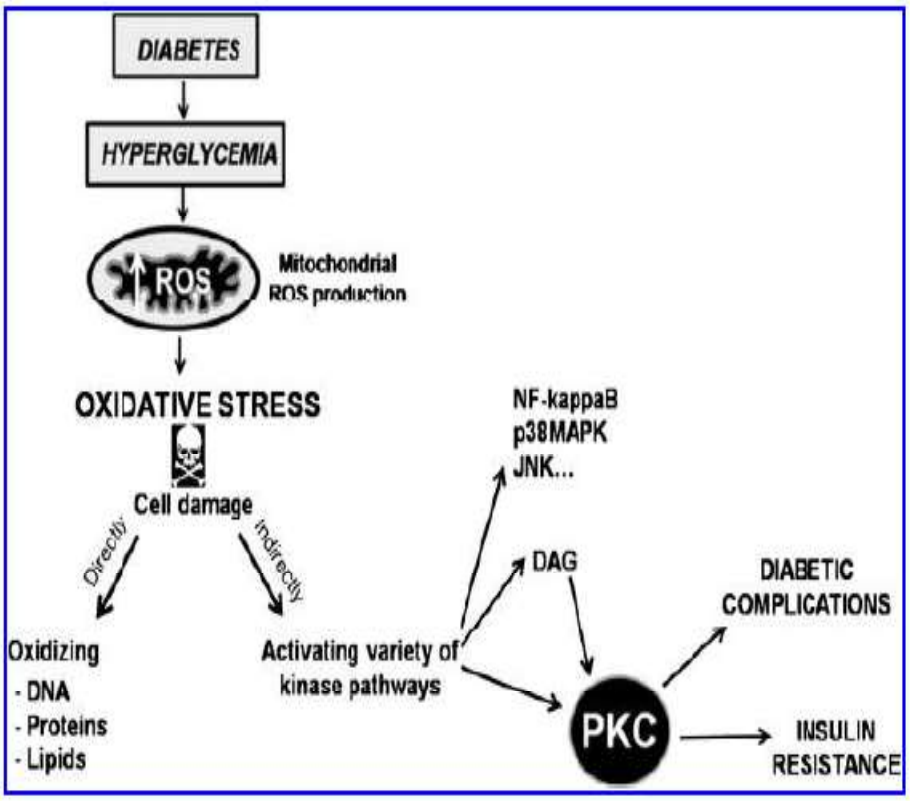


Figure 4: Increased PKC activation by hyperglycemia (Giorgi *et al.*, 2010).

4) The Hexosamine Pathway Flux in Diabetes

This pathway generates an amidated glucose derivative from fructose 6-phosphate in the presence of glutamine-fructose 6-phosphate amido transferase and this is subsequently converted to UDP-N-acetyl glucosamine (UDP-GluNAc). The UDP-GlcNAc is acted upon by O-linked β-N-acetyl glucosaminyl transferase, which transfers the GlcNAc to hydroxyl group side-chain of serine and threonine residues which produces glycosylated proteins (Mapanga and Essop, 2016). In diabetes, channeling of fructose 6-phosphate, the third reaction in glycolysis into the hexosamine pathway is exaggerated (Giocca and Brownlee, 2010). This increased flux cause glycosylation of certain proteins which results into vital enzymes dysregulation. Thereafter, vascular homeostasis, particularly the O-N-acetyl glucosamine acylation at the Akt site of eNOS protein would decrease eNOS activity and endothelial dysfunction (Paneni *et al.,*2013). Hyperglycemia has been revealed to induce the activity of glucosamine: fructose-6-phosphate amido transferase in the cells of the aortic smooth muscle while in the cardiomyocytes it affects calcium cycling through the increased nuclear acylation of O-GlcNAcylation (Giacco and Brownlee, 2010).

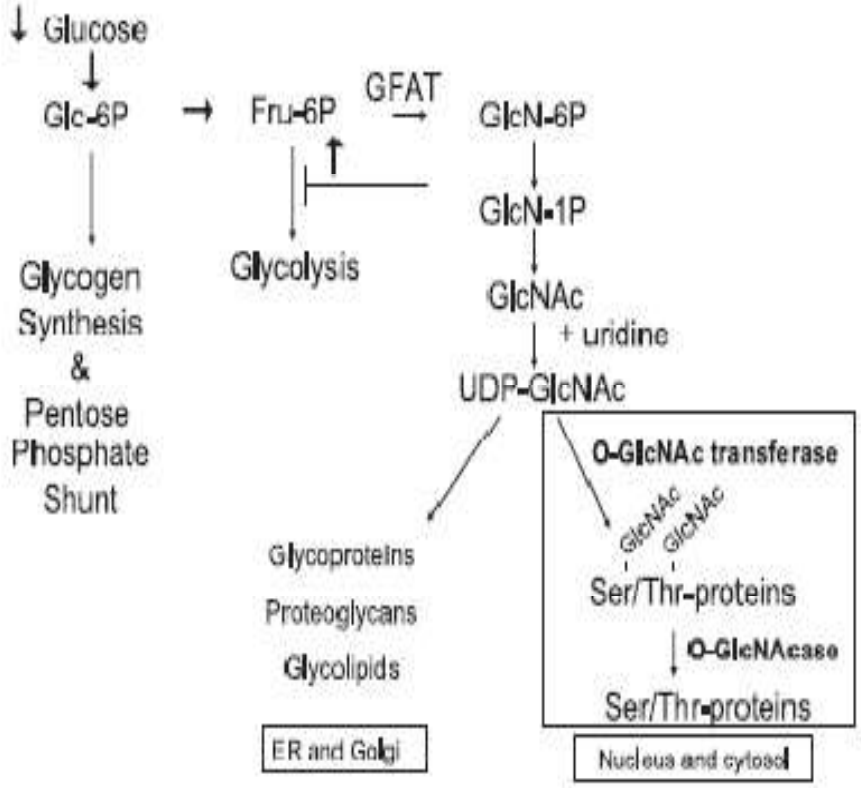


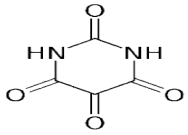
Figure 5: The hexosamine flux pathway in Diabetes Mellitus (Buse, 2006).

2.6 INDUCTION OF EXPERIMENTAL DIABETES

Chemical agents have been shown to be the most effective and easiest approach in experimental diabetes induction. This is due to the easily monitored and identified body changes, however other agents that induce diabetes include biological peptides, potentiators, and steroids. The commonest used chemical agents include alloxan, streptozotocin (STZ), dithizone, monosodium glutamate. These agents have been shown to alter and or completely destroy the function of the pancreas.

2.6.1 ALLOXAN

Alloxan (2,4,5,6 tetraoxypyrimidine: 2, 4, 5, 6 -pyrimidine tetron) is used to induce experimental diabetes, and its action features pancreatic β-cell death by necrosis. It can be administered intravenously, subcutaneously or intraperitoneally, while the required dosage is dependent on the animal model or strain. Alloxan modulates the blood glucose in three phases, in the first 30 minutes of induction, it stimulates insulin secretion and subsequent plasma concentration increase arising from higher ATP availability (Tripathi *et al.,*2014). The second phase happens after 1 hour of induction and this result in upsurge in hyperglycemia and simultaneous reduction in plasma insulin concentration. In the third phase, hypoglycemia ensues and lasts for about 8 hours after alloxan administration.



Alloxan (2,4,5,6 tetraoxypyrimidine: 2, 4, 5, 6 -pyrimidine tetron)

Mechanism of action

Alloxan generates an abrupt upsurge in insulin level irrespective of glucose concentration in the body, however this increase is transient because it is accompanied by suppression of the islet response to glucose (Tripathi *et al.,*2014). Alloxan interacts with the sulfhydryl group of glucokinase (to cause inactivation thereby inhibiting glycolysis) to cause dialuric acid formation, which subsequently re-forms alloxan in a reduction-oxidation round to generate ROS and superoxide radicals. The superoxide radical inturn produces hydrogen peroxide (H_2O_2) which eventually cause DNA fragmentation of the islet of the pancreas. Studies have also shown that elevated Ca^{2+} facilitates the alloxan-induced opening of the voltage dependent calcium channels facilitating its influx into the pancreatic cells (Tripathi *et al.,*2014).

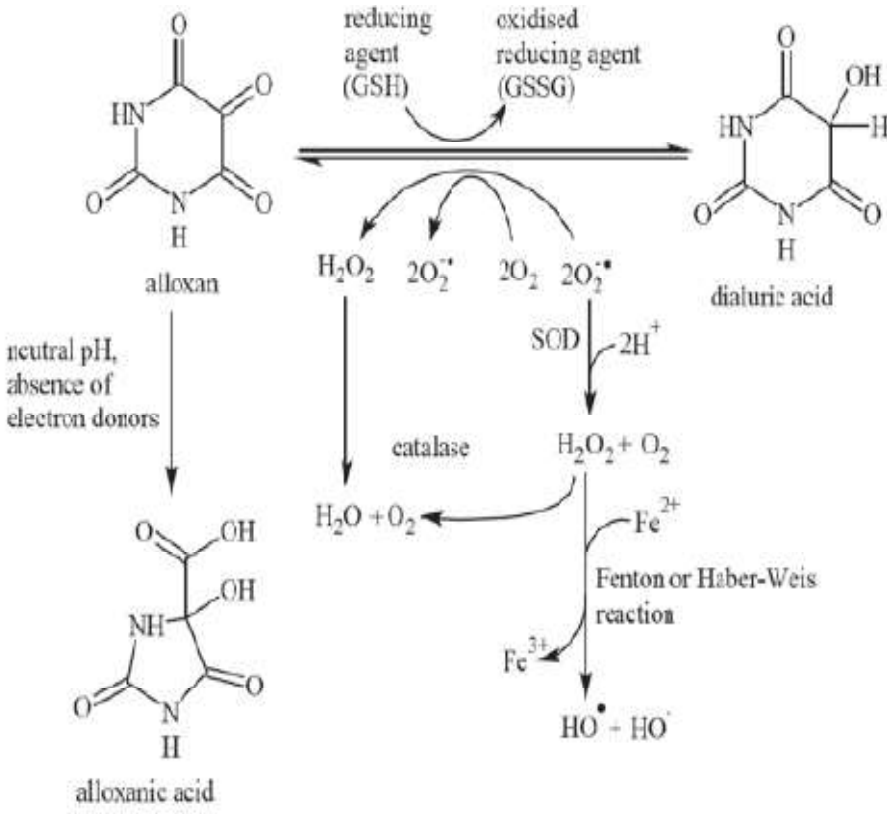
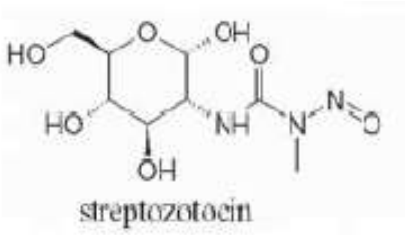


Figure 6: The mechanism of action of Alloxan (Soltesova and Herichova, 2011).

Alloxan is rapidly oxidized to alloxanic acid in the extracellular site, which in the intracellular site is oxidized to dialuric acid. This consumes oxygen and generates glutathione disulphide and superoxide anions which further produce peroxides.

2.6.2 STREPTOZOTOCIN

This compound is a glucosamine nitrosourea compound that alkylates, while causing toxicity to the cell by DNA damage. It is 2-deoxy-2-[3-methyl-3-nitrosoureido]-D-glucopyranose derivative of N-methyl-N-nitrosourea which is a Streptomyce achromogenes synthesis product.



streptozotocin

Streptozotocin (2-deoxy-2-[3-methyl-3-nitrosoureido]-D-glucopyranose)

This chemical can induce both Type 1 and 2 diabetes depending on the dose and duration. It can also cause partial or absolute pancreatic β-cell destruction depending on the dosage administered. In infant rat, induction of T2DM by STZ is achieved by injecting between 70-100 mg/kg doses (Soltesova and Herichova, 2011). In adult rats, Type 2 diabetes is induced by administration of low doses of up to 15 mg/kg of STZ and high fat diet (Soltesova and Herichova *et al.,*2011). The reason for this is that high fat diet will result into insulin resistance while the low dose will partially destroy the β-cells (Soltesova and Herichova, 2011). Studies by Deeds *et al.,*(2011) stressed that T2DM induction is acheived

by injection of moderate dose of 40 mg/kg of STZ without the use of high fat diet (Deeds *et al.,*2011). The β-cell is permeated by STZ through the glucose transport (GLUT) 2 found on the cell membrane (Soltesova and Herichova, 2011). There are 3 response phases in STZ induction which include:

1) Increase glucose level with decrease insulin secretion.
2) Hypoglycemic condition which is sustained for 2-4hrs after 4-8 hours of STZ injection (Soltesova and Herichova, 2011). This could lead to death of experimental animal by convulsion, due to temporary plasma insulin level elevation without damage to the β-cell.
3) In the last phase, about 12-24 hours after STZ administration, there is total degranulation and loss of β-cell integrity with the other part of the islet unaffected (Soltesova and Herichova *et al.,*2011).

Mode of action

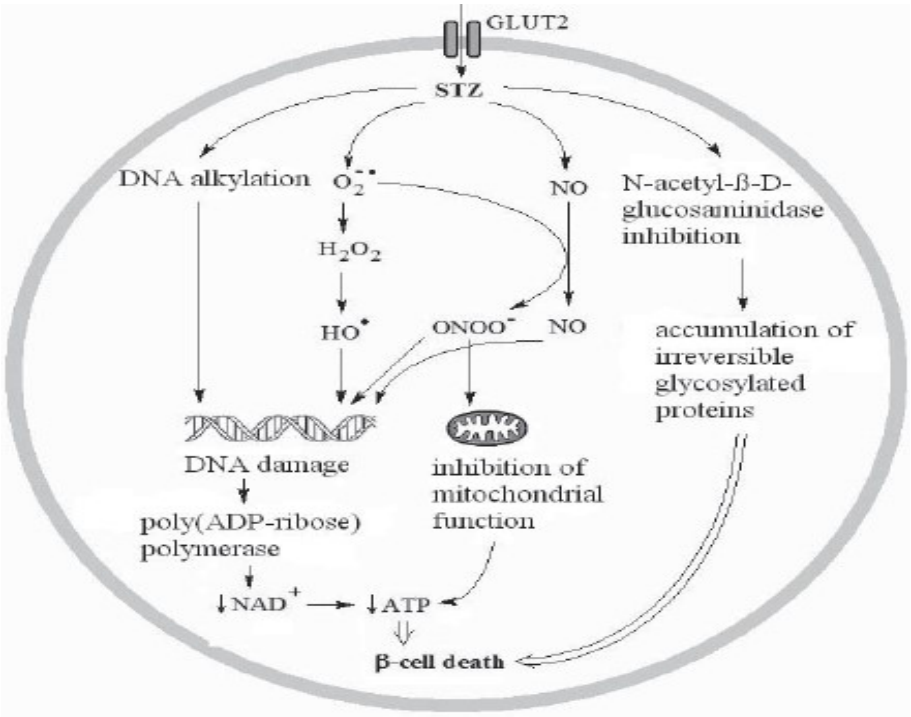


Figure 7: Action mode of Streptozotocin (Soltesova and Herichova, 2011).

Other agents that can be used to induce experimental diabetes include:

2.6.3 MONOSODIUM GLUTAMATE

This is derivative of naturally occurring non-essential amino acid that is easily water soluble, this induces T2DM without polyphagia. Monosodium glutamate act by causing high insulin response (Tripathi *et al.,*2014).

2.6.4 VIRUS

Studies have shown that Coxsackie virus and D- variant of encephalomyocarditis induce experimental T1DM. They act by destroying the β-cells of the pancreas (Tripathi *et al.,*2014).

1. Encephalomyocarditis (D-variant): this variant is administered to experimental animals and it consequently produces insulin dependent hyperglycemia

2. Coxsackie virus: this virus is capable of causing diabetes by causing the destruction of the acinar cells of the pancreas and subsequent induction of insulin dependent diabetes (Tripathi *et al.,*2014).

2.6.5 HORMONES

Diabetes induced by growth hormone: these hoormones induce diabetes by destruction of the pancreatic cells with complete absence or only traces of insulin remaining (Tripathi *et al.,*2014).

Corticosteroid induced diabetes: corticosteroid, commonly dexamethasone and prednisolone are capable of inducing steroid diabetes. These hormones act by inhibiting the action of insulin and activating gluconeogenesis leading to hyperglycemia, and hyperlipidemia (Tripathi *et al.,*2014).

2.7 BIOMARKERS OF DIABETES MELLITUS

2.7.1 BLOOD GLUCOSE

The blood glucose in the body is tightly regulated by certain organs which include liver, autonomic nervous system and endocrine glands. When the blood glucose is reduced in the body, it triggers the action of glucagon from the pancreatic cells while hyperglycemia in the body triggers insulin from the same site (Riaz, 2015). When the levels of glucose is dysregulated in the blood stream it causes hyper or hypoglycemia. There are different biochemical examinations that can be carried out on the blood glucose which include:

a) Fasting blood glucose

This is when the blood sample is obtained for analysis at least 8 hours after the last meal. This is used to know the severity of DM. The fasting blood glucose is usually done in the morning before breakfast and the value expected to be ≥ 200 mg/dL for diabetes (Ngugi *et al.,*2012).

b) Postprandial plasma glucose test

This diagnostic test is to investigate the capability of the body system to clear glucose after 2 hours of being challenged with carbohydrate load. This is to detect impairment in metabolism of glucose and a result of ≥ 1400 mg/dL glucose would usually be considered

diabetic. Peculiar limitation of this test is its high inaccuracy due to uncontrollable valuables like age, weight, state of health, the previous meal before the carbohydrate challenge, medications at that time, time of the day among others (Ngugi *et al.,*2012).

c) Oral glucose tolerance test

This measures glucose clearance under defined conditions that include previous fasting of 8-14 hours before challenge with a glucose solution within 5 minutes. After this, the blood glucose level is measured and diabetic condition showed a blood glucose level of about 2000 mg/dL. This level declines in the blood due to insulin unavailability that should mobilize the glucose to the site of utilization. The conditions attached to this test include:

i) Immobilisation of glucose that decreases its tolerance.

ii) Illness, use of hormones like cortisol, thyroxin, growth hormone among others.

d) Intravenous glucose tolerance

This is used for individuals with absorption disorders or those that have previously undergone intestinal surgery. An intravenous challenge of 20% glucose solution over a period of 30 minutes is utilised. Diabetic patients have an observed glucose concentration of ≥2500 mg/dL and when the infusion is discontinued, the glucose concentration return to normal at about 90 minutes (Ngugi *et al.,*2012).

e) O' Sullivan test

This test is to determine gestational diabetes. The fasting patient is challenged with 50 g load of glucose and after an hour, the O' Sullivan test is carried out to determine the glucose level and a result of above 1500 mg/dL is considered diabetic (Ngugi *et al.,*2012).

2.7.2 INSULIN

This is a peptide hormone of 86 amino acid residues secreted by the pancreatic β-cells. The secretion of the hormone is stimulated when the level of glucose in the blood exceeds 3.9 mmol/L. About 0.25-1.5 units of insulin is secreted during fasting (Raiz, 2015). Glucose regulate the synthesis of insulin through GLUT 1, 2, 3 and 4. In the body, GLUT 2 transports glucose from the blood to the liver cells, this causes the ion activity of the β-cells to change while insulin is secreted. The GLUT 1 and 4 are present in developing lungs, enhancing glucose uptake into fetal lungs. Glucose enters the insulin dependent tissues like skeletal, cardiac and adipose through GLUT 4, while GLUT 1 and 3 are foud in kidney brain, nerve and red blood cells. This also applies to the sulphonylurea receptor i.e the binding site for drugs that acts like secretagogues for insulin (Riaz, 2015). Glucokinase also regulates insulin secretion because the enzyme converts glucose to glucose-6-phosphate thereby lowering the level of glucose required for insulin secretion.

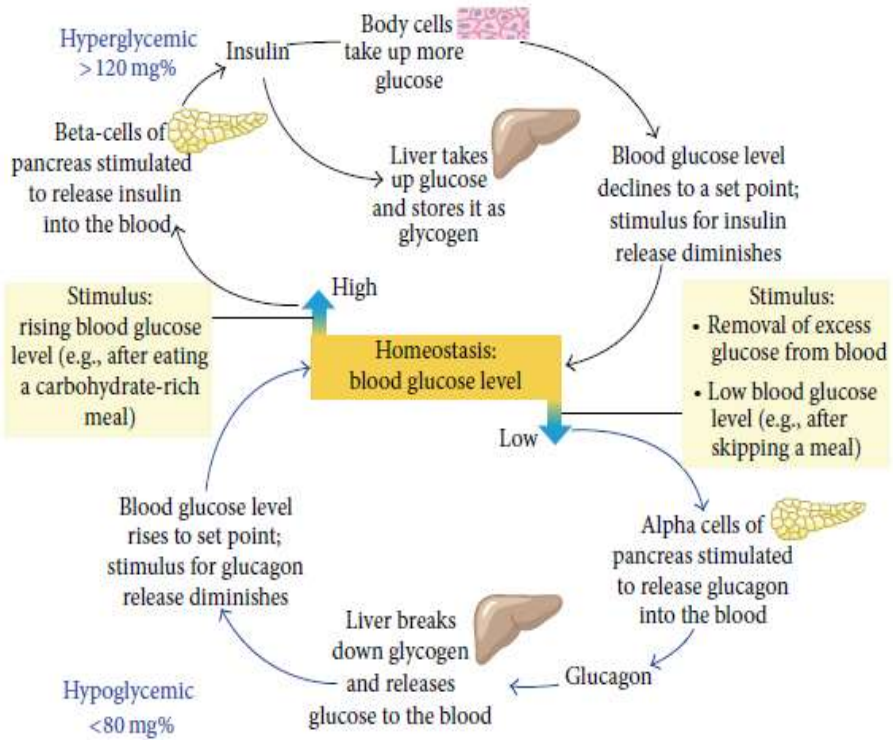


Figure 8: Tightly regulated glucose homeostasis in the body (Riaz, 2015)

2.8 MITOCHONDRIA

It is well established that mitochondria show early pathological feature of diabetes with numerous studies presenting altered membrane protein components and the resultant effect on membrane depolarization, uncoupling of the inner mitochondrial membrane protein, compromised respiratory function, Cyt C release and successive stimulation of initiator and executioner caspases (Nafaji *et al.,*2014). These observations would underscore the understanding of the connection between diabetes and the mitochondria.

The mitochondrion is the central hub of metabolic process; it produces the cell's metabolic energy by oxidative phosphorylation, therefore it is called the 'power house' of the cell (Mitchell, 1961). The organelle connects energy production to biosynthesis of other macromolecules in the cell. Mitochondria play indispensable roles in the biosynthesis of heme, lipid, iron-sulphur compounds, degradation of macromolecules like proteins, also in membrane potential generation and maintenance among others (Chandel, 2015; Kuhlbrandt, 2015). Structurally, the mitochondrion consist of the matrix, an outer- and inner-membrane and the inter membrane space (Willems *et al.,*2015). The mitochondrial outer membrane is separated from the cell by a dual phospholipid membrane. It is a porous structure that enables the influx and efflux of tiny particles through the pore-forming proteins and the voltage-dependent anion channel (VDAC) while larger particles are moved through specific translocases (Kuhlbrandt, 2015). The inner membrane of the mitochondria selectively allows the passage of cations, anions and small molecules via specific channel resulting in electrochemical membrane potential gradient built up of 180 mV. The third compartment of the mitochondria is the matrix that has a pH of about 7.9-8.0 (Llopis, 1998) which creates the transmembrane potential that drives the ATP

synthesis. Several biosynthetic reactions take place in the mitochondrial matrix. These include oxidative phosphorylation, kreb's cycle, β-oxidation, and pyruvate oxidation among others. The compartmentation of mitochondrion favors metabolism, for instance, the electron transport chain (ETC) coupled with the matrix's enzymes are utilized by the citric acid cycle which subsequently produce the reducing equivalents (NADH and FADH) that are utilised to produce ATP in oxidative phosphorylation (Mitchell & Moyle, 1967; Stryer *et al.,*2002; Voet*et al.,*2013).

In the urea cycle, two reactions take place in the liver mitochondrial; transamination reactions occurs in the mitochondria where α-ketoglutarate and oxaloacetate are converted to amino acids. Protein synthesis also takes place in the matrix where the mitochondrial DNA synthesizing proteins are located (Fox, 2012). Mitochondria are dynamic structures whose tubular network transforms from fission to division (Kuhlbrandt, 2015). When the organelle is isolated from the cell, it has the unique feature of resealing *in vitro* to carry out respiration and ATP synthesis (Alexandre *et al.,*1978). Studies by electron and light microscopy, cryo-et and cryo-em of unfixed, unstained organelle have shown the architecture of the organelle resolving their macromolecular component in situ (Perkins and Frey, 2000; Liao *et al.,*2013; Kuhlbrandt, 2014; 2015). The organelle play important role in health and death of multicellular organisms (Borutaite, 2010). It mediates cell death by permeabilising the mitochondrial pore resulting in the membrane potential breakdown and transverse movement of solutes of ≤1.5 kDa molecular mass, in and out of the organelle causing mitochondria opening especially when there is oxidative stress, decreased ATP and calcium overload (Halestrap *et al.,*2002). As a consequence, there is an energy-dependent apoptotic cell demise when there is movement of pro-apoptotic

molecules like apoptosis inducing factor (AIF), Cyt C, endonuclease G, second mitochondrial derived-activator of caspase (Smac)/Diablo into the cytosol. There is also an energy-independent cell demise called necrosis. Necrosis is described as a type of cell death whose occurrence is in an uncontrolled passive process that affects large fields of cells with an accompanied cell swelling, plasma membrane de-arrangement, release of chemotactic signals, recruitment of inflammatory cells and eventual release of the content of the cell which can cause cascade of reactions that execute the cells (Gustafsson and Gottlieb 2008; Borutaite, 2010). The mitochondrial pore is proposed to be assembled by the cyclophilin D (Cyp D), adenine nucleotide translocase (ANT) in the inner leaflet of the membrane and VDAC at outer leaflet of the membrane.

2.9 COMPONENTS OF THE OUTER MITOCHONDRIAL MEMBRANE

2.9.1 VOLTAGE-DEPENDENT ANION CHANNEL

This is an outer mitochondrial membrane resident protein, which is situated as a result of its electro physical properties with the mPT pore. The protein exist in different isoforms; 1, 2 and 3 respectively. Classically VDAC has been shown to be a mitochondrial Permeability Transition (mPT) pore constituent (Vyssokikh, 1999) but recent studies explained that the mPT pore is preserved in mouse whose VDAC 1 and 3 isoforms have been knocked out and VDAC 2 silenced. They also proved the irrelevance of VDAC to hexokinase II, Bax and t-Bid induced cell demise. The VDAC is regulated by change in mitochondrial membrane potential ($\Delta\Psi_m$) and when this increases, the mPT is maintained in the closed state (Zorov *et al.,*2009). However, the reliance of VDAC on the $\Delta\Psi_m$ is surprising since

there is no voltage regulation in the outer mitochondria membrane. Suggestions have arisen that the close contact of VDAC to the inner mitochondrial membrane may result in detection of high voltage existing across the inner mitochondria membrane thereby leading to its closure (Zorov *et al.,*2009). Regulatory proteins also interfere with conductance of VDAC, this causes efflux and influx of oxidative substrates like amino acids, small peptides into the mitochondria (Zorov *et al.,*2009). Konig's synthetic polyanion and phosphorothioate oligonucleotides act by causing permeability of VDAC to ATP and ADP (Zorov *et al.,*2009). Other modulators of VDAC are NADH and hexokinase (Liu and Columbini 1992; Zorov *et al.,*2009).

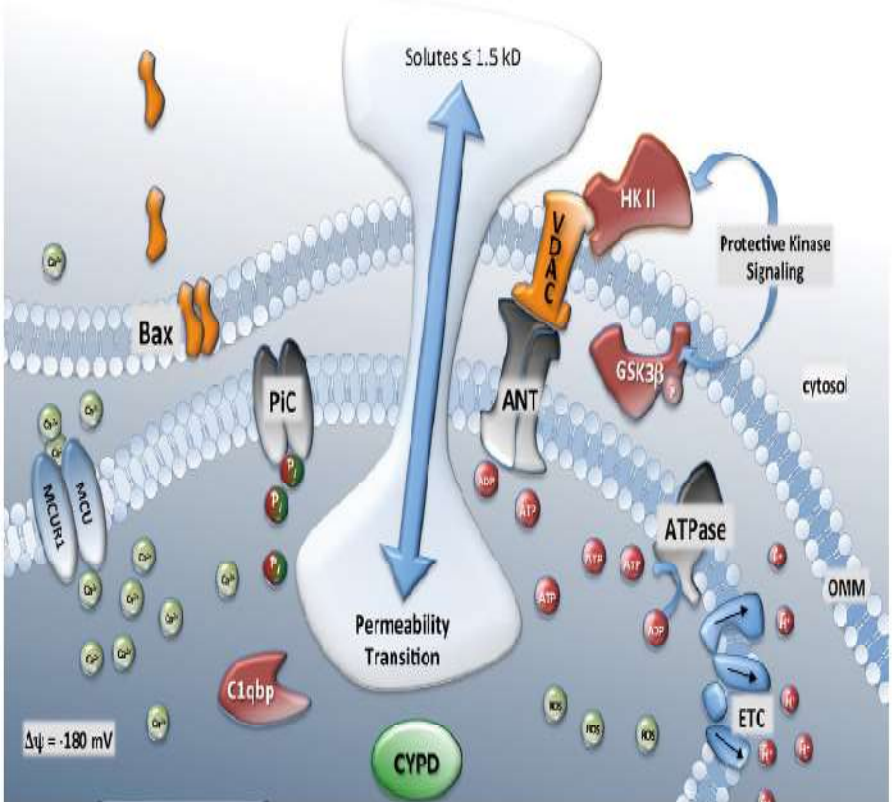


Figure 9: The mitochondrial inner- and outer- membrane (Elrod *et al.*, 2013).

2.9.2 ADENINE-NUCLEOTIDE TRANSLOCASE

This pore component is found jn the inner mitochondrial and it plays a vital role of ATP production in the mitochondria matrix (Klingenberg, 2008; Kwong and Molkentin, 2014). Bongkrekic acid has been shown to be an inhibitor of ANT, it prevents pore opening by stabilizing the 'm' conformation. While carboxyatracylate stimulates the pore opening by promoting the 'c' conformation (Kwong and Molkentin, 2014). Early studies on the ANT was carried out by Hunter and Haworth (1979; 2000) with more illumination proffered by the Le Quos and Halestrap and colleagues, they established that ANT is one of the component of the mPT pore and compounds that are capable of maintaining the c-conformation will sensitize it to [Ca^{2+}], while compounds that maintain the m-conformation will make it less sensitive to [Ca^{2+}] (Halestrap *et al.,*2002; Elrod and Molkentin, 2013).

The calcium trigger site sensitivity to calcium concentration is decreased by the presence of ADP. The ADP is an important modulator of the mPT pore opening which can bind two sites; the high and low affinity sites. When carboxyatracyloside binds to the high affinity site, there is inhibition of ADP binding causing sensitization of mPT pore opening (Halestrap *et al.,*2002). Simply put, presence of adenine nucleotide makes the mPT pore less sensitive to [Ca^{2+}]. The identity of the low affinity site has not been well deciphered but Halestrap and colleagues proposed that it could be an extra mitochondrial binding site for adenine nucleotides on ANT (Halestrap *et al.,*2002). The ADP, dADP and ATP have been shown to have high affinity for binding to ANT, and on binding, the ANT detects membrane potential (Halestrap *et al.,*2002). This is realizable because ANT catalyzes the electrogenic interchange process of ATP^{4-} in place of ADP^{3-} (Halestrap *et al.,*2002) and

this affects the binding of adenine nucleotides to ANT, both on the outer- and inner-mitochondria membrane site (Halestrap *et al.,*2002).

2.9.3 F_1F_0 ATPase

The F_1F_0 ATPase is a pore-forming protein that has the F_0-domain in the inner mitochondria and the F_1 domain found in the matrix. The identified regions are associated via the peripheral and central stalk. This complex possess a molecular mass of ~600 kDa which is made up of 15 subunits called the oligomycin sensitive-conferring protein (OSCP) which include A6L, F6, a, b, c, d, e, f, g, o, α, β, γ, δ, and ε subunits called the peripheral stalk (Johckheere *et al.,*2012; Bonora *et al.,*2014). This creates a bridge between F_1 and F_0 components of the complex.

2.9.3.1 ROLE OF F_1F_0 ATPASE SYNTHESIS

Peter Mitchell postulated the chemiosmotic model and hypothesized that the F_1F_0 ATPase would dissipate the generated electrochemical gradient in ETC in a controlled manner. This is responsible for ATP generation in oxidative phosphorylation of ADP by P_i (Mitchell, 1961; Bonora *et al.,*2014). The gradient established by the ETC is dissipated by the released of H^+ between subunit-aand the c ring that results into rotation in the c-ring which is subsequently transferred to the γ and ε subunits (Noji *et al.,*1997). The rotation of the three dimers of α and β complex induces a conformational change in F_1 domain accountable for ATP synthesis (Bonora *et al.,*2014). The F_1 domain can exist coordinately in three conformations namely the βDP (with high attraction for ADP), the βTP (with high attraction for ATP) and finally the βE (with reduced attraction for ATP) (von Ballmoos *et*

*al.,*2009). Robust data by Bonora *et al.,*(2014) and von Ballmoos *et al.,*(2009) shows that the central stalk of the F_1F_0 ATPase can rotate about 700 times in a second depending on substrate availability, temperature and other factors (von Ballmoos *et al.,*2009: Bonora *et al.,*2014). The F_0 peripheral stalk is connected to the outer domain of the F_1 subunit; this part of the complex opposes the possibility of the 3 dimers of αβ to revolve along c-ring and the central stalk. It also serve as anchorage to the subunit.

The chemiosmotic hypothesis, as proposed by Nobel Laurate, Peter Mitchell (1961), explains that the ETC is coupled to oxidative phosphorylation by an inner mitochondrial membrane proton gradient.

2.9.3.2 INHIBITORS OF THE F_1F_0 ATPASE

When ADP and Mg^{2+} bind concurrently to the F_1F_0 ATPase complex, inhibition ensues, however, these can be blocked by increasing the proton-motive force attainable by Mg^{2+} and ADP expulsion from the inhibitory site (Bonora *et al.,*2014). Another factor that can alleviate the Mg-ADP block is the availability of inorganic phosphate (P*i*). The F_1F_0 ATPase is regulated by binding of Cyp D to the OSCP region (Jonckheere *et al.,*2012; Kwong and Molkentin, 2014; Giogio *et al*., 2015); this binding can be inhibited byCsA (Bernardi and Di Lisa, 2015). Another inhibitor of the F_0F_1 ATPase in pathologies is inhibitor factor 1 (IF_1) (Faccenda and Campanella, 2012). The F_1F_0 ATPase is sensitive to oxidative substrates like Cys -294 and -103 in α and β subunit respectively (Bonora *et al.,*2014). The interaction with these substrates result in disulfide bridge formation; change in the pH and Ψ_m affect the hydrolytic activity. Proteins like ANT interact with F_1F_0

ATPase to form super-complexes, while Cyp D interacts by binding via the peripheral stalk, thereby reducing its synthetic and catalytic activity. Studies have shown that Bcl-xL protein stimulates the activity of F_1F_0 ATPase on binding to it (Alvian *et al.,*2011; Bonora *et al.,*2014). Intense wave of rigorous researches have also connected the F_1F_0 ATPase with the mPT suggesting that oligomycin which inhibits the activity of F_1F_0 ATPase when it binds to F_0, also inhibit mPT pore opened by erufosine as well as Bax (Shchepina *et al.,*2002; Pucci *et al.,*2008; Bonora *et al.,*2014). Similarly, studies of Bernardi and colleagues have proposed that the mPT pore may consist of F_1F_0 ATPase dimers (Szabadkai and Chinopoulos 2013; Bonora *et al.,*2014).Studies by Gorgio *et al.,*(2013) explained the similarity in activity of the F_1F_0 ATPase to the mPT pore but Alvian *et al.,*(2011) and Bonora *et al.,*(2011) revealed that the F_1F_0 ATPase is only a requirement for the mPT pore. Despite their findings, more work is still needed to validate two different models that showed that F_1F_0 ATPase dimer is responsible for forming the mPT pore and that monomers of F_1F_0 ATPase couples with the c unit ring are responsible for formation of the pore (Gorgio *et al.,*2013; Alvian *et al.,*2014; Bonora *et al.,*2014).

2.10 COMPONENTS OF THE INNER MITOCHONDRIAL MEMBRANE

2.10.1 CYCLOPHILIN D

This is a class of peptidyprolyl cis-transisomerase (PPIase), that is found in the mitochondria matrix, it was discovered by the inhibitory action of cyclosporine A (CsA), an immunosuppressant on the PPIase activity of CypD (Rao *et al.,*2014). Generally, cyclophilins are ubiquitous proteins found in man and the mitochondrial homologous is the

Cyp D coded by *Ppfil* gene in mouse (Bernardi and Di Lisa, 2015). Experimental work by Baines *et al.,*and Nagagawa *et al.,*(2005) described the regulatory function of Cyp D on the mPT, they explained that the mitochondrial devoid of Cyp D showed reduced sensitivity to Ca^{2+} and pore opening even when there is oxidative stress (Baines *et al.,*2005; Nagagawa *et al.,*2005; Kwong and Molkentin, 2014). Nevertheless, at high Ca^{2+} concentration there is still pore opening. This shows that Cyp D inhibition may be of pharmacological potential in diseases where mPT pore is implicated (Kwong and Molkentin, 2008). Bernardi and Di Lisa (2015) have argued that Cyp D is just a regulator of mPT not a component of it. They further described it as directly interacting with ANT but not in the presence of cyclosporine A, where the dissociation leading to desensitization and delay in the mPT pore opening rather than completely blocking it (Zorov *et al.,*2009). It is also known that Cyp D regulates mPT pore by interacting with phosphate carrier. Chinopoulous and colleagues proposed two mechanism by which Cyp D regulates mPT:

i) regulation of matrix ANT level and

ii) inhibition of mPT pore by increased H^+ pumping by ATP hydrolysis resulting in increased maintenance of $\Delta\Psi_m$.

The CsA has been shown to be the most specific inhibitor of the mPT pore but it lacks clinical significance due to its immunosuppressant characteristics and inability to transverse the blood brain barrier. It has been shown that Sangliferin A (SfA), a derivative of CsA does not inhibit the pore but prevents Cyp-D binding to ANT thereby inhibiting apoptosis. Despite the fact that these derivatives inhibit the mPT pore, they have enormous side effects like nephrotoxicity, neurotoxicity and hepatotoxicity (Rao *et al.,*2014).

2.10.2 THE PHOSPHATE CARRIER

The phosphate carrier (PiC) found in the mitochondrial is a phosphate group carrier to the matrix hence playing a major role in oxidative phosphorylation (Kwong and Molkentin, 2014). Studies by Alcalia and colleagues showed that it is also one of the key regulators of mPT opening and apoptosis (Alcalia *et al.,*2008). This carrier is also an inner mitochondrial component, since it is a co-binder with Cyp D, however, studies have questioned PiC as a component of the inner mitochondria (Varanyuwatana and Halestrap, 2012; Gutierrez-Aguilar, 2014,). Kwong and Molkentin (2014) suggested that the PiC is only able to alter matrix phosphate levels and that it secondarily resulted in pore opening.

2.11 THE MITOCHONDRIAL MEMBRANE PERMEABILITY TRANSITION PORE

The mPT pore can be described as a channel obtained due to the breakdown of the chemiosmotic gradient found in the inner mitochondrial membrane which is facilitated by 'opening of a large conductance pore' that is called the mPT pore (Elrod and Molkentin, 2013). The mPT pore is a structure that transverses the outer and inner mitochondrial with its components not well defined. Classical studies shows that owing to Ca^{2+} overload, there is enormous swelling of the mPT pore (Gunter and Pfeiffer, 1990). However, more recent studies described the pore as a molecular entity whose structure allows the flow of solutes ≤1500 Dalton via the mitochondrial inner membrane causing colloidal swelling, depolarization of the membrane potential, transient of pro-apoptotic factors into the

cytosol, increased ROS generation, release of Cyt C, oxidative phosphorylation uncoupling and eventually cell death (Halestrap *et al.,*2002). Several experimental procedures can be used to determine the opening of the mPT pore, which spans from measuring of mitochondrial swelling using spectrophotometry to measuring TMPT electrode collapse of membrane potential in mPT (the technique is at disadvantaged where the $\Delta\Psi_m$ is compromised). Similarly, measurement using Ca^{2+} sensitive electrode that determines accumulated calcium in mitochondria is a dependable technique that requires definite apparatuses (Halestrap *et al.,*2002).

The mPT opening is undoubtedly a feature of cell death and the mechanism underlying the pore opening genders from the organized execution of numerous interdependent steps (Kroemer *et al.,*2007). In the mPT pore opening, certain events may occur which include outer mitochondrtial membrane permeabilisation involving discharge of Cyt C, AIF and adenylate kinase (ADK) from the inner mitochondria to the extra mitochondrial compartment. Therefore the mitochondrial outer membrane permeabilization involves detection of the translocated proteins. However, the inner mitochondrial membrane is impervious to ions and water, therefore its permeabilization is determined through assessment of the electrochemical gradient (Kroemer *et al.,*2007). The AIF and Cyt C are released alongside with other proteins like Smac/DIABLO, Omi/Htr A_2, Endonuclease G and procaspase 9.Studies have revealed that Cyt C and AIF release are not dependent on the release of these other proteins. Biochemical examinations have also been employed to determine the integrity of the mitochondria, they include determination of phosphocreatine (a product of creatine kinase), NADH oxidase, Cyt C oxidase respectively.

2.12 THE MITOCHONDRIAL MEMBRANE PERMEABILITY TRANSITON PORE AND THE ATPase COMPLEX

Studies have shown that the mPT pore opens when the F_1F_0 ATPase is compromised; this gives a new perspective to the molecular elucidation of the pathophysiology of diseases caused by mPT pore opening and uncontrolled action of F_1F_0 ATPase. The F_0 section of the enzyme is found in the peripheral portion of the cytoplasm while F_1 domain projects into the mitochondrial matrix. The F_1F_0 ATPase is called proton-translocating ATPase synthase; it is a multifaceted protein found in the cristae. The protons moving via the F_0 complex drives the eight (8) subunits while the central stalk projects the C-ring, connecting the F_1 crown where the generation of ATP ensues. The F_1 domain can hydrolyze ATP but cannot synthesize it that is why it is referred to as ATPase.

Mechanism of Proton Transport in the F_1F_0 ATPase

1. Translocation of proton by F_0
2. Catalysis of ATP by F_1
3. Coupling of the dissipation of proton gradient with ATP synthesis requiring interaction of F_1 and F_0.

2.13 MECHANISM OF OUTER MITOCHONDRIAL MEMBRANE PERMEABILISATION

1. Bax/Bak- mediation of mPT pore opening

The Bcl 2 homology (BH) family has been divided into the antiapoptotic domain, BH 1234 (Mcl 1, Bcl W, Bcl 2, Bcl xL, and A1A), the proapoptotic domain, BH 123 (Bak and Bax) and the proapoptotic BH 3 only proteins (Bad, Bid, Bik, Bim, Puma, Noxa, Hrk). The site of the BH3 protein action is the mitochondria. The BH 1234 protein resides on the outer mitochondrial membrane where they bind and neutralize the effects of BH 123 proteins. The anti-apoptotic proteins protect the mitochondria from permeabilisation while the proapototic proteins induce permeabilization. Under normal physiological condition, Bak is found on the outer mitochondrial membrane and Bax is found in the cytosolic region. When there is pathology, resulting in apoptosis, Bax transverses the cytosol onto the outer mitochondrial membrane where oligomerization and insertion unto the membrane occurs. Bax could also destabilizes the lipid bilayer to create a multimer of giant protein pore. Bak which was inactive on the outer membrane colocalizes itself with Bax to initiate pore formation through insertion into the mitochondrial. This interaction with the outer mitochondrial can be inhibited by Bcl-xL. Bax remains in the cytosol bound to Ku autoantigen of 70 kDa or its derived peptides when apoptosis is not induced (Sawada *et al.,*2003; Kroemer *et al.,*2007). The Bad protein (BH 3only protein) is referred to as the facilitator of membrane permeabilisation. It acts by interacting with the BH 1234 proteins. The tBid is the only BH3-only protein that acts as the activator of mitochondrial permeabilization because it induces the BH 123 proteins to initiate mitochondrial permeabilisation either by causing Bax to translocate to the outer mitochondria or Bak to insert onto the outer mitochondria membrane (Kroemer *et al.,*2007). Whereas other studies have shown that Bax can initiate pore opening independent of VDAC and ANT, Kroemer *et al,* (2007) believed that these two hypotheses may co-exist in specific cell death,

however, the researchers reiterated that the Bax-mediated mPT opening may be dependent on the concentration of Bax and its capability to oligomerize onto the outer mitochondrial (Pastorino *et al.,*1999; Kroemer *et al.,*2007).

2. VDAC-Mediation of mPT pore opening

The VDAC protein is a complex channel that allows the diffusion of substances of ~5 kDa to move through it. Tsujimoto and colleagues were the first to discover the role of VDAC in apoptosis (Tsujimoto and Shimizu, 2000). They hypothesised that Bax/Bak induces VDAC opening and consequent loss of mitochondrial potential and Cyt C release (Shimizu *et al.,*2004). This effect was circumvented by binding of recombinant Bcl-xL and Bcl-2 (Kroemer *et al.,*2007). Another hypothesis by Pastorino *et al.*among others have shown that when hexokinase I and II binds to VDAC isoform, it prevents association with Bax and this inhibits mPT pore opening. However, different isoforms of VDAC responds to the proapoptotic proteins in different manners. For instance, VDAC-1 interacts with Bax while VDAC-2 interacts with Bak (Pastorino *et al.,*2002).

3. Cyclophilin D mediation of mPT pore opening

The CypD protein regulates mPT pore opening by interaction with PiC, ANT and F_1F_0 ATPase by two mechanism explained by Chinopoulous *et al.,*(2011): see page 45.

4. The Translocator-Protein (TSPO) mediation of mPT pore opening

The 18 inches protein is resident on the outer mitochondrial membrane. It was first discovered as the primary binding site of benzodiazepine anxiolytic drug (Liu *et al.,*2016). Studies by Bonora, KlaffSchenkel and colleagues have confirmed the ability of TSPO

blocking antibody to inhibit the mPT pore opening demonstrating its role on the pore opening in cell demise. (Klaffschenkel *et al.,*2012; Bonora *et al.,*2015)

5. Kinases mediation in mPT pore opening

Certain kinases like creatine kinase mitochondrial 1 (CRMT1), hexokinase I and II (HK I and II), PKC and glycogen synthase kinase 3β (GSK3β) have been shown to interact with the mPT pore without phosphorylating the protein substrates (Bonora *et al.,*2014). However, CKMT 1 catalyzes the phosphorylation of creatine to give phosphocreatine in a reaction closely coupled to oxidative phosphorylation therefore depleting the phosphate available for ATP synthesis (Bonora *et al.,*2014). The glycolytic enzyme, (hexokinase, which catalyzes glucose phosphorylation), HK I & II interact with VDAC thereby accessing the ATP exiting the mitochondria (Bonora *et al.,*2014). It has been suggested by Chiara *et al.,*Schindler and Foley that the mPT regulatory function of hexokinase is as a result of metabolic effect (Chiara *et al.,*2008; Schindler and Foley, 2013). The GSK 3β and the PKC show activities on the mPT that have been partially linked to phosphorylating core components unlike the CKMT I and HK I and II (Bonora *et al.,*2014). Reports have shown that on phosphorylation of VDAC 1 by GSK 3β, there is a resultant removal of HK II (Bonora *et al.,*2014) thereby increasing ATP utilization by mitochondria (Das *et al.,*2008; Bonora *et al.,*2014). However, when GSK 3β is phosphorylated on the inactive site, it inhibit mPT pore opening by altering the ANT 1 and Cyp D interaction (Bonora *et al.,*2014). The PKC phosphorylates VDAC I thereby facilitating HK II binding to it (Bonora *et al.,*2014).

2.14 APOPTOSIS

This is an organised cell death vital for the normal physiology of the multicellular organism especially during embryogenesis and metamorphosis. The dysregulation of this process leads to tumoriogenesis and/or degenerative diseases like Alzheimer, Parkinson disease, diabetes among others. Despite the complexities of apoptotic signal at several points leading to self-destruction of the cells, autophagy and apoptosis are the prominent forms of cell death that play physiological roles (Lockshin, 1964). Necrosis is another kind of cell death that results due to fatal insult in an energy independent manner (Webster, 2012). 'Apoptosis' was first coined by Kerr and colleague in 1972 (Kerr *et al.,*1972) which means falling off. Apoptosis enhances development, morphogenesis, proliferation /homeostasis (i.e majority of the B and T cells generated die during maturation) and deletion of damaged and dangerous cells in brain development. In nerve cells, about 50% of the cells die before metarmorphosis into adulthood (Hutchins, 1998).

Apoptosis is also very crucial in the development of the reproductive organ (Meier, 2000). Thus when the process is dysregulated, pathological condition results. Apoptosis is therefore a common occurrence which takes place in nematodes, insects, prokaryotes, eukaryotes, mammals, nematodes, cnidana and even in plant (Ameisen,2002; Richardson and Kumar,2002). The features of apoptosis exemplifies some morphological characteristics which include:

a) Cellular shrinkage that allows deformation and loss of contact and communication with other neighboring cells.

b) Condensation of chromatin leading to margination of the nuclear membrane.

c) Plasma membrane blebs or buds.

d) Fragmentation of cells which are later engulfed by macrophages and cleared without initiating any inflammatory response.

2.15 CASPASES AS DEATH SIGNALS IN APOPTOSIS

Caspases are the proteases of cysteine activated in almost all circumstances of apoptotic cell demise. Cell death towing the path of caspase mode of execution is classically termed apoptosis (Leist, 2001). Caspase means aspartate-specific protease which is cysteine-dependent, their activities reliant on the extremely conserved active-site that contains cysteine residue and also a QACRG pentapeptide. Caspases that generally relieves their substrates of inactivity after the aspartate residue, include:

a. Proteolytic maturating caspases (caspases -1,-4,-5, -11, -12): this maturates the pro-inflammatory (pro-IL 18 and pro-IL 1β) cytokines proteolytically.
b. Caspases involved in apoptotic signaling machinery: these include caspases -3, -9, -8, -2, -6, -7 and -10. Caspases are generally referred to as procaspases when they are still in their inactive zymogens but as they mature, they evolved to a heterotetramer comprising of two small and two large subunits that form an active caspase. They include:

i. Initiation caspase (-2, -8, -9, and -10)

ii. Executioner caspase (-3, -6, and -7)

2.16 INITIATION CASPASES

These are caspase 2, 8, 9 and 10, they exist as zymogens that are inactive and only activated by dimerization and not by cleavage as observed in executioner caspase. Once dimerization has occurred, there is facilitation of autocatalytic cleavage of caspase monomer to small and large subunit (Mcllwain *et al.,*2016). This mediates apoptosis as a result of stimuli inside and outside of the cell (Elmore, 2007).

2.17 EXECUTIONER CASPASE

These consist of caspase -3,-6 and -7, which are synthesized in the inactive forms i.e as procaspases. These caspases must be cleaved by the initiator caspases, between the small and large domains. This consequently brings the two domains side by side inducing a structural change that makes the two active site of the executioner caspase to interact. When an executioner caspase is activated it results in the subsequent activation of other ones (Mcllwain *et al.,*2016).

2.18.1 INTRINSIC PATHWAY OF APOPTOSIS ACTIVATION

The intrinsic apoptotic pathway originates from the mitochondria. The downstream proapoptotic event occurs when apoptosis activating factor-1 (Apaf-1) binds to Cyt C exiting the mitochondrial, this binding results in increased affinity for dATP (Kroemer*et al.,*2007). This recruits procaspases 9 which is dimerized at the Apaf-1 scaffold resulting in

its activation. At this point, a wheel-like multi-protein complex apoptosome is formed (Kroemer *et al.,* 2007). At the instance of initiator caspase activation, there is proteolytical activation of the executioner caspases which specifically cleave protein substrate orchestrating the mediation, amplification and eventual execution of cell death. Studies of Beresewicz *et al.,*(2006) have shown that Cyt C can also induce apoptosis by binding to inositol 3-triphosphate receptors resulting in calcium release into the cytosol with subsequent activation of calpain and release of AIF (Beresewicz *et al.,*2006; Kroemer*et al.,*2007). Cytotoxic stress, DNA damage, suppression of growth factor hormones, cytokines, radiation, hypoxia, toxins, free radical are some of the intracellular signals that activate apoptosis (Elmore, 2007; Wu *et al.,*2014). These intracellular signal cause the permeabilisation of the mPT pore, membrane potential loss and Cyt C, serine protease Htr A2/Omi and Smac/DIABLO release (Elmore, 2007). These proteins are responsible for activating the caspase family. Endonuclease G, AIF and CAD are also proteins released due to mPT pore compromise, when the cell is already committed to death. The AIF cleaves the DNA while endonuclease G cleaves the nuclear chromatin to give oligonucleotide DNA fragments and these effects are independent of caspases (Elmore, 2007).

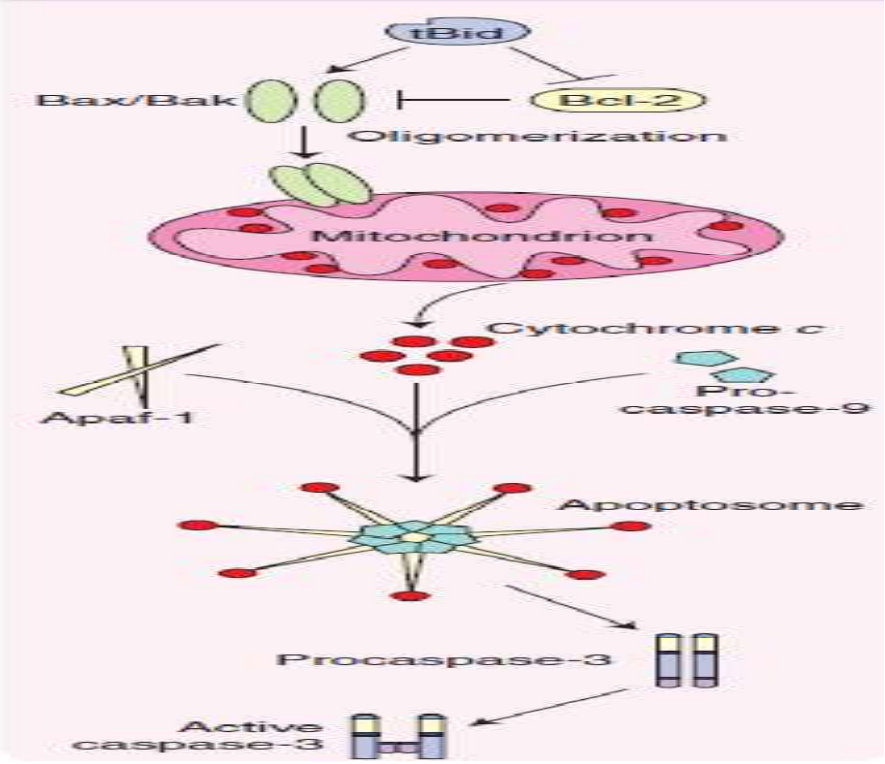


Figure 10: The caspase activation in intrinsic apoptotic pathway (Parrish *et al.*, 2013).

2.19 EXTRINSIC PATHWAY OF APOPTOSIS ACTIVATION

Extrinsic stimuli involved in the initiation of apoptosis include the transmembrane receptor-mediated interaction that are TNF members having about eighty (80) amino acids referred to as the death domains. These death domains are found on the extracellular side and they transmit signals that initiate apoptosis. They include the Fas L/Fas R, TNF α/TNFR 1, Apo 3L/DRS, Apo 2L/DR4 and Apo 2L/DR5 (Elmore, 2007). Although extrinsic apoptosis is best characterized by FasL/ FasR and TNF-α/ TNFR 1 model, upon stimuli receipt, the adapter proteins which constitute the death domains are recruited and these bind the receptors. The FasR has an intracellular death domain that permits the conscription of adapter protein like Fas associated death domain (FADD) from the cytosol. When TNF ligand binds TNFR, an adapter protein TNF receptor associated death domain (TRADD) is recruited. The FADD then recruits procaspase 8 through dimerization of its death domain to death inducing signaling factor, (DISC), a receptor complex found on the cell membrane that is formed due to the ligation of a member of TNF family. Procaspase 8 binds DISC thereby bringing so many zymogens together and this auto catalyzes one another. Caspase 8 activation initiates activation of caspase 3 (execution stage) or cleavage of Bid that will accelerate the release of Cyt C from the mitochondria (Parrish *et al.,*2013).

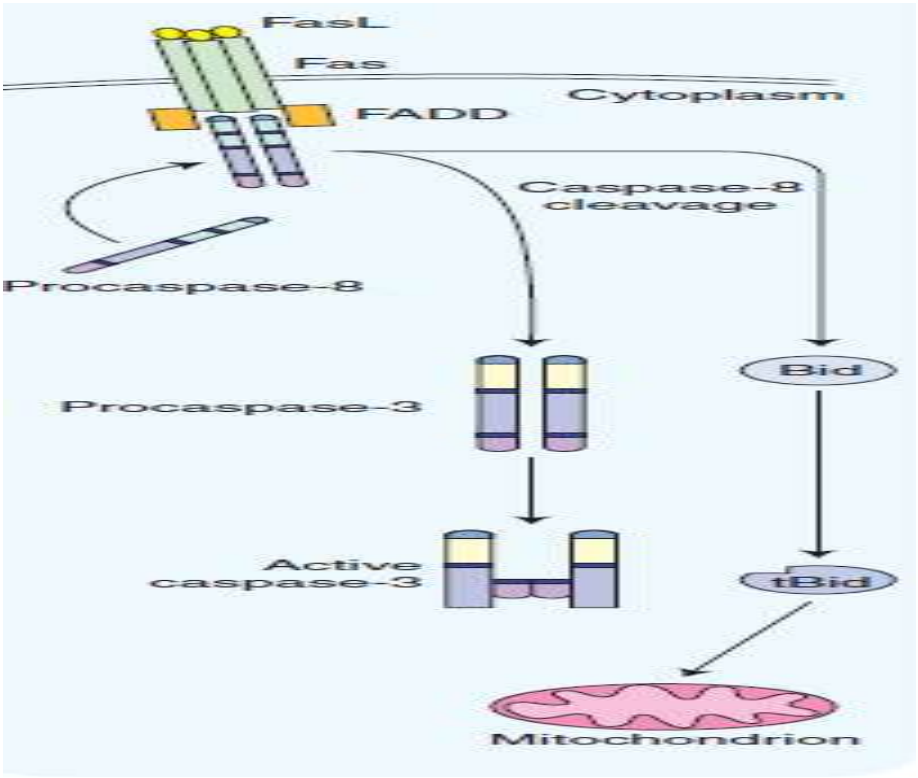


Figure 11: Activation of caspase 8 in extrinsic apoptosis (Parrish *et al.*, 2013).

2.20 REGULATION OF EXTRINSIC APOPTOSIS

This is achieved by binding of c-FLIP protein binding to FADD and caspase -8 causing inhibition of their activities (Scaffidi, 1999). Toso protein inhibit the Fas-induced apoptosis in T-cells (Hitoshi *et al.,*1998).

2.21 DYSREGULATION OF APOPTOSIS IN DISEASES

The mPT pore had been speculated to be a modulator of cell death; therefore researches have been directed to decipher how the pore can be manipulated to treat certain pathologies where apoptosis is dysregulated (Bonora and Pinton, 2014). In a case study where apoptosis is upregulated, findings have revealed how the mPT pore is activated causing increased apoptosis. Similarly, in pathologies where apoptosis is down-regulated like cancer, there is inactivation of the pore thereby impeding apoptosis and multiplicity of mutagenic cells. Excessive apoptosis have been shown to be a major hallmark of diabetes, neurodegenerative diseases, Alzheimer among others.

INTRA-MITOCHONDRIAL CALCIUM

This is the best mPT pore inducer and when elevated it activates the pore and apoptosis ensues (Bonora and Pinton, 2014). Classically, it has been known that cytosolic concentration of Ca^{2+} is responsible for an extensive variety of physiological processes like mitotic cell division, neurotransmission, metabolism amongst others. However, it mediate cell death in apoptosis through regulated mild insult and in necrosis by massive insult (Pinton *et al.,*2008). There are other second messenger in the cell that include cAMP,

cGMP, inositol-3-phosphate but amongst all, Ca^{2+} has a unique characteristic of low diffusion rate than others giving intracellular heterogenicity. Ca^{2+} is normally supplied to the mitochondria by the endoplasmic recticulum via some domains and the two organelles interrelate through mitochondrial-associated membranes (Patergnani *et al.,*2011) Robust evidences by Bonora and Pinton have shown that when Ca^{2+} effluxes from the endoplasmic reticulum into the mitochondria, AKT kinase mediates the phosphorylation of inositol-3-phosphate receptor (IPR) and results in excessive apoptosis (Bonora and Pinton, 2014). In normal cells, phosphorylation of IPR is regulated by promyelocytic leukemia protein (PML) and by homologue of phosphatase and tensin (PTEN) (Pinton *et al.,*2000; Bonora and Pinton 2014).

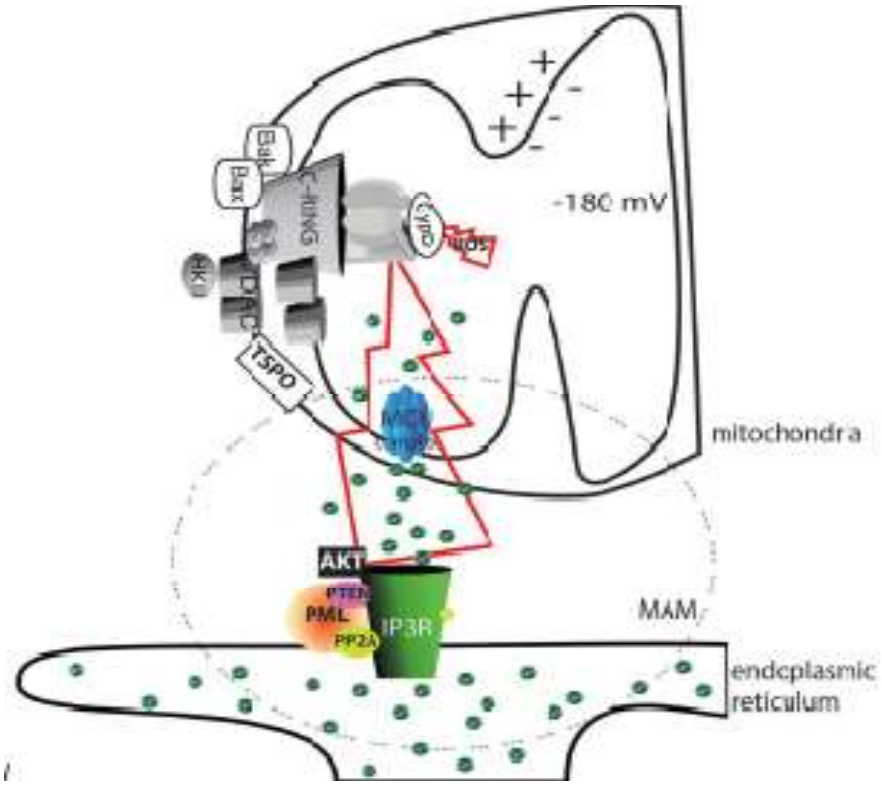


Figure 12: Ca^{2+} release via the endoplasmic reticulum into the mitochondria in normal cells (Bonora and Pinton, 2014)

In diseases where the activation of mPT pore is circumvented like cancer, there is loss of the PML, PTEN and protein phosphatase regulators thereby leading to reduction in the cytotoxic Ca^{2+} available for activating the mPT pore (Bonora and Pinton, 2014).

2.22 REACTIVE-OXYGEN SPECIES

Reactive-oxygen species (ROS) possess radicals of oxygen, where the most abundant is $O_2^{\bullet -}$, while H_2O_2 is obtained by spontaneous dismutation of $O_2^{\bullet -}$ (Fukai and Ushio-Fukai, 2011; Zorov *et al.,*2014). The ·OH is the most potent oxidant responsible for the damage of DNA bases and this radical is obtained from H_2O_2 and $O_2^{\bullet -}$through the Haber-Weiss reaction (Zorov *et al.,*2014) catalyzed by Fe^{2+}. Other oxygen radicals that can also cause oxidation of cellular structures include but are not limited to; nitric oxide (NO), radical of alkoxyl group (RO·), radical of sulfate group ($SO_4^{\bullet -}$), and the metal-ion complexes. Presence of ROS in the cell serve signaling roles by regulating biochemical reactions but when it exceed the threshold level, pathological damage to the cell ensues; the mitochondrial regulates levels of ROS in the living cell. When ROS in the cell is above the threshold level and not compensated by the antioxidant it results into oxidative stress. Mitochondria was first observed to generate ROS by Jensen, he discovered that the oxygen consumed in complex I of the ETC i.e substances oxidizing succinate or NADH was converted to H_2O_2 (Jensen, 1966; Zorov *et al.,*2014). Findings have shown that complex III participates in generation of ROS in the ETC and this happens when the mitochondrial proton gradient is enormous with a concomitant low oxygen consumption (Webster, 2012).

CHAPTER THREE

3.1 EXPERIMENTAL RATS

Wistar strain albino rats (male) weighing about 100-120 g were acquired from the Veterinary Medicine Animal Husbandry, Veterinary Department, University of Ibadan, Ibadan, Nigeria. Ventilated cages were used in housing the rats at 12 hours light/dark cycling and were given food and water *ad libitum*. Acclimatization by the rats was allowed for two weeks before experimental use.

STUDY DESIGN

The rats were separated into various groups and treated for 28 days as follows:

Group 1: Normal rats + fresh water

Group 2: Rats injected with Streptozotocin (STZ) to induce diabetes

Group 3: Diabetes + Quercetin(30mg/kg)

Group 4: Diabetes + Quercetin(10mg/kg)

Group 5: Pretreatment with 30 mg/kg quercetin for 3 days before diabetes induction and then continued for 28 days

Group 6: Diabetes + Quercetin + Vitamin E (10mg/kg each of quercetin & vitamin E)

Group 7: Diabetes + Glibenclamide(0.6mg/kg)

Group 8: Diabetes + Vitamin E(10mg/kg)

Group 9: Normal + Quercetin alone(30mg/kg)

Group 10: Normal + Quercetin alone (10mg/kg)

Group 11: Normal + Vitamin E alone (10 mg/kg)

Group12: Normal + Quercetin + Vitamin E (10 mg/kg each)

Group 13: Normal + pretreated quercetin (30 mg/kg)

3.2 INDUCTION OF DIABETES

Diabetes Mellitus was induced with a single intraperitoneal dose of streptozotocin (40 mg/kg) after an overnight fast. Animals with fasting blood glucose ≥ 250 mg /dL (13.8 mmol) were considered diabetic and selected for use (Srinivasan and Indumathi, 2003; Deeds *et al.,*2011)

3.3 DETERMINATION OF BLOOD GLUCOSE

Fasting blood glucose was determined using Blood glucose Assay Kit (RANDOX).

Principle

Glucose oxidase is obtained from *Aspergillus niger* growth medium. β-D-glucose is oxidized to hydrogen peroxide and D-glucono-1,5-lactone by *Aspergillus niger*, this is gradually hydrolyzed to D-glucuronate while hydrogen peroxide eventually produces oxygen and water by the action of peroxidase. When toluidine is introduced into the reaction medium, a colored compound is generated and measured at 500 nm by spectrophotometry.

β-D-glucose $\longrightarrow$ D-glucono 1,5 lactone + H_2O_2

D-glucono 1,5 lactone $\longrightarrow$ D-gluconic acid

H_2O_2 $\xrightarrow{\text{peroxidase enzyme}}$ H_2O + O_2

O_2 + Toluidine $\longrightarrow$ Colored compound

Procedure

Reagents

R1a: Buffer

R1b: GOD-PAP Reagent

Reagent R1 (one container) is reconstituted with 20 mL of Buffer 1a. This reagent is steady at 2 to 8°C for a period of three months or at 15 to 25°C for a period of 5 days.

Procedure for blood glucose determination 500 nm

Test-tubes	Standard or Test sample	Reagent-Blank
Test or Standard	50 µL	50 µL
Reagent-blank	5000 µL	5000 µL

Mix thoroughly, then leave at 15-25°C for 25 min or 10 min at 37°C. The absorbance was read within 60 minutes as against the reagent-blank.

Calculation

Concentration of glucose =

$$\frac{Absorbance\ of\ sample}{Absorbance\ of\ standard} \times Standard\ concentration$$

Equation 1: Calculation of glucose concentration

3.4 DETERMINATION OF PLASMA ASPARTATE AMINOTRANSFERASE (AST) ACTIVITY

Plasma AST was determined by using AST assay kit (RANDOX).

Principle

There is formation of L-glutamate and oxaloacetate by reversible transamination reaction between L-aspartate and α-ketoglutarate catalyzed by AST. Malate is obtained from oxaloacetate in a reductive process by malate dehydrogenase and NADH. The absorbance changes directly proportionately to the level of AST in the test solution. This is determined by spectrophotometry at 340 nm (Reitman and Frankel, 1975).

L - Aspartate + 2 oxoglutarate $\xrightarrow{\text{AST}}$ L - glutamate + Oxaloacetate

Oxaloacetate + NADH + H^+ $\xrightarrow{\text{Malate Dehydrogenase}}$ D-Malate + NAD^+

Reagents

Tris hydroxymethyl aminomethane (Tris) buffer 80 mmol/L, pH 7.65 at 37^{o}C (Sigma Chemical Co, USA), lactate dehydrogenase ≥0.9 kU/L, 240 mmol/L L-Aspartate, 0.2 mmol/L NADH, 12 mmol/L α-Oxoglutarate, Malate dehydrogenase ≥0.6 kU/L

Procedure

Plasma (sample) of 0.1 ml was added into 2.9 ml of the reagent mixture while distilled water was added for blank sample. The resulting solution was thoroughly agitated, then incubated for 30 minutes at 37^{o}C. The blank content was transferred to the cuvette and read by spectrophotometry at 340 nm.

3.5 DETERMINATION OF PLASMA ALANINE AMINOTRANSFERASE (ALT) ACTIVITY

Plasma ALT was determined by using ALT assay kit (RANDOX).

Principle

Alanine aminotransferase catalyzes amino transfer from L-alanine to 2-oxoglutarate, yielding L-glutamate and pyruvate. Elevated ALT in the reaction mixture is an indication of high pyruvate concentration. This is read at 510 nm using spectrophotometry (Reitman and Frankel, 1975).

L - Alanine + 2 Oxoglutarate $\xrightarrow{\text{ALT}}$ L-Glutamate + Pyruvate

Reagent

Reagent A (Buffer A), Phosphate buffer (100 mmol/L), 2-Oxoglutarate (6 mmol/L), DL-Alanine (200 mmol/L), Sodium Azide (12 mmol/L).

Reagent B (Buffer B)

2, 4-dinitrophenyhydrazine (2.0 mmol/L), 0.4 mol/L Sodium hydroxide.

Procedure

Pipette Buffer A (0.5 ml) into a test-tube containing equal volume of reagent either blank and plasma (100 µL) separately, the same volume of distilled water is added to both test-tubes and incubated for 30 minutes at 37^{o}C. Add Buffer B (0.5 mL), then leave at 20 minutes for 20-25^{o}C. After this, add 5.0 ml of Sodium hydroxide, then read the resulting

solution at 546 nm after 5 minutes against reagent blank in a spectrophotometer. Activity of plasma ALT would be obtained from a standard table.

3.6 DETERMINATION OF PLASMA CHOLESTEROL LEVELS

Plasma cholesterol and triglyceride levels were determined by using cholesterol and triglyceride assay kit (RANDOX).

Principle for Cholesterol Determination

Enzymatic hydrolysis of cholesteryl esters with the concomitant oxidization of 3-OH group of cholesterol yields hydrogen peroxide, indicated by a color change that is read by spectrophotometry at 500 nm.

Cholesteryl-ester + Water $\xrightarrow{\text{cholesteryl-ester hydrolase}}$ Cholesterol + Acyl group

Cholesterol + Oxygen $\xrightarrow{\text{cholesterol oxidase}}$ Cholest-4-en-3-one + Hydrogen peroxide

2 Hydrogen peroxide + 4-aminophenazone + phenol $\xrightarrow{\text{peroxidase}}$

4-(p-benzoquinone-monoimino)-phenazone + $4H_2O$

Reagents

Reagent A

Piperazine-N, N'-bis (2-ethanesulfonic acid) (PIPES) (50 mmol/L), Phenol (24 mmol/L), Buffer (pH 6.9)

Reagent B

Cholesterol esterase (250 U/I), NaCl (2.5 mmol), aminophenazone (0.5 mmol/L), cholesterol oxidase (250 U/I).

Reagent C

Cholesterol standard (2.585 mmol/L=100 mg/50 ml)

Reagent B was dissolved in Reagent A to give Reagent C

Procedure

TABLE 1: PROTOCOL FOR ESTIMATION OF CHOLESTEROL ACTIVITY

Test-tubes	Test	A	B	C	D
1	Reagent C	2 mL	2 mL	2 mL	2 mL
2	Standard cholesterol	-	40 µL	-	-
3	Test sample	-	-	40 µL	-
4	Distilled water	40 µL	-	-	-

Agitate test-tubes and leave for 5 minutes at 37^{o}C. The readings are taken at 500 nm against content in tube D using spectrophotometry.

Calculation

Concentration =

$$\frac{A\ plasma\ x\ n}{A\ standard}$$

Equation 2: Cholesterol concentration

3.7 DETERMINATION OF TRIGLYCERIDES LEVELS

Plasma triglyceride level was determined by using triglyceride assay kit (RANDOX).

Principle

The reactions involved various steps where glycerol is obtained from the hydrolysis of triglyceride, the glycerol obtained is further oxidized by an oxidase and hydrogen peroxide.

Triglycerides + $3H_2O$ $\xrightarrow{\text{lipase}}$ Glycerol + Acyl group

Glycerol + Adenosine triphosphate $\xrightarrow{\text{glycerol kinase}}$ Glycerol-3-phosphate + Adenosine triphosphate

Glycerol-3-P + Oxygen $\xrightarrow{\text{glycerol-3-P oxidase}}$ Dihydroxyacetone-P + Hydrogen peroxide

Hydrogen peroxide + 4-Aminophenazone + ESPAS $\xrightarrow{\text{peroxidase}}$ 4-(-p-benzoquinone-monoimino)-phenazone + $2H_2O$ + HCl

ESPAS= N-ethyl-N-sulfopropyl-m-anizidine

Reagents

Reagent A

N-ethyl-N-sulfopropyl-m-anizidine (1 mmol/L), PIPES buffer pH 7.5 (50 mmol/L)

Reagent B

Glycerol kinase (800 U/I), lipoprotein lipase (1100 U/I), glycerol-phosphate oxidase (3000 U/I), ATP (0.3 mmol/L), peroxidase (350 U/I), 0.7 mmol/4 aminophenazone.

Reagent C

Glycerol standard (2.28 mmol/L=200 mg/100 ml)

Reagent B was dissolved in 10 ml of Reagent A to obtain Reagent C

TABLE 2: COMPOSITION OF REAGENTS FOR TRIGLYCERIDE LEVEL

Test	Solution	A	B	C	D
1	Reagent C	2 mL	2 mL	2 mL	2 mL
2	Standard triglyceride	-	40 µL	-	-
3	Test	-	-	40 µL	-
5	Distilled H_2O	40 µL	-	-	-

Agitate tube and leave at 37^{o}C for 5 minutes. Take readings against solution in tube 5 at 545 nm using spectrophotometry.

3.8 DETERMINATION OF PLASMA HORMONE LEVELS

3.8.1 DETERMINATION OF PLASMA LEVELS OF INSULIN HORMONE

Assay kit obtained from RANDOX was used for plasma insulin level determination. The reference methods in the kit were adhered to and values were estimated using ROBONIK 11-2000 ELISA reader (ROBONIK, Mumbai, India).

Principle

The enzyme-linked immunosorbent assay (ELISA) technique used is based on direction of monoclonal antibodies on different antigenic determinant present on molecule of insulin. In the course of incubation in the micro-titer well, anti-insulin antibodies bind to insulin molecules in a reaction. Enzymes that are not bound are washed off and the bound conjugate identified with 3, 3', 5, 5'-tetramethylbenzidine (TMB). Micro plate reader is used to take readings at 450 nm.

Reagents

Reagent kit, ELISA insulin kit, coated plate, anti-insulin monoclonal antibody, conjugate enzyme-1X and buffer, peroxidase conjugated-monoclonal anti-insulin, wash buffer-21X, TMB substrate, stop solution, 0.5 M H_2SO_4.

Procedure

The enzyme conjugate-1X solution, wash buffer, samples and insulin control were prepared, while the microplate wells for samples and calibrators were made ready. In each well was added 25 µL each of calibrators and sample, subsequently, 100 µL solution of enzyme conjugate-1X was introduced. The plate shaker was incubated for 2 hours at 700-

900 rpm and the wells washed with wash buffer-1X solution 6 times, thereafter, 350 µL of wash solution was added to each well. This was subsequently discarded, then inverted over absorbent paper, eliminating all liquid. Furthermore, 200 µLof substrate and TMB was added into all wells and left for 15 minutes at 18-25^{o}C, while the reaction was stopped by 50 µL of stop solution and mixed thoroughly on a shaker.Readings were taken within 30 minutes by spectrophotometry at 540 nm.

Calculations

i. Absorbances obtained for calibrators were plotted against concentration of insulin.
ii. The sample concentration was obtained from the calibration curve.

Factor for conversion

1 µg = 174 pmol

3.8.2 DETERMINATION OF PLASMA LEVELS OF LUTEINIZING HORMONE

Assay kit obtained from RANDOX was used for determination of plasma luteinizing hormone level. The reference methods in the kit were adhered to and values were estimated using ROBONIK 11-2000 ELISA reader (ROBONIK, Mumbai, India).

Principle

This assay uses anti-LH antibody for the micro-titer well and horseradish peroxidase (HRP) conjugate as the antibody-enzyme which is another mouse anti-LH antibody. Reaction of the test samples and antibodies occurred and antibodies not bound are washed off from the

wells after 60 minutes of incubation at room temperature. Afterwards, incubation with TMB causes color formation intercepted by 2 N hydrochloric acid, a color transit from blue to yellow was observed and readings by spectrophotometry at 450 nm was carried out. The intensity of the yellow color is an indicator of the concentration of LH (Knobil, 1980).

Reagents

Conjugate enzyme (12 mL), 12 mL of Stop solution, 12 mL TMB substrate, Wash Buffer concentrate (15 mL).

Procedure

The data sheet was made for identification of sample.Standard solution and test samples of equal volumes were put in suitable wells, mixed for 30 seconds and then left at 18-22^{o}C for 60 minutes. The plate content were flicked into the sink to remove incubation mixture and rinsed with washing buffer to get rid of remaining droplets of water. This was left at room temperature for 20 minutes after TMB addition to the wells. Stop agent (100 µL) was used to interject the reaction, while mixing caused yellow coloration that was quite distinct from the initial blue.Finally, readings were obtained from a micro-titer plate at 450 nm within 15 minutes.

Calculations

Mean absorbance for all the samples and standard were calculated, and then a curve with plot of absorbance mean (standard) versus concentration was obtained. This was used in obtaining the concentration of LH.

3.8.3 DETERMINATION OF PLASMA LEVELS OF FOLLICLE-STIMULATING HORMONE

Assay kit obtained from RANDOX was used for determination of plasma follicle stimulating (FSH) hormone level. The reference methods in the kit were adhered to and values were estimated using ROBONIK 11-2000 ELISA reader (ROBONIK, Mumbai, India).

Principle

The immunometric experiment where streptavidin coats the microwell plate, while the samples, biotinylated capture antibody, and HRP are all incubated inside the microwell plates. The biotinylated capture antibody will bind streptavidin and the introduced FSH, while the detection antibody will only bind a different epitope on the FSH molecule. All these are immobilized onto the wells by the streptavidin-biotinylated antibody interaction. Afterwards, this is left at room temperature for about 45 minutes, followed by distilled water washing to get rid of unbound labeled antibodies. After this TMB is introduced and left at room temperature for 20 minutes. This step causes a blue color to develop; the reaction is stopped by 1 N HCl which is indicated by a yellow color that is assessed using spectrophotometry at 450 nm. The amount of bound anti-FSH-HRP is directly proportional to the color developed and this is proportional to the amount of FSH.

The absorbance is directly proportional to [Anti-FSH-HRP] which is directly proportional to [FSH]

Reagents

TMB, Anti-FSH, HRP and Anti-FSH-Biotin Conjugate, 1 N HCl, Mouse monoclonal alpha antibodies, Mouse monoclonal beta antibodies.

Procedure

As for LH

3.84 DETERMINATION OF PLASMA LEVELS OF TESTOSTERONE

Assay kit obtained from RANDOX was used for determination of plasma testosterone level. The reference methods in the kit were adhered to and values were estimated using ROBONIK 11-2000 ELISA reader (ROBONIK, Mumbai, India).

Principle

Here, there is competition of sample's antigen and conjugate antigen for inadequate binding sites for antibody present on the microwell. Distilled water is used to remove excess antibodies and unbound substances. Thereafter, the enzyme-substrate (TMB) is added, then left at 15 minutes at ambient temperature, subsequently, the reaction was intercepted by 1 N HCl solution. Microtiter reader was employed in determining the absorbance at 450 nm within 20 minutes. It was observed that the concentration of testosterone was corresponding to the color intensity.

Reagents

Testosterone (free), TMB, HRP conjugate concentrate, 1 N HCl, antibody coated microwell plate (anti-free testosterone).

Procedure

Pipette 25 µL of sample into labeled wells, then add 4 times HRP solution, agitate the plate and leave at 37^{o}C for 1 hour. Thereafter wash three times with distilled water and add TMB into each well. Incubate again at same temperature for 1 hour, then add 1 N HCl, measure the readings at 540 nm on a microwell plate reader.

This was done by strict adherence to the manufacturer's instruction.

3.9 TISSUE PREPARATION FOR IMMUNOHISTOCHEMISTRY ANALYSIS OF CYTOCHROME C

Normal healthy albino rats were sacrificed by cervical dislocation with the heart and liver sections were placed in 10% phosphate-buffer formalin, while the testes were placed in bouin's reagent for proper fixation. This method provides in situ information on the position of antigen on the organs, an information that gives more substantial investigational result.

Principle

The binding between the primary antibody and the 'specific antigen' forms a complex which is introduced to the 'secondary antibody'. Chromogen and substrate reaction is catalyzed and colored deposits at the binding site are obtained.

Procedure

Before sectioning, the tissues were placed in paraffin wax or cryomedia and sliced with microtome to 4-40 μm. These were dehydrated with alcohol wash of different concentrations, they were then cleared using detergent like xylene. Digitonin (100 μL) was added to harvested cells on ice for 5 minutes. Cells of the heart, liver and testes were fixed in 4% paraformaldehyde (PBS), washed in same three times and left in blocking buffer (0.05% saponin in PBS, 3% BSA) for 1 hour. These were left over the night at 4^{o}C with 1:200 anti-cytochrome c monoclonal antibodies (Elabscience, China), then, washed thoroughly and left again for 1 hour at room temperature in 1:200 PE-labelled secondary antibodies (Elabscience, China) that acts as a blocking buffer. The binding sites were identified by 3'-3'-diaminobenzidine tetrahydrochloride stain and hematoxylin was employed for counter staining. Cells were then viewed by microscopy.

3.10 TISSUE PREPARATION FOR IMMUNOHISTOCHEMISTRY ANALYSIS OF CASPASES 3 and 9

Principle

This technique recognize proteins by binding of antigen to antibody through labeled conjugates.

Reagents

Monoclonal antibodies for caspases 3 and 9 (Elabscience, China), 1 mmol/l EDTA (Sigma Aldrich, Inc., USA), Avidin-biotin-peroxidase complex, Tris-Triton (dissolve 0.1 M Tris HCl buffer in 0.01% Triton X-100 and adjust pH to 7.6), secondary antibody (biotinylated), 0.05% Diaminobenzidine, 0.03% Hydrogen peroxide, 0.25% $NiSO_4.6H_2O$, Cytoseal 60, Xylene.

Procedure

The testes were fixed in bouin's buffer while heart and liver tissues were fixed in and phosphate buffer. These were cut into 6 μm, dewaxed, and afterwards submerged in H_2O_2: methanol solution (1:19) for 30 minutes, to stop the activity of endogenous peroxidase. Thereafter, the fixed tissues were boiled in EDTA/Tris buffer solution (pH 8.0) for 10 minutes, and cooled at room temperature. Blocking was done with horse serum/Tris triton, then mixed in caspase 3 or 9 antibodies (1:1200) and left over the night at 4°C. This was rinsed with the buffer, while secondary buffer added for 1 hour at 25^{o}C. Thereafter, avidin-biotin peroxidase was added 10 minutes in Tris triton that have 0.05% diaminobenzidine and 0.03% hydrogen peroxide.

Xylene was used in clearing the tissues, while activated caspase 3 and 9 were used for blocking and stains were observed by photographing and Fiji software package was used to calculate the extent of stains.

3.11 TISSUE PREPARATION FOR HISTOPATHOLOGY

Fixations of heart, liver and testes sections were done in 10% formalin, and subsequently put in paraffin before cutting with microtome, dehydration by varying concentration of alcohol and clearing by xylene detergent was carried out. These were then placed in paraffin wax, then deparaffinized and dehydrated. The paraffin blocks were stained with hematoxylin, washed and embedded in acid alcohol to remove the excess stain and counter stained by 10% eosin before microscopic examination.

3.12 ISOLATION OF RAT LIVER MITOCHONDRIA

Low ionic-strength mitochondria acquired from the rat liver were obtained by centrifugation process using a revision of the procedure of Johnson and Lardy (1967).

Reagents

Buffer C (Isolation buffer: 210 mM Mannitol, 70 mM Sucrose, 5 mM (2-[4-(2-hydroxymethyl] piperazin-1) (HEPES)-KOH (pH 7.4), 1mM EGTA)

Mannitol (7.66 g) (BDH Chemicals Ltd, Pools, England), HEPES (0.24 g) (May and Baker Lab, USA), EGTA (0.038 g) (Sigma Chemical Co, USA) and Sucrose (4.8 g) (BDH Chemicals Ltd, Pools, England) were all dissolved in 140 mL distilled water, stirred properly and pH adjusted to 7.4 by KOH (Sigma Aldrich, USA) before making up to 200 mL with distilled water and kept at 4˚C.

Buffer D (Washing buffer: 210 mM Mannitol, 70 mM Sucrose, 5 mM HEPES-KOH (pH 7.4), 0.5%BSA)

Sucrose (4.8 g) (BDH Chemicals Ltd, Pools, England), 0.24 g of H-E-P-E-S (May and Baker Lab, USA), 7.66 g of Mannitol (BDH Chemicals Ltd, Pools England) and 1 g of Bovine Serum Albumin (BSA) (Sigma Aldrich, USA) were all dissolved in 140 mL distilled water, stirred properly and pH adjusted to 7.4 by KOH (Sigma Aldrich, USA) before making up to 200 mL with distilled water. The solution was spun gently in order for the BSA to dissolve and prevent foaming. The buffer solution was kept at 4˚C.

MSH buffer (Suspension buffer: 210 mM Mannitol, 70 mM Sucrose, 5 mM HEPES-KOH (pH 7.4))

0.24 g of HEPES (May and Baker Lab, USA) was dissolved in about 100 mL distilled water, standardized to pH 7.4 with KOH (Sigma Chemical Co, USA). Thereafter, 7.66 g of Mannitol (BDH Chemicals Ltd, Pools England) and 4.8 g of Sucrose (BDH Chemicals Ltd Pools, England) was added into 140 mL distilled water and stirred properly before making up to 200 mL with distilled water.

Procedure

Male Wistar rats were sacrificed by cervical dislocation, opened up and liver straightaway removed. This was washed several times with buffer C, then weighed and shredded with a pair of scissors. A 10% suspension was made in a Teflon glass cup and homogenised. The solution was quickly transferred into a refrigerated MSE centrifuge and spanned 2,300 rpm twice for 5 minutes each time. Supernatant was centrifuged again at 13,000 rpm for 10 minutes to obtain the mitochondria. Mitochondria pellet was washed twice at 12,000 rpm for 10 minutes. The obtained mitochondria were immediately suspended in buffer (MSH), then dispensed in eppendorf tubes as aliquots and kept on ice until use.

Low ionic-strength liver mitochondria used for determination of ATPase activity and Lipid peroxidation were isolated as defined above except that Buffer C and Buffer D were substituted with 0.25 M Sucrose.

3.13 ISOLATION OF RAT HEART MITOCHONDRIA

Low ionic-strength mitochondria acquired from the rat heart were obtained by differential centrifugation using a revision of Mela and Seitzk (1969) and Sanz *et al.,*(2007) with slightmodifications.

Reagents

Buffer E (Isolation buffer: 220 mM Mannitol, 70 mM Sucrose, 1 mM EDTA, 10 mM Tris pH 7.4)

Sucrose (2.4 g) (BDH Chemicals Ltd, Pools, England), 3.64 g Mannitol (BDH Chemicals Ltd, Pools, England), and 0.039 g EDTA (Sigma Chemical Co, USA) were dissolved in 70 mL solution of 0.16 g Tris HCl at pH 7.4, stirred properly before making up to 100 mL with distilled water and kept at 4^{o}C.

Buffer F (Washing buffer: 220 mM Mannitol, 70 mM Sucrose, 1 mM EDTA, 10 mM Tris (pH 7.4), 0.2% BSA)

Sucrose (4.98 g) (BDH Chemicals Ltd, Pools, England), 7.66 g Mannitol (BDH Chemicals Ltd, Pools, England), and 0.078 g EDTA (Sigma Chemical Co, USA) were dissolved in 140 mL solution of 0.32 g Tris HCl at pH 7.4 and 0.4 g BSA, then stirred properly before making up to 200 mL with distilled water and kept at 4^{o}C.

MST buffer (Suspension buffer: 220 mM Mannitol, 70 mM Sucrose, and 10 mM Tris-HCl (pH 7.4))

Mannitol (3.64 g) (BDH Chemicals Ltd, Pools England) and 2.46 g of Sucrose (BDH Chemicals Ltd Pools, England) were added to the solution of 0.16 g of Tris (May and Baker Lab, USA) dissolved in 70 ml of distilled water and standardized to a pH of 7.4 with HCl (Sigma Chemical Co, USA). This was made up to 100 ml in a standard volumetric flask.

Procedure

Mitochondrial isolation was done according to the method described by Johnson and Lardy (1967) on page 79. The rats were sacrificed by cervical dislocation, dissected, the tissue removed and briefly rinsed with the isolation buffer [220 mM mannitol, 70 mM sucrose, 10 mM Tris and 1 mM EGTA adjusted to pH 7.4 using KOH]. This was weighed, finely minced, and homogenized in a 10% suspension of the buffer in a glass/Teflon cup homogeniser. The homogenate obtained was centrifuged at 3,000 rpm for 5 minutes at 4^{0}C twice to remove cellular debris and unbroken cells. The supernatant was centrifuged again at 11,000 rpm for 10 minutes to obtain the mitochondria. Mitochodria pellets were washed twice with washing buffer [220 mM mannitol, 70 mM sucrose, 1 mM EDTA, 10 mM Tris, and 0.2% BSA] at a speed of 10,500 rpm in a refrigerated centrifuge for 10 minutes. The mitochondria obtained were re-suspended in a buffer [220 mM mannitol, 70 mM sucrose, and 10 mM Tris-HCl], dispensed as aliquots into eppendorf tubes and kept on ice until use. All procedures were done at 4^{0}C.

Low ionic-strength heart mitochondria used for determination of ATPase activity and Lipid peroxidation were isolated as described above except that Buffer E and Buffer F were substituted with 0.25 M Sucrose.

3.14 ESTIMATION OF MITOCHONDRIAL MEMBRANE PERMEABILITY TRANSITION PORE OPENING IN RAT HEART AND LIVER MITOCHONDRIA

Principle

This method is based on the permeabilisation of the mitochondrial membrane causing swelling of the mitochondria, and thus altering the refractive index and thereby producing a

decrease in the absorbance of the mitochondria suspension at 540 nm. Isolated mitochondria undergoing Ca^{2+}-induced permeability transition allow entry of water and solutes, loss of $\Delta\Psi_m$, and massive amplitude matrix swelling which culminate in a decrease in photometric absorption at 540 nm (Lapidus and Sokolove, 1992). Membrane permeability transition was evaluated by measuring the swelling of mitochondria based on the principle that as mitochondria swell, there is a concomitant discharge of inner mitochondrial proteins (Lapidus and Sokolove, 1992). This is measured as a decrease in light absorbance using a spectrophotometer. Complication may arise due to the changes in the redox state of the respiratory chain components, therefore the wavelength of the incident light should be at the isobestic point for cytochromes and this is 520 nm or 540 nm (Lapidus and Sokolove, 1992).

Reagents

0.8 µM Rotenone

Rotenone (0.00079 g) (Sigma-Aldrich, Germany) was dissolved in 50% Ethanol (v/v). This was kept at 4^0C in a dark container because of its photosensitivity.

5 mM Succinate

Succinate (1.3508 g) was dissolved in about 10 mL of distilled water and made up to 20 mL mark using a standard volumetric flask.

12 mM $CaCl_2.2H_2O$

Calcium chloride dehydrate (0.03528 g) dissolved in about 10 mL of distilled water, made up to 20 mL mark using a standard volumetric flask and kept at 4^oC.

250 mM Sodium Succinate

Sodium succinate (1.3501 g) was dissolved in dissolved in about 10 mL of distilled water, made up to 20 mL mark using a standard volumetric flask and kept at 4^{o}C.

1 mM Spermine

Spermine (0.00809 g) was dissolved in distilled water dissolved in about 10 mL of distilled water, made up to 20 mL mark using a standard volumetric flask and then left at 4^{0}C in a black container because of its photosensitivity.

Procedure

The integrity of the isolated mitochondrial for membrane permeability transition swelling was determined by incubating mitochondria (0.4 mg/mL) in suspension buffer containing rotenone (0.8 μM) for 3½ minutes. Afterwards, succinate (5 mM) was added and the absorbance change was monitored over a time scan of 12 minutes at 30 second interval (Lapidus and Sokolove 1993). For large amplitude swelling assay, calcium chloride (12 mM) was added to induce mitochondria swelling at 30 seconds before succinate addition. Mitochondria isolated from diabetic rats, various treatment groups and normal control were subjected to the mPT assay with no addition of calcium, while the absorbance readings were determined using a spectrophotometer at 540 nm at 30 seconds interval for 12 minutes.

Table 3: Protocol for Mitochondrial swelling using quercetin (*in vitro*)

Samples	Swelling Buffer (µL)	Rotenone (µL)	Spermine (µL)	Mitochondria (µL)	$CaCl_2$(µL)	Quercetin (µL)	Sodium Succinate (µL)
Blank	2500	-	-	-	-	-	-
No triggering agent	2410	10	-	30	-	-	50
Triggering agent	2385	10	-	30	25	-	50
Spermine	2322.5	10	62.5	30	25	-	50

Quercetin	2400	10	-	30	-	10	50
Quercetin	2380	10	-	30	-	30	50
Quercetin	2360	10	-	30	-	50	50
Quercetin	2340	10	-	30	-	70	50

Note: Readings were taken at 540 nm

Table 4: Protocol for Mitochondrial swelling for normal and STZ-induced diabetic rats and treatment groups

Samples	Swelling buffer (µL)	Rotenone (µL)	Mitochondria (µL)	Sodium succinate (µL)
Blank	2500	-	-	-
Normal rat mitochondria	2410	10	30	50

STZ-induced rat mitochondria	2410	10	30	30
STZ-induced rat mitochondria from treatment group	2410	10	30	50

Note:

1. Readings are taken at 540 nm in a spectrophotometer.
2. Treatment groups are 10, 30 mg/kg quercetin, 10 mg/kg vitamin E, 10 mg/kg quercetin and vitamin E each, 0.6 mg/kg glibenclamide, 30 mg/kg pretreatment with quercetin.

3.15 PROTEIN DETERMINATION BY LOWRY'S PROCEDURE

Mitochondrial protein content was estimated according to the method of Lowry *et al.,* (1951) using BSA as standard.

Principle

The peptide nitrogen reacts with copper II ion in an alkaline condition of pH 10-10.5 and this causes the reduction of Folin-Ciocalteau phosphomolybdic-phosphotungstic acid to heteropolymolybdenum blue by aromatic acids oxidation catalyzed by copper. This procedure is sensitive to small amount of protein concentration (0.1-2 mg protein/mL).

Graded concentrations of BSA were used to generate a standard curve

Reagent

- Reagent A: 2% Na_2CO_3 in 0.1 M NaOH [2 g Na_2CO_3(BDH Chemicals Ltd, England) and 0.4 g NaOH (Sigma Chemical Co, USA) were dissolved in 100 mL distilled water and kept at room temperature.
- Reagent B: 2% Na-K-tartrate (Hopkins and Williams England) was dissolved in 100 mL distilled water and kept at room temperature.
- Reagent C: 1g $CuSO_4.5H_2O$ (Sigma Chemical Co, USA) was dissolved in 100 mL distilled water and kept at room temperature.
- Reagent D: This is an alkaline $CuSO_4$ solution prepared just before use. This is constituted of 50 mL of Reagent A, 0.5 mL of Reagent B and 0.5 mL of Reagent C (100:1:1 of Reagent I, II and III).
- Reagent E: The stock solution of Folin-Ciocalteau reagent (Sigma Chemical Co, USA) was diluted with equal amount of distilled water (1:1) to obtain Reagent E.

3.16 PREPARATION OF FOLIN-CIOCALTEAU REAGENT

Sodium Molybdate (25 g) ($Na_2MoO_4.2H_2O$) and 100 g of Sodium Tungstic ($Na_2WO_4.2H_2O$) were dissolved in about 700 ml of distilled H_2O. Concentrated HCl (100 ml), 50 ml of 85% phosphoric acid and few drops of bromine was added to the mixture and refluxed for 10 hours in an all-glass apparatus. This mixture was thereafter cooled at room temperature, made up to 1 L with distilled water and then filtered. This was stored at 4^0C and color of the resulting solution to give a golden yellow. A green color indicates an unsatisfactory result. The reaction may be regenerated by boiling with a few drops of

bromine. Reagent preparation must be in the fume cupboard and a black container must be used for storage due to its photolytic nature. The 2 N solution was usually diluted to 1 N using distilled water just before experimental use.

3.17 STANDARD PROTEIN SOLUTION

Preparation of BSA (1 mg/mL) (Sigma Chemical, USA): this was done by dissolving 5 mg of BSA in 5 mL distilled water. Thereafter, 1 mL of the stock solution was dissolved in 19 mL distilled water to give a 200 µg/ml concentration mixture with an absorbance of 1.140 at 279 nm. The molecular extinction coefficient (E) of BSA is 45,000 and its molecular weight is 65,000.

Procedure

To the protein samples were added 3 ml of Reagent D, this was mixed and left for 10 minutes at room temperature. Thereafter Reagent E was added to the mixture, mixed vigorously and left for 30 minutes at room temperature for color development. Absorbance readings were taken at 750 nm in a CamSpec M106 Spectrophotometer, these were plotted against the respective BSA concentration to generate a standard curve.

3.18 SAMPLE PREPARATIONS.

Mitochondria (10 µL) obtained from all groups of animals were dissolved in 990 µL of distilled water in duplicate test tubes, while the blank test tube contained 1000 µL of distilled water. 3.0 ml (3000 µL) of Reagent D was added to all test tubes (duplicates and blank) and left for 10 minutes at room temperature. Thereafter, 3 mL (300 µL) of Reagent

E(Folin-C) was added to all test tubes and allowed to stand for 30 minutes after which readings were taken at 750 nm wavelength on a Camspec M105 spectrophotometer.

Table 5: Protocol for Protein Estimation (Lowry *et al.*,1951)

Test tubes	I	II	III	IV	V	VI	VII	VIII	IX
Standard BSA (µL) solution	-	100	200	300	400	500	600	700	800
Distilled H_2O (µL)	1000	900	800	700	600	500	400	300	200

Reagent D (µL)	3000	3000	3000	3000	3000	3000	3000	3000	3000
Incubate at room temperature for 10 minutes									
Folin E (µL)	300	300	300	300	300	300	300	300	300
Incubate at room temperature for additional 30 minutes									

Note: Readings were taken at 750 nm

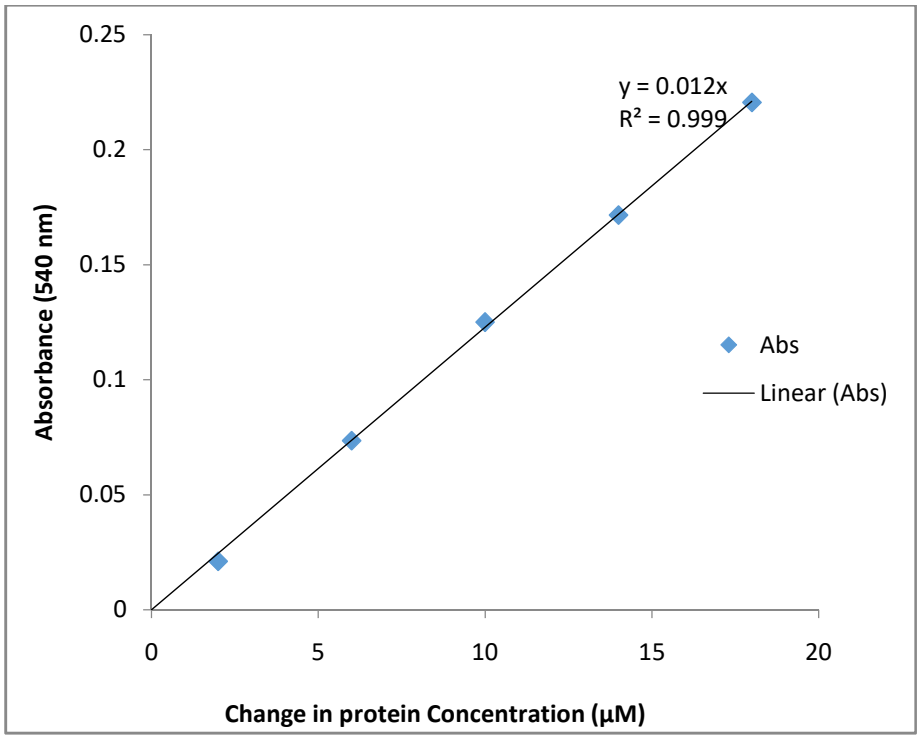


Figure 13: The Standard Protein Curve

3.19 LIPID PEROXIDATION DETERMINATION

The lipid peroxidation level was determined using mitochondria as the source of lipid media was conducted according to the method described by Ruberto *et al.,*(2000).

Principle

The 2-thiobarbituric acid (TBA) interacts with one of the products of lipid peroxidation (LPO), malondialdehyde (MDA). Boiling at 96^{o}C for 1 hour in acid solution gives a pink colored complex which absorbs maximally at 532 nm and fluoresces at 533 nm. The product of the reaction dissolves easily in butanol.

Reagents

0.8% Thiobarbituric acid

Thiobarbituric acid (0.8 g) (BDH Chemicals, Ltd., Poole, England) was dissolved in 50 mL distilled water and the solution transferred into a 100 mL standard flask and then made up to the mark with distilled water.

1.1% Sodium deodocyl sulphate

Sodium deodocyl sulphate (8.1 g) (BDH Chemicals, Ltd., Poole, England) was dissolved in 50 ml distilled water and the solution transferred into a 100 mL standard flask and then made up to the mark with distilled water. Equal volumes of TBA and SDS were mixed and used in the assay.

20% Acetic acid

Glacial acetic acid (20 mL) (BDH Chemicals, Ltd., Poole, England) was added to distilled water in a 100 mL standard flask and solution made up to the mark.

Procedure

Mitochondria (0.4 mL) isolated from all groups were incubated in a mixture containing 1.5 mL of 20% acetic acid, 1.5 mL of 0.8% thiobarbituric acid and 1.1% SDS (1:1). The reaction tubes were placed in a water bath and heated at 95^{0}C for 1 hour. This was subsequently cooled under a running water tap and 5mL of butanol added. The solution was centrifuged at 3,000 rpm for 10 minutes and the organic upper layer read against a distilled water as blank at 532 nm in a spectrophotometer.The level of malondialdehyde released as a product of lipid peroxidation was quantified and calculated using an extinction coefficient of 0.156 μM^{-1} .cm^{-1} Adám-Vizi and Seregi (1982).

Lipid peroxidation (nmole MDA/mg protein) =

$$\frac{Absorbance\ x\ Volume\ of\ mixture}{E532\ nm\ x\ Volume\ of\ sample\ x\ mg\ protein/mL}$$

Equation 3: Calculation of Lipid peroxidation (nmole MDA/mg protein)

Table 6: Protocol for Determination of Lipid Peroxidation Level

Groups	Mitochondria (µL)	H_2O (µL)	Acetic acid (µL)	TBA:SDS (µL)	Butanol (mL)
Normal rat	35	1015	1500	1500	5
STZ-induced diabetic rat	35	1015	1500	1500	5
STZ-induced diabetic rat + Quercetin and Vitamin E (10mg/kg each)	35	1015	1500	1500	5
STZ-induced diabetic rat + 10mg/kg Quercetin	35	1015	1500	1500	5
STZ-induced diabetic rat + 30mg/kg Quercetin	35	1015	1500	1500	5
STZ-induced diabetic rat + 10mg/kg Vitamin E	35	1015	1500	1500	5
STZ-induced diabetic rat +0.6mg/kg Glibenclamide	35	1015	1500	1500	5
Normal rat +10mg/kg each of Quercetin and Vitamin E	35	1015	1500	1500	5
Normal rat +10mg/kg Quercetin	35	1015	1500	1500	5
Normal rat +30mg/kg Quercetin	35	1015	1500	1500	5
Normal rat +10mg/kg Vitamin E	35	1015	1500	1500	5
Normal rat + 0.6mg/kg glibenclamide	35	1015	1500	1500	5

3.20 DETERMINATIONof ATPase ACTIVITY

Assessment of the ATPase activity was done according to the procedures of Lardy and Wellman (1953) as modified by Olorunsogo and Bababunmi (1979), while the concentration of inorganic phosphate released was determined as described by Bassir (1963).

Reagents

A. 0.1 M Tris HCl pH 7.4

Tris (hydroxyl methyl) aminomethane (Sigma Aldrich Inc., USA) (1.2 g) was dissolved in about 60 mL of distilled water and the pH adjusted to 7.4 with HCl. The final solution was made up to 100 mL in a standard volumetric flask and stored in the refrigerator at 4^{o}C.

B. 0.25 M sucrose

Sucrose (Sigma Aldrich Inc., USA) (8.56 g) was dissolved in 60 mL of distilled water and made up to 100 mL mark in a standard volumetric flask and kept in the refrigerator at 4^{0}C.

C. 5mM KCl

Potassium chloride, KCl, (37.25 mg) was dissolved in 60 mL distilled water and then made up to the 100 mL mark with distilled water in a standard volumetric flask.

D. 0.01 M ATP (pH 7.4)

Disodium salt of ATP (Sigma Aldrich Inc., USA) (0.2757 g) was dissolved in 50 mL distilled water, the pH adjusted to 7.4 and then made up to 100 mL mark in a standard volumetric flask with distilled water. This was stored in eppendoff tubes at -4^{o}C.

E. 9% Ascorbate

Ascorbic acid (BDH Chemicals, England) (9 g) was dissolved in 80 mL of distilled water and stored in brown reagent bottle at 4^{o}C. This reagent is usually prepared fresh.

F. Ammonium Molybdate:

Ammonium molybdate (Hopkins and Williams Ltd. England)(1.25 g) was dissolved in 100 mL of 6.5% H_2SO_4. This reagent is usually stored in plastic container at room temperature.

G. 10% Trichloroacetic acid:

Trichloroacetic acid (BDH Chemicals, England) (10 g) was dissolved in 60 mL distilled water and made up with distilled water to the 100 mL mark in a standard volumetric flask. This was transferred into a reagent bottle and stored at 4^{o}C.

Procedure

The activity of mATPase was determined according to the methods described by Lardy and Wellman (1953). The mitochondria used for the assay were isolated following the steps outlined in the mPT assay but for 0.25 M sucrose buffer used as isolation buffer. Assay involving mitochondria obtained from normal control, diabetic, and treatment groups were performed in a test tube containing 0.25 mM sucrose, 5 mM KCl, and 0.1 M Tris-HCl buffer adding up to 2 mL with distilled water as required. Thereafter, ATP (0.01 M) was added to the tubes implored into a shaker water bath. Mitochondria (0.4 mg/mL protein) were added to another test-tube containing the buffer and reaction subsequently stopped by the addition of 1 mL of 10% sodium dodecyl sulfate (SDS). ATP hydrolysis devoid of the enzyme was assessed in a set of tubes that contained only ATP without mitochondria,

while some tubes contained mitochondria isolated from normal control, diabetic, and treatment groups were incubated with ATP. Another tube contained mitochondria from normal rat and subsequent addition of 25 μM 2, 4-dinitrophenol used as uncoupler. All reaction tubes were incubated in a shaker water bath for 30 minutes at 27^{θ}C. Thereafter, the reaction was terminated by the addition of 1 mL 10% SDS sequentially, the way mitochondria were added. Furthermore, 1 mL ammonium molybdate and ascorbate were added to the mixture, incubated at room temperature for 30 minutes and absorbance readings taken at 660 nm in a spectrophotometer. The release of inorganic phosphate concentration was determined from the phosphate standard curve.

Table 7: Protocol for Determination of Mitochondrial ATPase Activity

	Sucrose (µL)	KCl (µL)	Tris (µL)	H_2O (µL)	UCP (µL)	ATP (µL)	Mit (µL)	SDS (µL)	NH_4Mb (µL)	Ascorbate (µL)
Blank	200	200	1300	300	-	-	-	1000	1000	1000
Normal rat	200	200	1300	265	-		35	1000	1000	1000
STZ-induced diabetes	200	200	1300	260	-	40	35	1000	1000	1000
Quercetin 10 mg/kg	200	200	1300	225	-	40	35	1000	1000	1000
Quercetin 30 mg/kg	200	200	1300	225	-	40	35	1000	1000	1000
Quercetin + Vitamin E 10 mg/kg	200	200	1300	225	-	40	35	1000	1000	1000
Vitamin E 10 mg/kg	200	200	1300	225	-	40	35	1000	1000	1000
Glibenclamide 0.6 mg/kg	200	200	1300	225	-	40	35	1000	1000	1000
0 Time	200	200	1300	225	-	40	35	1000	1000	1000

Note:

1. Readings were taken at 660 nm
2. UCP is the uncoupler

3.21 DETERMINATION OF MITOCHONDRIAL INORGANIC PHOSPHATE CONCENTRATION

Principle

Inorganic phosphate concentration was determined by the procedure described by Bassir (1963). The reaction produces a yellow color when there is interaction of molybdate and inorganic phosphate was reduced in ascorbic acid to blue coloration. The intensity of the color measured in a spectrophotometer at 680 nm, is comparative to the inorganic phosphate concentration.

Reagents

A. 1 mM Na_2HPO_4

Disodium hydrogen phosphate (Sigma Aldrich Inc., USA) (0.143 g)was dissolved in 1 mL distilled water.

B. 9% Ascorbic acid

Ascorbic acid (BDH Chemicals, Poole, England) (9 g)was dissolved in a little quantity of distilled water and made up with same to the mark on a 100 mL standard volumetric flask.

C. 1.25% Ammonium molybdate in 6.5% H_2SO_4

Ammonium molybdate (Hopkins and Williams Ltd., England) (1.25 g) was dissolved in 100 mL of 6.5% H_2SO_4. The reagent is stored at 25^{o}C in a plastic bottle.

Procedure

Ammonium molybdate (400 µL) was added to 5 ml of deproteinized supernatant, then followed by the addition of 0.2 mL of 2% freshly prepared ascorbate solution with gentle shaking and incubated at room temperature for 20 minutes. The same procedure was repeated for potassium dihydrogen phosphate standard solution (0.2 mg/5 mL inorganic phosphate). A resulting solution of blue coloration was obtained and read in a spectrophotometer at 680 nm against a water blank.

Table 8: Protocol for Determination of Inorganic Phosphate Concentration

Test in duplicates	Blank	1	2	3	4	5	6	7	8
1 mM Na_2HPO_4 (uL)	-	20	40	60	80	100	120	200	300
Distilled water (µL)	1000	980	960	940	920	900	880	800	700
1.25% Ammonium molybdate (mL)	1	1	1	1	1	1	1	1	1
9% Ascorbate (mL)	1	1	1	1	1	1	1	1	1

Calculation

Mg inorganic phosphate =

$$\frac{Reading\ of\ test}{Reading\ of\ standard} x \frac{0.02\ x1}{10000}$$

Equation 4: Calculation of inorganic phosphate

Mole of inorganic phosphate released =

$$\frac{mg\ of\ inorganic\ x\ 1000}{molecular\ mass\ of\ Pi}$$

Equation 5: Calculation of mole of inorganic phosphate released

Therefore, mole of inorganic phosphate (Pi) released per minute per milligram of mitochondrial protein is given by the expression mole/min/mg protein.

$$\frac{mg\ Pi\ released\ per\ mL\ x\ 1000}{\frac{Molecular\ mass\ of\ pi\ x1000}{mg\ protein\ x\ 30}}$$

Equation 6: Mole of inorganic phosphate (Pi) released per minute per milligram of mitochondrial protein

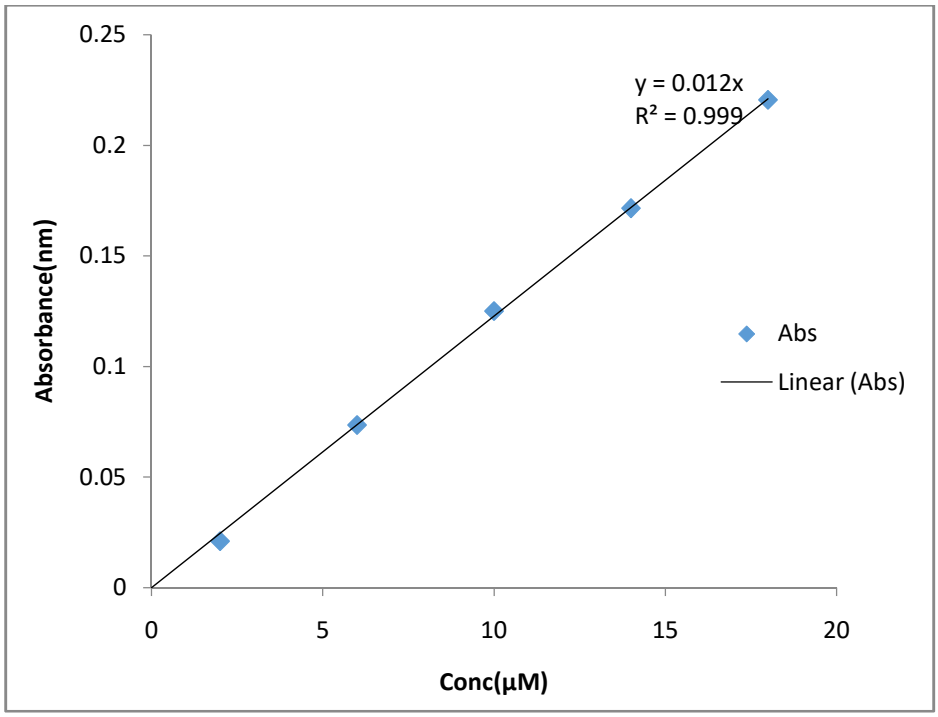


Figure 14: The Standard Phosphate Curve

CHAPTER FOUR

4.0 EXPERIMENTS AND RESULTS

EXPERIMENT 1: DETERMINATION OF THE PLASMA LEVELS OF BLOOD GLUCOSE, CERTAIN HORMONES, LIVER ENZYMES ACTIVITY AND BODY WEIGHT IN STZ-INDUCED DIABETIC RATS

INTRODUCTION

Hyperglycemia-induced oxidative stress is a main player in increasing ROS insults in cells. ROS–mediated hyperglycemia occurs via diverse mechanisms including the polyol pathway that converts glucose to sorbitol in the presence of NADPH and glutathione causing depletion of antioxidant equivalents making cells vulnerable to attack by reactive species. Hyperglycemia also enhances AGEP formation causing cell damage via glycation of vital proteins in the body altering their functions. Glycation further modifies the extracellular matrix causing abnormal function. Modification of plasma proteins leads to wrongful binding of the AGEP receptors thereby eliciting ROS generation. Hyperglycemia also activates PKC pathway via the conversion of dihydroxyacetone-3 phosphate (an intermediate in the glycolytic pathway) to diacylglycerol, this activates PKC isoforms leading to phosphorylation of specific proteins meant for vital functions (Giacco and Brownlee, 2010). Poly-ADP ribose polymerase (PARP) pathway is also activated by

hyperglycemia. Increase hexosamine pathway is also another mechanisms by which hyperglycemia result into oxidative stress in the cell. Hence, targeting blood glucose level in diabetes could be a therapeutic regimen in decreasing diabetes-induced complications (Mapanga and Essop, 2016).

Insulin is a hormone that plays vital function in tissue development and glucose homeostasis; it is produced by the pancreatic β-cells (Rains and Jain, 2011). Insulin is released subsequent to increased blood glucose concentration causing amplified glycolytic flux, glucose transport to muscles, generation of reducing equivalent that increases superoxide production that eventually generates mitochondrial hydrogen peroxide and hydroxyl radical (Karunakaran and Park, 2013). Insulin enhances glucose mobilization into adipose tissues causing reduced hepatic glucose concentration by gluconeogenesis and glycogenolysis in diabetes. Insulin resistance and or decreased secretion can activate stress sensitive pathways. Reports have shown that β-cells have higher susceptibility to oxidative stress due to reduced antioxidants availability (Karunakaran and Park, 2013). It is therefore logical to hypothesize that increasing the antioxidant capacity of diabetic subjects may alleviate ROS-induced oxidative damage in the β-cells.

Reports have shown that sex steroids function principally by acting on the reproductive system. The level of FSH, a pre-requisite for maturation of follicles and regulation of estrogen synthesis in women; regulation of spermatogenesis in men by its action on the testes' sertoli cells has been shown to be reduced in diabetic condition (Bahey *et al.,* 2014). There is paucity of information on the influence of insulin action on the levels of FSH in diabetic rats administered quercetin and vitamin E. We investigated whether the variation

in FSH level is associated with insulin modulation in diabetic rats administered quercetin and vitamin E.

Diabetes has been shown to affect LH responsible for production of leydig cells, a pre-requisite for male fertility. The level of LH has been shown to be modulated by insulin action, however, there are conflicting results from total recovery by Benitez and Perez Diaz (1985) to lack of recovery by Huston *et al.* (1999). Data from numerous studies are still inconsistent showing unclear role of insulin in male reproduction in DM (Ballester *et al.,* 2013). We aimed to evaluate the effect of diabetes on correlation between insulin and LH levels and how quercetin and vitamin E administration alter the testicular function.

Testosterone is crucial in the structure-function development in male sex organ and studies have shown reduction in the levels in diabetes (Beatrice *et al.,*2014; Bahey *et al.,*2016). About 30-50% of patients with T2DM have low circulating levels of testosterone (Gianatti *et al.,* 2014) with evidence showing that testosterone treatment decreases insulin resistance (Grossmann *et al.,* 2010). Testosterone has been documented to promote the transformation of pluripotent stem cells to myogenic lineage, thereby inhibiting their differentiation into adipocytes (Gianatti *et al.,* 2014). Additionally, testosterone regulates the metabolic function of mature adipocytes and myocytes and in effect reduce insulin resistance. We aimed to test the hypothesis that testosterone level has a positive correlation with insulin level in T2DM and by extension determining the effect of quercetin and vitamin E treatment on serum testosterone level.

Diabetes complications can also be characterized by tissue wastage, arising from increase apoptosis; reports by researchers have shown body mass loss in diabetes (Khaki *et*

*al.,*2010; Chatterjee *et al.,*2012; Yokono*et al.,*2014). The study evaluated the effect of weight loss on the progression of complications in DM and how administration of quercetin and vitamin

For the pancreatic β-cells to defend themselves against reactive species, they must metabolize these species via the enzymatic or non-enzymatic antioxidants (Anuradha *et al.,*2014). Antioxidants have demonstrated effective capability in altering the course of diseases, therefore in the present study, quercetin, a widely distributed flavonoid in nature (Dai *et al.,*2012), whose bioactivities have been documented to include antioxidant, anti-inflammatory, antitumor, anti-allergic among other roles possess beneficial potentials that may make it safer than available commercial drugs.

In T2DM, lipid abnormalities can be defined by high triglyceride and cholesterol concentration which is facilitated by insulin resistance (Bitzur *et al*., 2009). Reports have shown the correlation of high triglyceride and cholesterol level with CVD risk (Bitzur *et al*., 2009). A treatment model that would maintain regulated glycemic and lipid control may prevent micro and macro-vascular complication in DM (Naqvi *et al*., 2017). Common therapy of dyslipidemia is statin, a HMG-CoA reductase inhibitor therapy, niacin or insulin among others (Jialal *et al*., 2010) with all showing limitations. Treatment therapies aimed at controlling dyslipidemia in DM are quite conflicting, therefore the goal of this study is to determine whether quercetin and vitamin E would be more effective treatment therapies.

Elevated levels of alanine and aspartate aminotransferase are biomarkers of hepatocellular damage in DM. this may suggest liver abnormalities associated with DM. It has been

shown that elevation of these enzymes are associated with metabolic syndrome and not glycemic control (Saligram *et al*., 2012).

Vitamin E is an antioxidant present in most edible foodstuffs. It has been reported to have potential therapeutic benefit in down-regulation of tumor cells survival, inhibition of PKC, reduction of the levels of 3-hydroxyl-3-methyl glutaryl CoA (HMG CoA) reductase and regeneration of glutathione (Aggarwal *et al.,*2010; Anuradha *et al.,*2014).

Procedure

The procedure for blood glucose, liver enzymes, cholesterol, triglyceride and certain hormones determination are as discussed under materials and methods (page 59-72).

Results

The protective effect of co-administration of Q10&VitE, Q10, VitE and glibenclamide on STZ-induced diabetes was evaluated by determining the level of blood glucose. The data presented in Figure 13 showed a statistically significant elevation of 72% in the blood glucose level of STZ-induced diabetic rats when compared with the normal control. Oral co-administration of Q10&VitE in STZ-induced diabetic rats showed that significantly reduced blood glucose levels were obtained in STZ-induced diabetic rats. The blood glucose lowering outcome of this treatment on diabetes was more than all other treatment group with a statistical difference when compared with glibenclamide. Treatment of STZ-induced diabetes with quercetin alone showed effect similar to the Q10&VitE formulation, and this lowers the blood glucose than 30 mg/kg dose of quercetin. Treatment with vitamin E showed no statistically significant difference from the effect obtained in the co-administration of quercetin and vitamin E.

Conclusion

Administration of antioxidants considered in the study possessed blood glucose lowering activities with the highest effects coming from co-administration of quercetin and vitamin E.

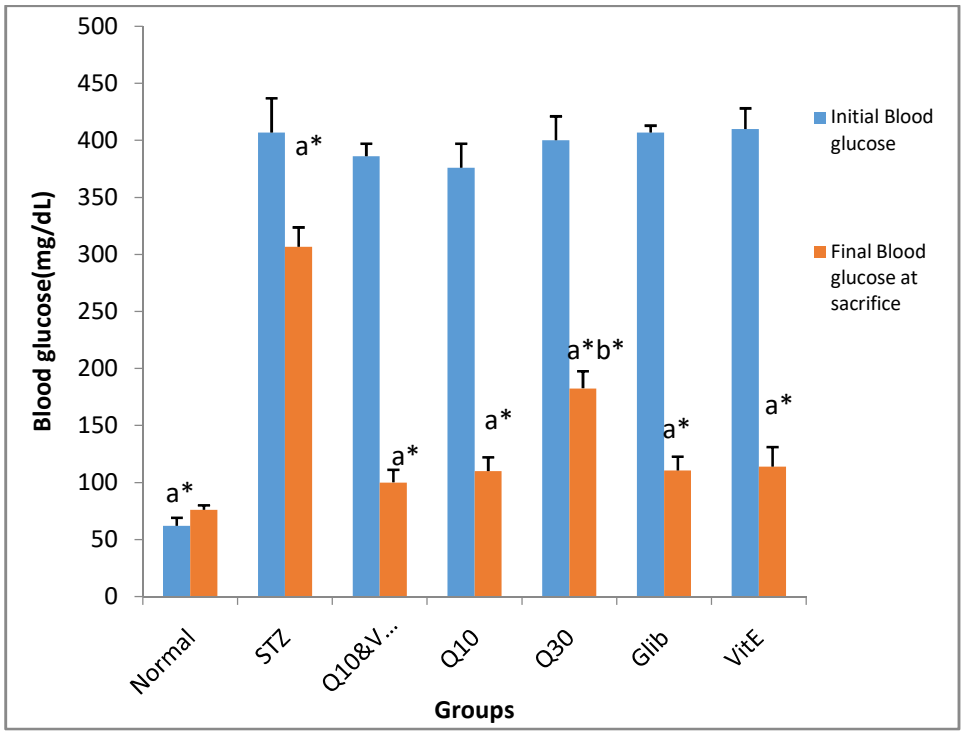


Figure 15: The blood glucose levels of STZ-induced diabetic rats and their various treatment groups

Normal: Normal rat
STZ: STZ- induced diabetes rats
Q10&VitE: STZ –induced diabetes rats orally administered 10 mg/kg quercetin and 10mg/kg vitamin E
Q10: STZ –induced diabetes rats orally administered 10 mg/kg quercetin
Q30: STZ –induced diabetes rats orally administered 30 mg/kg quercetin
VitE: STZ –induced diabetes rats orally administered 10 mg/kg vitamin E
Glib: STZ –induced diabetes rats orally administered 0.6 mg/kg glibenclamide
a* = Statistically significant from STZ –induced diabetic rats
b* = Statistically significant from normal rats

PLASMA INSULIN LEVEL DETERMINATION

Procudure

The procedure for plasma insulin level determination is as discussed under materials and methods (page 69).

Results

Treatment of STZ-induced diabetic rats with Q10&VitE increased the level of insulin availability more effectively than 10 mg/kg or 30 mg/kg (Figure 16), when compared with diabetic rats. Similarly, treatment with vitamin E significantly increased the plasma insulin level when compared with the untreated diabetic rats.

Conclusion

All treatment formulations showed increased plasma insulin level than glibenclamide but Q30 dose of quercetin.

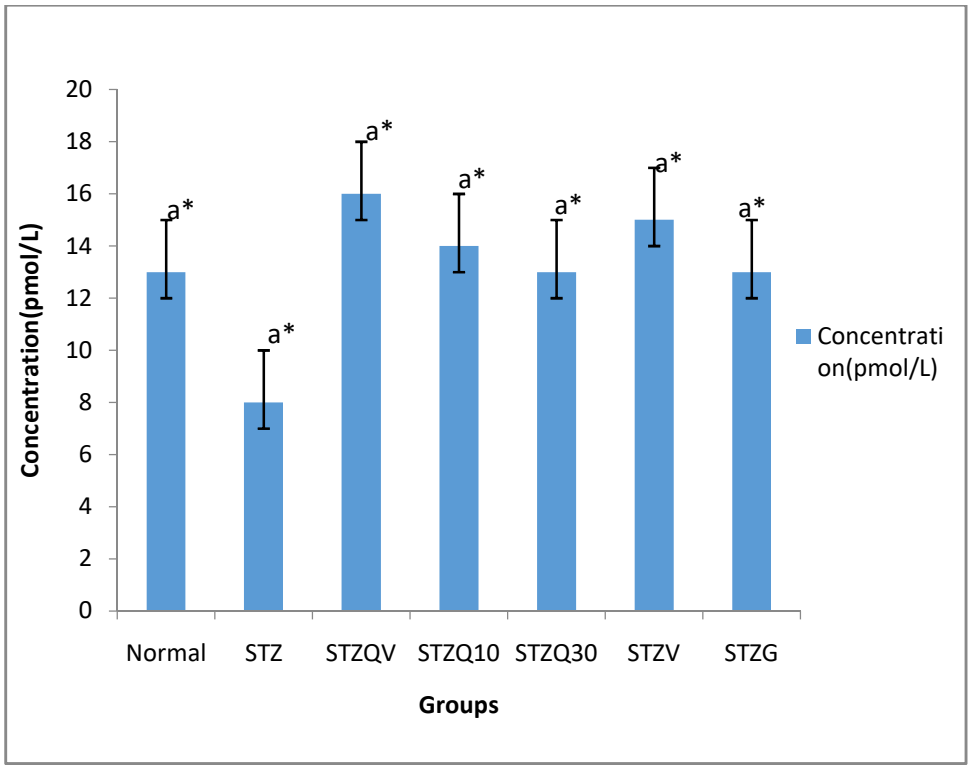


Figure 16: The plasma insulin levels of STZ-induced diabetic rats and the various treatment groups

Normal: Normal rat
STZ: STZ induced diabetes rats
Q10&VitE: STZ-induced diabetes rats orally administered 10 mg/kg quercetin and 10mg/kg vitamin E
Q10: STZ-induced diabetes rats orally administered 10 mg/kg quercetin
Q30: STZ-induced diabetes rats orally administered 30 mg/kg quercetin
VitE: STZ-induced diabetes rats orally administered 10 mg/kg vitamin E
Glib: STZ-induced diabetes rats orally administered 0.6 mg/kg glibenclamide
a* = Statistically significant from STZ –induced diabetic rats
b* = Statistically significant from normal rats

BODY-WEIGHT DETERMINATION

Procedure

The body weights of the rats were recorded after acclimatization as week 0 and this was used to calculate the dose of the antioxidants that were orally administered. Subsequently the weights were observed and recorded weekly until the 4^{th} week.

Results

In Figure 17, evaluation of the body weight of the rats showed a statistically decrease in the body-weight of STZ-induced diabetic rats when compared with the normal ones. Treatment with Q10&VitE effectively reversed the weight loss in STZ-induced diabetic rats. Treatment with Q10, Q30 and VitE also compare favorably with glibenclamide.

Conclsion

This suggests that the tissue wastage observed in diabetes could be abrogated by treatments with the antioxidants considered in the study.

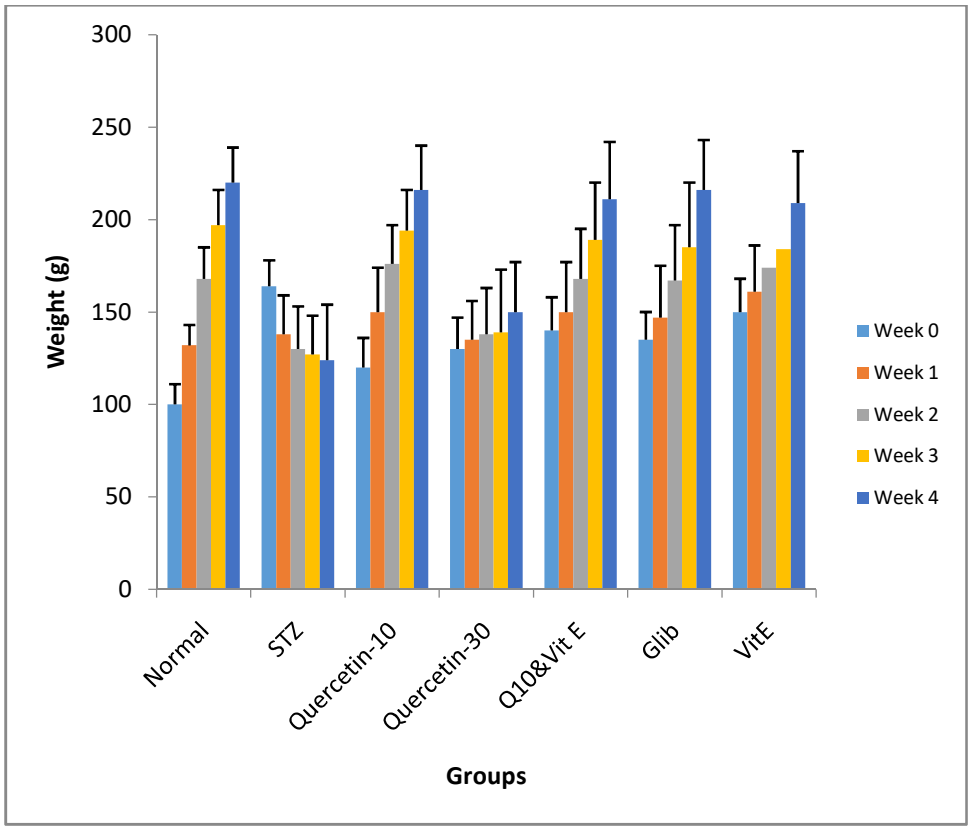


Figure 17: The weekly body weight of STZ-induced diabetic rats and the treatment groups.

Normal: Normal rat
STZ: STZ induced diabetes rats
Q10&VitE: STZ-induced diabetes rats orally administered 10 mg/kg quercetin and 10mg/kg vitamin E
Q10: STZ-induced diabetes rats orally administered 10 mg/kg quercetin
Q30: STZ-induced diabetes rats orally administered 30 mg/kg quercetin
VitE: STZ-induced diabetes rats orally administered 10 mg/kg vitamin E
Glib: STZ-induced diabetes rats orally administered 0.6 mg/kg glibenclamide

PLASMA ENZYME ACTIVITIES DETERMINATION

Prcedures

The procedures for plasma AST and ALT activities determination are as discussed under materials and methods (page 60-61).

Rrsults

In response to oxidative insult caused by diabetes in the hepatocytes and other cells. It was shown in Figure 18 that AST and ALT levels were statistically elevated in diabetic rats ($P>0.05$) while compared with control indicating a possible liver insult. To determine whether the blood lowering effect of quercetin and vitamin E on STZ-induced diabetes was related to its ability to reduce marker enzymes of liver damage, the levels of AST and ALT were determined on diabetic rat plasma. Figure 18 showed that diabetes increased the plasma levels of AST and ALT, however, treatment with either Q10 or Q30 and Q10&VitE decrease the elevation of AST and ALT levels in a statistically significant manner ($P>0.05$) and this compares favorably with glibenclamide.

CONCLUSION

The antioxidants considered altered the levels of plasma aminotransferases and may possess hepato-protective effect.

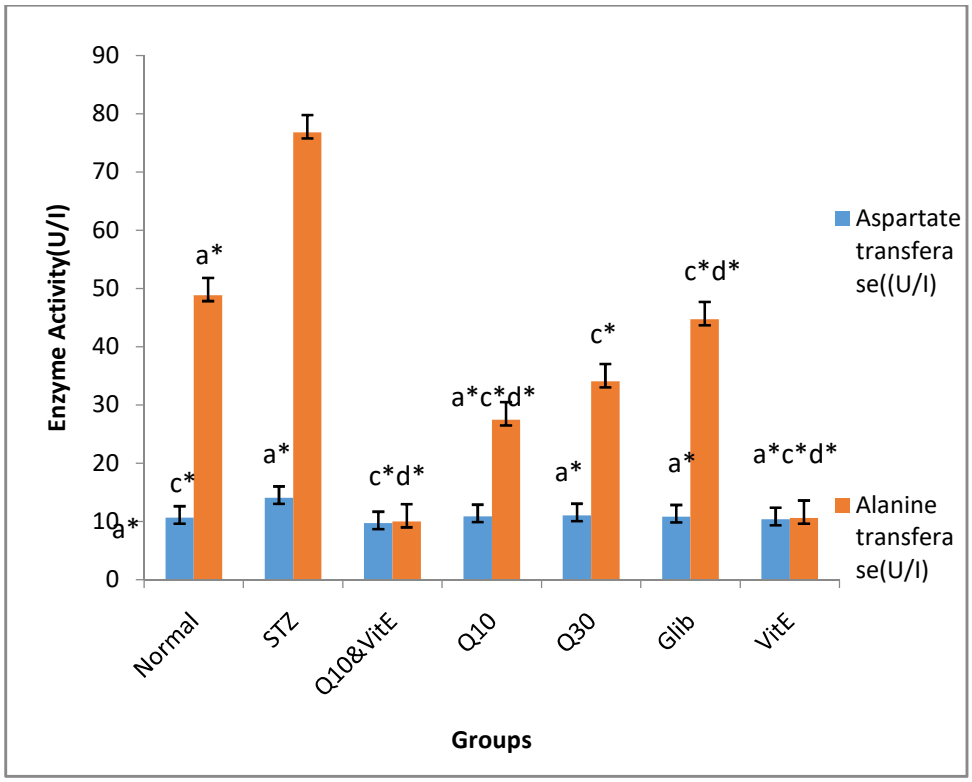


Figure 18: Aspartate and Alanine amino transferase activities in STZ-induced diabetic rats and the treatment groups

Normal: Normal rat
STZ: STZ induced diabetes rats
Q10&VitE: STZ-induced diabetes rats orally administered 10 mg/kg quercetin and 10mg/kg vitamin E
Q10: STZ-induced diabetes rats orally administered 10 mg/kg quercetin
Q30: STZ-induced diabetes rats orally administered 30 mg/kg quercetin
VitE: STZ-induced diabetes rats orally administered 10 mg/kg vitamin E
Glib: STZ-induced diabetes rats orally administered 0.6 mg/kg glibenclamide
a* = Statistically significant from STZ –induced diabetic rats
b* = Statistically significant from normal rats

CHOLESTEROL DETERMINATION

Perocedur

Plasma level of cholesterol activities were determined using commercially available kits and adhering strictly to manufacturer's instruction as described under materials and methods (Page 64).

Results

Results in Figure 19 showed that there was an increase in the level of cholesterol in STZ-induced diabetic rats ($P>0.05$) compared with normal control. Treatment with Q10&VitE, Q10, Q30 and VitE (Figure 1e) significantly reduced the levels of cholesterol in comparison with STZ-induced diabetic rats ($P>0.05$).

CONCLUSION

Diabetes has been known to increase susceptibility to cardiovascular diseases. High plasma cholesterol is associated with increased CVD risk. The antioxidants considered in the study ameliorated the levels of plasma cholesterol; therefore may have the possibility of alleviating cardiovascular complications.

TRIGLYCERIDE DETERMINATION

Procedure

Plasma activity of triglyceride was determined using commercially available kits and adhering strictly to manufacturer's instruction.

Results

The effect of varying doses of quercetin on plasma triglyceride level is depicted in Figure 19. A maximum reduction ($P>0.05$) in the elevated levels of triglyceride activity was achieved with Q10&VitE formulation, treatment with VitE alone also showed more activity than glibenclamide. The effect of Q10 and glibenclamide treatment on plasma triglyceride in diabetic rats showed similar effects ($p>0.05$). The increase cholesterol level correlates positively with increase triglyceride level in diabetes as shown in the Figure 17.

Conclusion

All treatment formulations reduced the triglyceride level in STZ-induced diabetic rats, therefore they may be good therapeutic regimen for reducing CVD risk factors in DM.

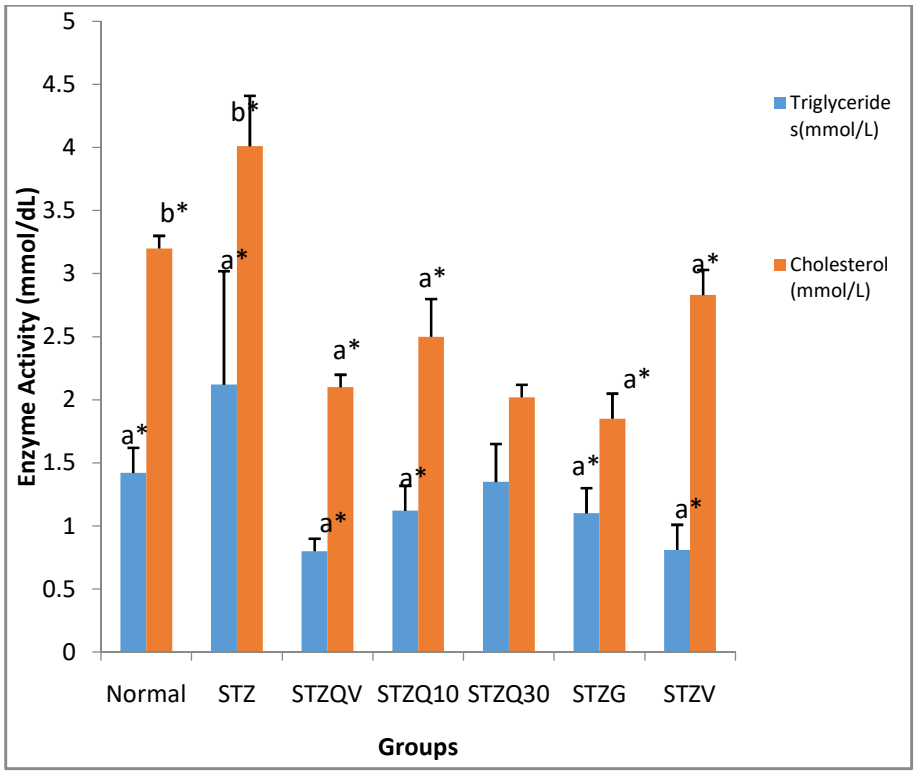


Figure 19: Triglyceride and Cholesterol Activities in STZ-induced diabetes rats and the treatment groups

Normal: Normal rat
STZ: STZ induced diabetes rats
Q10&VitE: STZ-induced diabetes rats orally administered 10 mg/kg quercetin and 10mg/kg vitamin E
Q10: STZ-induced diabetes rats orally administered 10 mg/kg quercetin
Q30: STZ-induced diabetes rats orally administered 30 mg/kg quercetin
VitE: STZ-induced diabetes rats orally administered 10 mg/kg vitamin E
Glib: STZ-induced diabetes rats orally administered 0.6 mg/kg glibenclamide
a* = Statistically significant from STZ –induced diabetic rats
b* = Statistically significant from normal rats

LUTEINIZING AND FOLLICLE-STIMULATING HORMONE DETERMINATION

Procedure

The levels of LH and FSH were quantified using ELISA technique. Here, ELISA uses antibodies or antigens sensitivity joined to an easily-assay enzyme described under materials and methods (page70-72).

Results

Plasmatic LH and FSH levels were reduced in STZ-induced diabetic rats when the normal control rats were used as reference($P>0.05$) as show in Figure 20. Treatment with Q10&ViE, Q10, Q30, VitE and glibenclamide all increased the levels of LH and FSH in a statistically significant manner ($p>0.05$).

Conclusion

Antioxidants considered in the study increased the levels of LH and FSH and this compares favorably with the glibenclamide.

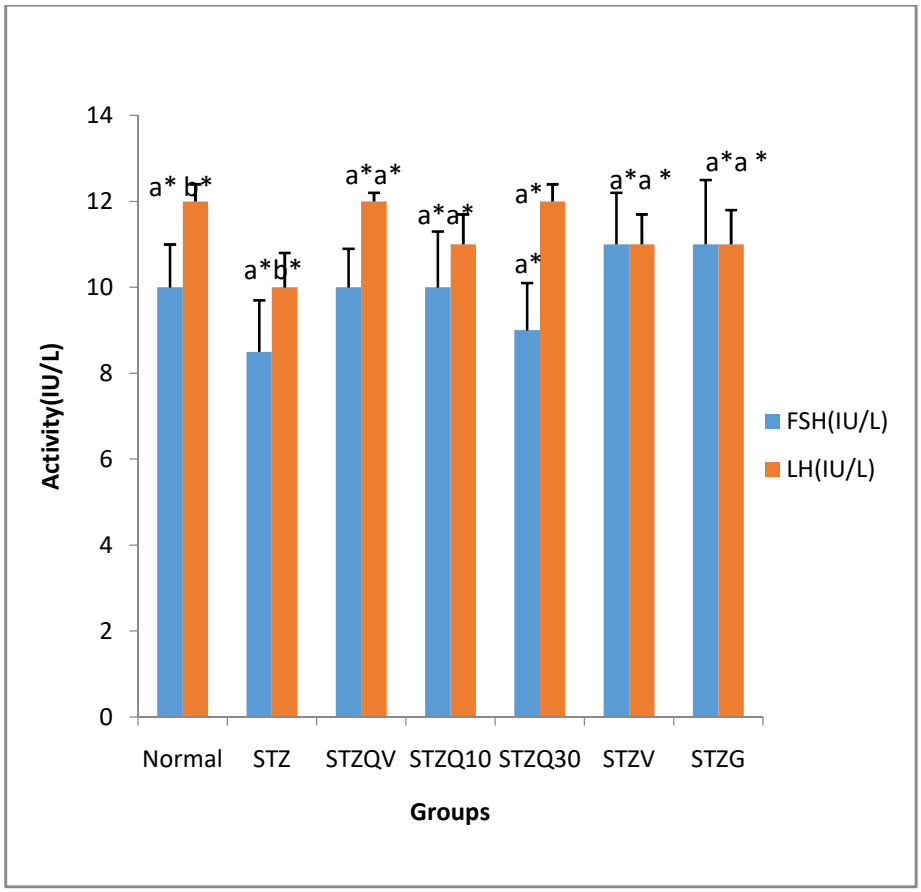


Figure 20: Follicle stimulating and luteinizing hormone levels in STZ-induced diabetic rats and treatment groups

Normal: Normal rat
STZ: STZ induced diabetes rats
Q10&VitE: STZ-induced diabetes rats orally administered 10 mg/kg quercetin and 10mg/kg vitamin E
Q10: STZ-induced diabetes rats orally administered 10 mg/kg quercetin
Q30: STZ-induced diabetes rats orally administered 30 mg/kg quercetin
VitE: STZ-induced diabetes rats orally administered 10 mg/kg vitamin E
Glib: STZ-induced diabetes rats orally administered 0.6 mg/kg glibenclamide
a* = Statistically significant from STZ –induced diabetic rats
b* = Statistically significant from normal rats

EXPERIMENT 2: EVALUATION OF THE EFFECT OF CALCIUM AND SPERMINE ON RAT HEART AND LIVER MITOCHONDRIAL MEMBRANE PERMEABILITY TRANSITION PORE

INTRODUCTION

The mPT pore is an enormous protein complex of ~1.0-1.3 nm in diameter. This complex would non-selectively allow solutes of molecular masses less than 1.5 kDa entrance. Under stress condition, a non-specific pore opens that leads to subsequent loss of ionic-homeostasis, swelling of the matrix and rupture of the outer mitochondrial membrane (Halestrap *et al.,*2003). The VDAC and the ANT do not interact but when there is activation of the mitochondrial permeability by the development of the mPT pore, the inner mitochondrial membrane loses its integrity with concomitant oxidative phosphorylation uncoupling.

When the mPT pore opens, all molecules of low molecular weight gain passage through it. High molecular weight proteins and the likes utilize a colloidal osmotic pressure that causes swelling and rupture of the mitochondria. Though the inner mitochondria membrane matrix is unfolded, rupture does not occur but the outer mitochondrial membrane opens. This opening results in swelling and rupture of the inner mitochondrial matrix. This leads to release of AIF, Cyt C, endonuclease G, and SMAC/Diablo (pro-apoptotic factors) (Rasola and Bernardi, 2014). Research has also shown that spermine, a polyamide inhibits mPT pore (Lapidus and Sokolove 1992). This acts by rearranging the phospholipid component of the mitochondrial and stabilizing the inner membrane. Additionally, it prevents the collapse of mitochondrial membrane potential, Mg^{2+} release,and adenine

nucleotidesobserved when mitochondria are exposed to deleterious inorganic phosphateand Ca^{2+} concentration. Another strong inhibitor of the mPT pore is trifluoperazine, whose activity is possible under energized conditions. Trifluoperazine acts by influencing mPT pore voltage sensitivity via alteration of the surface membrane charge (Halestrap *et al.,*2004).

Mitochondria are determinants of the cell's fate in oxidative stress or injury. Under normal physiologic conditions, the organelle is expected to have a matrix pH of about 7.9 to 8 creating a trans-membrane electrochemical gradient that allows ATP synthesis. Inhibition of the mPT is of great importance in certain pathological conditions, however, drug toxicity and resistance have motivated the drive for discovery of natural molecules that would be able to overcome these limitations, since this would be of pharmacological relevance.

Calcium has been reported to be the best mPT pore inducer that causes apoptosis(Bonora and Pinton, 2014). Cytosolic Ca^{2+}concentration is responsible for an extensive variety of physiological processes that ranges from mitotic cell division, neurotransmission, metabolism amongst others. However, it mediate cell death in apoptosis through regulated mild insult and in necrosis by massive insult (Pinton *et al.,*2008). Other second messengers in the cell like cAMP, cGMP, inositol-3-phosphate are capable of performing the role of Ca^{2+}, but Ca^{2+}has a unique characteristic of low diffusion rate than others giving it intracellular heterogenicity.

In this study, the inhibitory effect of spermine on calcium-induced mPT pore opening in normal rat heart and liver mitochondria was assessed. This was carried out to ascertain the

integrity of the isolated mitochondria since they are delicate organelles. Similarly, the inhibitory effect of quercetin was determined on the mPT pore (*in vitro*).

Procedure

Low ionic-strength mitochondria acquired from normal healthy rats essentially by the procedure described by Johnson and Lardy (1967), as described under materials and methods (page 76-77).

Assay for mitochondrial swelling in the absence of Ca^{2+} (triggering agent) involved pre-incubation of mitochondria in MSH (swelling) buffer, with 0.8 µM rotenone for 3½ minutes in a 2.5 mL glass cuvette at 37^{o}C, thereafter, the reaction was energized by 5 mM succinate. In the presence of a triggering agent, 120 mM calcium was added 3 minutes after addition of mitochondria, immediately followed by 5 mM spermine. Thereafter, succinate was added after 30 seconds to energize the reaction. The purpose of this is to show that the isolated mitochondria were intact and after pore opening by Ca^{2+}, they were inhibited by spermine. The mitochondria permeability transition was quantified at 540 nm over a period of 12 minutes at 30 seconds intervals in a M105 Spectrophotometer. A significant decrease in absorbance of the liver mitochondria is an indication of permeability transition.

Results

The data presented in Figure 21 depicted that whereas there was no significant variations in the volume of intact mitochondria respiring on sodium succinate in the presence of rotenone, addition of Ca^{2+} caused large amplitude mitochondria swelling which was reversed by spermine, a standard inhibitor of mPT pore opening.

The data presented in Fiure 22 depicted that although there was no significant variations in the volume of intact mitochondria respiring on sodium succinate in the presence of rotenone, addition of Ca^{2+} caused highly significant ($p<0.05$) increase in mitochondria swelling (58%) which was reversed through spermine (22%), a standard inhibitor of mPT pore opening. This showed that the mitochondria used in the study were intact.

Conclusion

Results indicated that calcium induced the mPT pore opening in isolated rat heart and liver, while spermine inhibited this induction, however, the effects were more in the liver than the heart. This showed that the mitochondria were intact, and not uncoupled and therefore suitable for use.

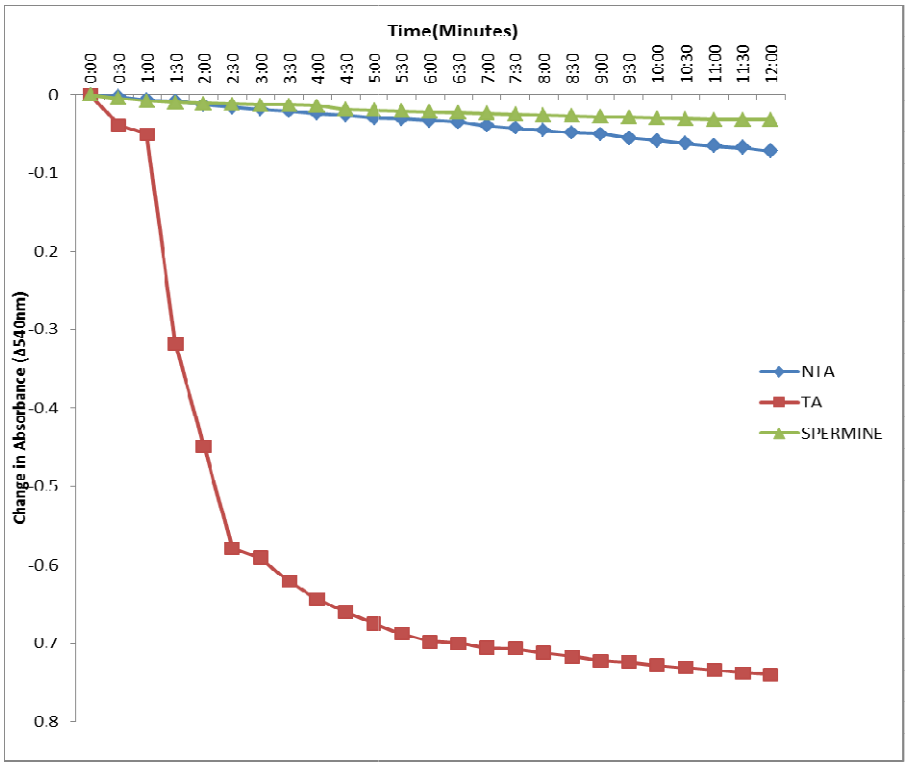


Figure 21: Calcium-Induced opening of the Liver Mitochondrial Membrane Permeability Transition pore and reversal with spermine energized by sodium succinate.

NTA=No triggering agent
TA=Triggering agent
INH= Inhibitor (Spermine)

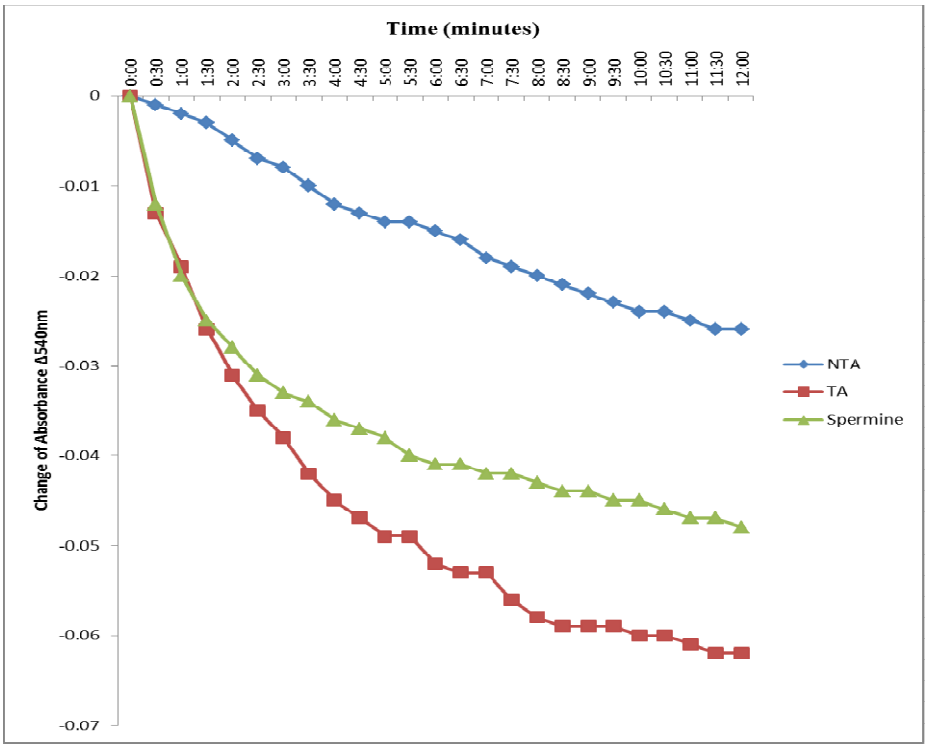


Figure 22: Ca^{2+}-induced opening of the heart Mitochondrial Membrane Permeability Transition Pore and reversal by spermine energized by sodium succinate

TA: In the presence of exogenous calcium

NTA: in the absence of exogenous calcium

Spermine: Inhibitor

Results are reported as mean of decrease in absorbance at 540nm at 30 seconds interval for 12 minutes ± standard deviation.

EXPERIMENT 3: EFFECTS OF VARYING CONCENTRATIONS OF QUERCETIN ON RAT LIVER MITOCHONDRIAL MEMBRANE PERMEABILITY TRANSITION PORE IN THE ABSENCE AND PRESENCE OF EXOGENOUS CALCIUM

Introduction

Mitochondria are determinants of the cell's fate in oxidative stress or injury. Under normal physiologic conditions, the organelle is expected to have a matrix pH of about 7.9 to 8 creating a trans-membrane electrochemical gradient that allows ATP synthesis. The matrix Ca^{2+} is crucial for mPT pore opening, this moves into the mitochondria when the organelles inter-relate through the mitochondrial associated membranes resulting in excessive apoptosis (Patergnani *et al.,* 2011). This excessive apoptosis is mediated by the phosphorylation of IPR by AKT kinase (Bonora and Pinton, 2014).Insights by Lemasters and colleagues have shown that Ca^{2+} may be released from cells as a result of bioenergetics failure. In some other situations Ca^{2+} may be released as a consequence of mPT pore opening or synergistic interaction with ROS to induce mPT pore opening (Lemaster *et al.,*2009). Studies have shown that Ca^{2+} among other reactive chemicals induce the mPT pore opening leading to mitochondrial membrane depolarization, large amplitude osmotic swelling and uncoupling of oxidative phosphorylation (Lemaster *et al.,*2009). In general, it has been shown that Ca^{2+} enters the mitochondrial electrophorectically via the Ca^{2+} uniporter and exists through the Na^{+}/Ca^{2+} carrier.

Several agents have been shown to inhibit the mitochondrial mPT pore including CsA but they show different limitations that ranges from pharmacokinetic to pharmacodynamics parameters (Morin *et al.,*2010). Therefore researchers are interested in mitochondria-

targeted antioxidants which may play a role in limiting and or completely inhibiting mPT pore (Morin *et al.,*2010).Inhibition of the mPT pore is of great importance in certain physiological conditions, however, drug toxicity and resistance have motivated the drive for the discovery of natural molecules that would be able to overcome these limitations, since this would be of pharmacological relevance. We hypothesised that dietary substances that would inhibit the mPT pore could inhibit apoptosis. Quercetin is a plant flavonoid found in vegetables, wine, tea and fruits, it has been reported to possess anti-histamine, anti-inflammatory and anti-oxidant activities among others (Punithavathi and Prince, 2010). Quercetin exerts its antioxidant effects via multiple mechanisms like chelating metal ions like Cu^{2+} and Fe^{2+}, inhibiting nitric oxide production and directly scavenging free radicals among others (Punithavati and Prince, 2010).

Hence, this experiment was designed to examine the effect of quercetin on rat liver mitochondrial membrane permeability transition.

Procedure

Low ionic strength liver mitochondria were obtained from normal rats as described under materials and methods (page 76-77). Mitochondrial protein was determined as described by Lowry *et al.,* (1951) using BSA as standard (page 85-87). Change in absorbance was quantified at 540 nm on a M105 Cam Spectrophotometer. Assay for mitochondrial swelling was estimated by the procedures of Lapidus and Sokolove, (1965) as described under materials and methods (page 80-82).

Results

Results in Figure 23 showed effect of quercetin on rat liver mPT pore in the absence of exogenous calcium. Here, addition of quercetin to succinate energized mitochondria caused

maximum inhibition of mitochondrial pore opening at 750 µg/ml higher than the inhibitory effects of spermine. In Figure 24, results showed the effect of quercetin on rat liver mPT pore in the presence of exogenous calcium. Here, the trend of calcium-induced opening of the pore was shown and in this regard, quercetin inhibited the opening of the pore in a concentration dependent manner.

Maximum inhibition of pore opening to the tune of 96% was achieved at 750 µg/mL, comparable to the inhibition by the standard mPT pore inhibitor, spermine (95%). Minimum inhibitory effect of 68% was obtained at 150 µg/ml while 78% degree of inhibition was obtained at 450 µg/ml. In summary, all the concentrations of quercetin used reversed the pore opening effectively.

Conclusion

All the concentrations of quercetin considered in the study had no effect on the mitochondria in the absence of calcium in the absence of calcium. Whereas, experiment in the presence of Ca^{2+}, there was a concentration-dependent increase in the reversal of calcium-induced pore opening.

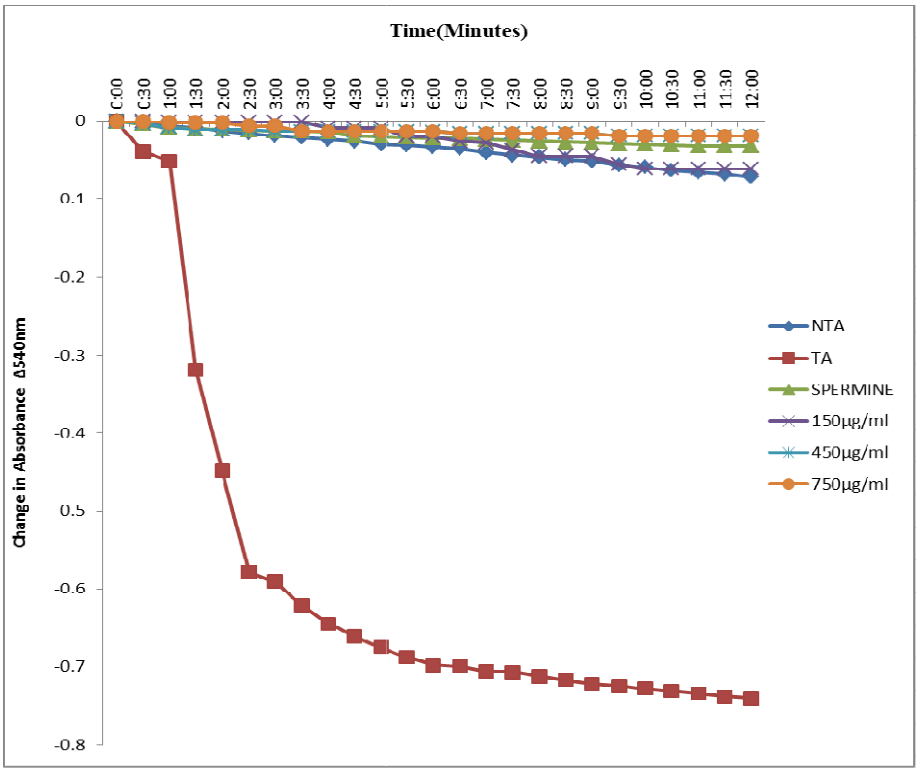


Figure 23: Effects of Quercetin on the rat liver Mitochondrial Membrane Permeability Transition pore in the Absence of exogenous Ca^{2+}

NTA=No triggering agent TA=Triggering agent SPERMINE= Inhibitor
150 µg/mL= 150 µg/mL of quercetin 450 µg/mL=450 µg/mL of quercetin
750 µg/mL=750 µg/mL of quercetin

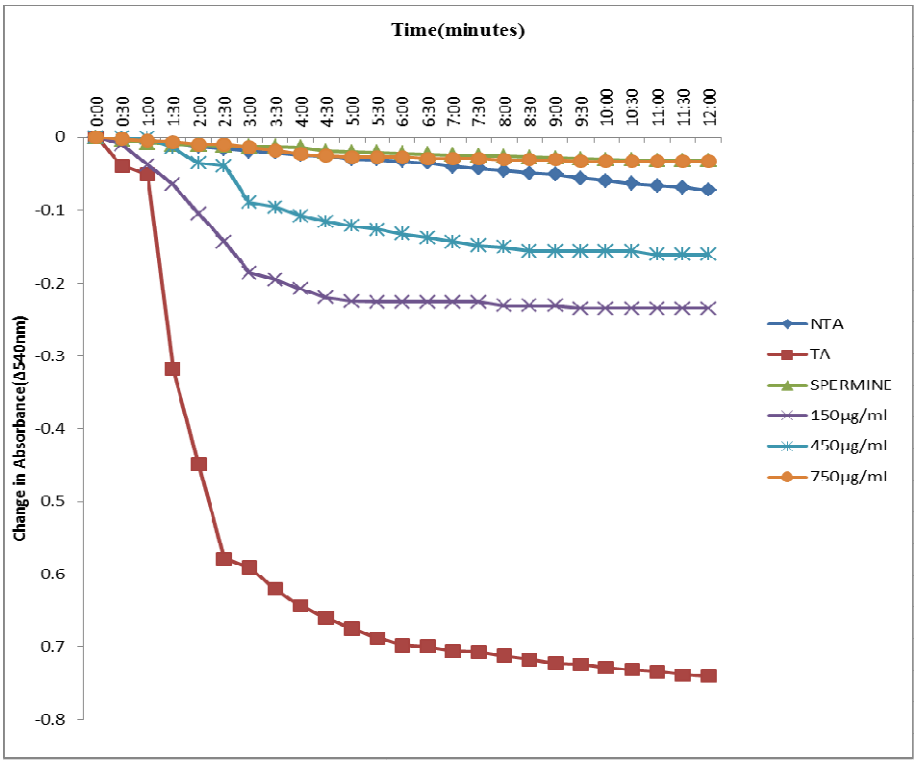


Figure 24: Effects of quercetin on rat liver mitochondrial membrane permeability transition pore in the presence of exogenous Ca^{2+}

NTA=No triggering agent TA=Triggering agent SPERMINE= Inhibitor

150 µg/mL= 150 µg/mL of quercetin 450 µg/mL=450 µg/mL of quercetin

750 µg/mL=750 µg/mL of quercetin

EXPERIMENT 4: ASSESSMENT OF THE INTEGRITY OF THE HEART AND LIVER MITOCHONDRIAL MEMBRANE PERMEABILITY TRANSITION PORE IN STZ-INDUCED DIABETIC RAT

Introduction

In the pathophysiology of diabetes, the mitochondrion have evolved as an entity to be given enormous attention. Disruptions in the integrity of this organelle have been identified as the origin of complications arising from diabetes (Nafaji *et al.,*2014). The altered mitochondrial function leads to mitochondrial mPT pore-opening caused by interaction of numerous mitochondrial-proteins; this process is a reversible one (Sivitz and Yorek, 2010). This pore is involved in activation of cell-death programmes. Considering the status of the pore will be quite informative in searching for innovative therapies that would be able to confer protection and delay complications in the progression of the disease. On induction of diabetes, there is β-cell destruction which causes no/or reduced insulin production resulting in increased glucose concentration in the cell. Hyperglycaemia mediated ROS production occurs via diverse mechanisms in the body, including the polyol pathway (which culminates into the depletion of NADPH and glutathione in the cell), enhanced formation of AGEP which damage the cell by glycating vital cellular components thereby altering vital proteins function. Diabetes-induced hyperglycemia also causes modification of plasma proteins and activation of protein kinase C pathway among other complications (Giacco and Brownlee, 2010). Due to the clinical relevance of the relationship between DM, liver and heart diseases and insufficient information in this area, the study examined the integrity of the mPT pore in STZ-induced diabetes on the rat heart and liver.

Procedure

Rats that were intra-peritoneally injected with a single dose of 40 mg/kg STZ, having a fasting blood glucose level of ≥250 mg/dL after 72 hours of diabetes induction were selected for use. These rats were given fresh water *ad libitum* and fed with standard diet for 28 days. Rats were sacrificed by cervical dislocation and the livers were quickly removed. Low ionic strength mitochondrial were isolated essentially by the methods of Mela (1969) and Sanz *et al.*(2007) in heart and by the method of Johnson and Lardy (1969)in liver with slight modifications. Method of Lowry *et al.,*(1951) was used in determining mitochondrial-protein content, using BSA standard. Assay for mitochondrial swelling was performed as previously described in materials and method section (page 80-82).

Results

Figure25 showed that in the normal rat liver mitochondria, no substantial variation was observed in the volume of intact mitochondria respiring on succinate in the presence of rotenone but STZ-induced diabetes caused highly significant ($p<0.05$) increase in liver mitochondria swelling (99%) when compared with the control. In this regard, the effect of diabetes on the mitochondria cause large amplitude swelling.

Figure 26 showed that the normal heart mitochondria showed no substantial variations whatsoever in the volume of intact mitochondria respiring on succinate however, STZ-induced diabetes caused significant ($p<0.05$) increase of 80% in heart mitochondrial swelling when likened to control. This also showed inductive effect caused by diabetes.

Conclusion

Results showed that the mPT pore of the heart and liver were opened in STZ-induced diabetes. The inductive effect of diabetes on the liver was more than that observed in the heart showing that mitochondrial-mediated cell death occurs more in the hepatocytes than the cardiac cells.

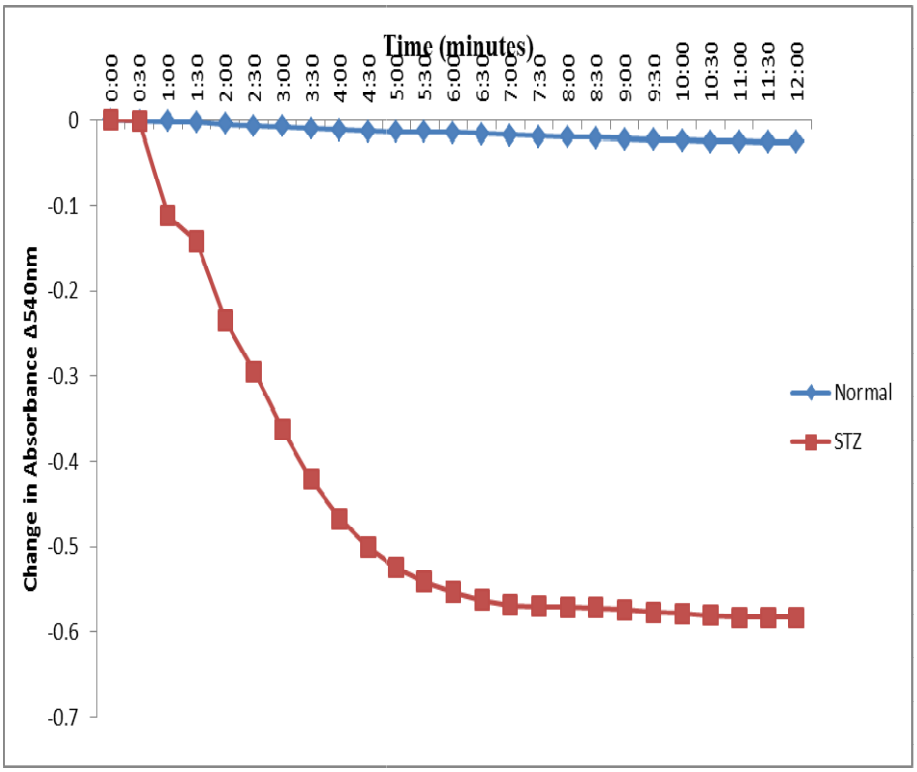


Figure 25: Assessment of the Integrity of the mitochondrial membrane permeability transition pore in STZ-induced diabetic rat liver

Results are reported as mean of decrease in absorbance at 540nm at 30 seconds interval for 12 minutes. Normal: mitochondria from normal rat; STZ: mitochondria from streptozotocin-induced diabetic rat liver

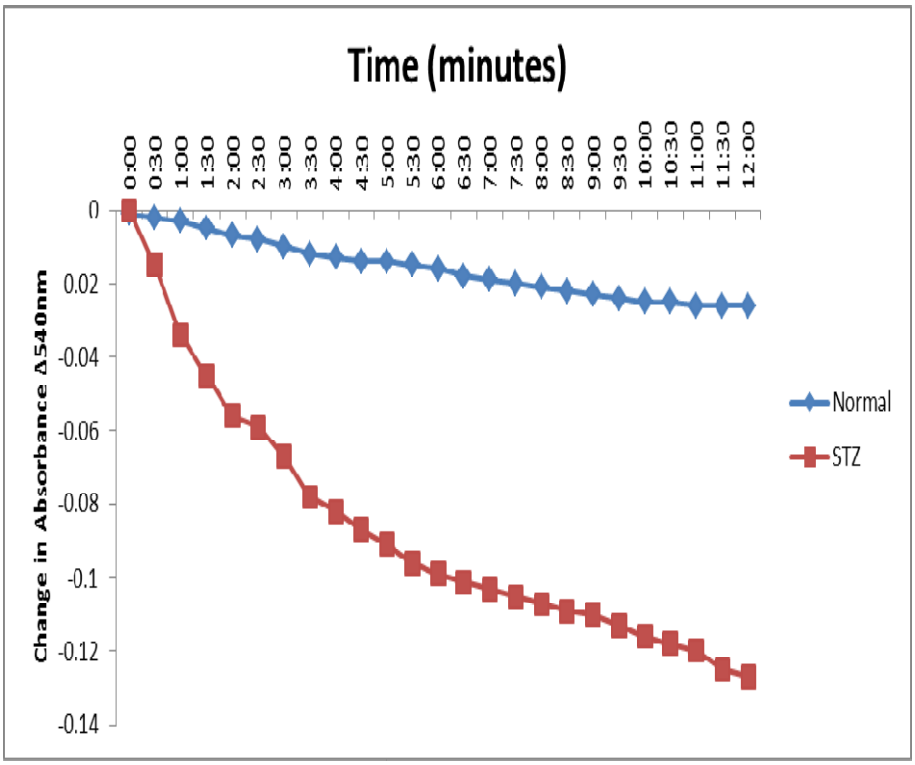


Figure 26: Integrity of the mitochondrial membrane permeability transition pore in STZ-induced diabetic rat heart

Results are reported as mean of decrease in absorbance at 540nm at 30 seconds interval for 12 minutes.

Normal: mitochondria from normal rat; STZ: mitochondria from streptozotocin-induced diabetic rat heart.

EXPERIMENT 5: EFFECT OF VARYING DOSES OF QUERCETIN, GLIBENCLAMIDE, VITAMIN E AND CO-ADMINISTRATION OF QUERCETIN AND VITAMIN E TREATMENT ON NORMAL RAT LIVER MITOCHONDRIAL PERMEABILITY TRANSITION PORE.

Introduction

Mitochondrion is an organelle that performs expanding role in biosynthesis, homeostasis, bioenergetics, mediate cell death among other functions (Borutaite, 2010). In cellular homeostasis, this organelle generate intermediates for biosynthetic reactions, it is site of ROS production, signal transduction, with specific role in ATP production during oxidative phosphorylation (Cheng and Ristow, 2013). Mitochondria mediate generation of ROS and reactive nitrogen species, energy, non-specific mPT pore, Cyt C, apoptosome, and downstream proteins like caspases (Borutaite, 2010).

Antioxidants have the unique capability of protecting the mitochondria from damage, however, endogenous antioxidants can be depleted due to overwhelming ROS presence in pathological conditions. Some antioxidants exhibit protective properties on the mitochondria, they include resveratrol which works by influencing the transcriptome to upregulate the availability of anti-oxidants defence. They also activate genes that increase the mass of the mitochondria thereby decreasing apoptosis (Ferretta*et al.,*2014). An antioxidant derivative of ubiquinone, MitoQ_{10} is a mitochondria-targeted antioxidant that protects endothelial function and ameliorate hypertrophy in rats that are hypertensive and prone to stroke (Ferretta*et al.,*2014). Generally, antioxidants demonstrate protective capabilities in the mitochondria, however they are dose dependent, and this may explain in

part their failure sometimes in crossing the clinical trial hurdles (Nunez-Cordoba and Martinez-Gonzalez, 2011). The aim of the experiment therefore is to determine if administration of certain antioxidants will be able to protect the mitochondria function and maintain the integrity of the organelle in diabetes.

Procedure

Low ionic strength mitochondria were isolated from normal health male Wistar strain albino rats that have been orally administered Q10, Q30, VitE, Q10&VitE, glibenclamide and fed purina chow and water *ad libitum* for 28 days as described on page 63. Rats were sacrificed by cervical dislocation, livers were removed, minced, homogenized and then centrifuged to isolate mitochondria as described previously (page 76-77).

Assay for mitochondrial swelling, in the absence and presence of no triggering agent was done as described on page 80-82. Absorbance values were read at 540 nm over a period of 12 minutes at 30 seconds intervals in a M105 Spectrophotometer.

Results

The data presented in Figure 27 showed that the normal rat liver mitochondria respiring on succinate in the presence of rotenone showed intactness of 93% while treatment with co-administration of Q10&VitE had intactness of 89% indicating that it inhibited the mPT pore as almost as that observed in normal rat.

Similarly, Q10 showed mPT pore intactness of 85%, however, Q30 showed intactness of only 66%, thus indicating that this dose is less effective in protecting the liver mitochondria from pore opening.

The data in Figure 27 also showed that vitamin E had intactness of 77%, showing less effect on the mPT pore than glibenclamide with 87% intactness. Results obtained also showed that only Q30 may have interacted with the integrity of the organelle and therefore this concentration may have to be reconsidered again for its safety on the organelle.

Conclusion

The results showed that only 30 mg/kg quercetin had the least effect on the mPT pore in terms of permeability induction.

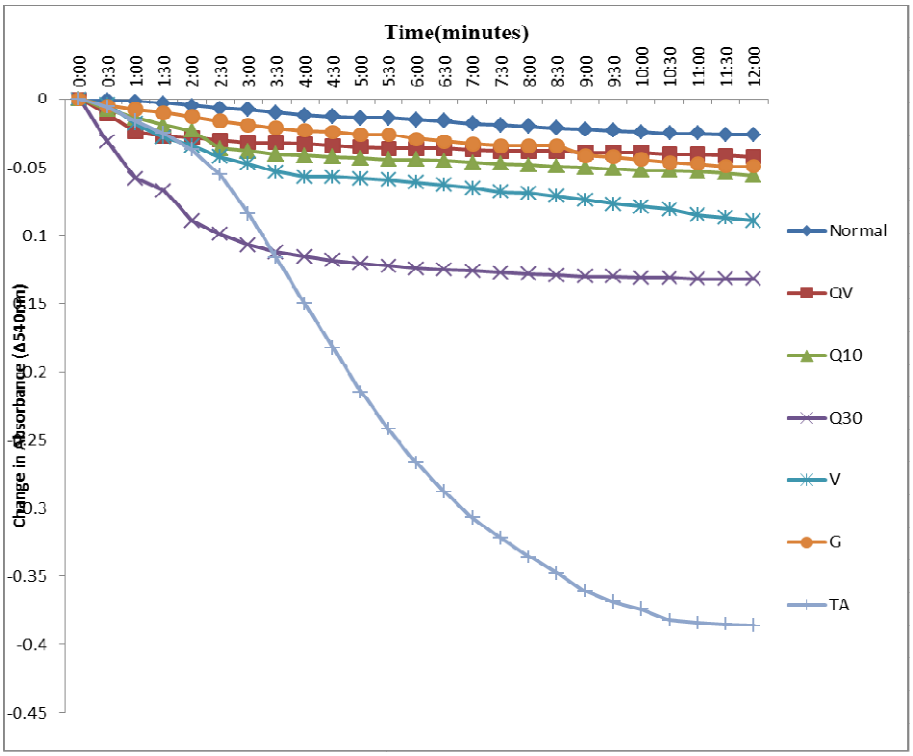


Figure 27: Effect of co-administration of quercetin, vitamin E, and glibenclamide on mitochondrial membrane permeability transition pore of normal rat liver

Normal= In the absence of exogenous calcium for normal rats
TA=Presence of exogenous calcium
nQ10= Rats orally treated with 10 mg/kg quercetin.
nQ30= Rats orally treated with 30 mg/kg quercetin.
nVitE= Rats orally treated with 10 mg/kg vitamin E
nQ10&VitE= Rats orally treated with 10 mg/kg quercetin and vitamin E.
nGlib= Rats orally treated with 0.6 mg/kg glibenclamide

EXPERIMENT 6:EFFECTS OF VARYING CONCENTRATIONS OF QUERCETIN, GLIBENCLAMIDE, VITAMIN E AND CO-ADMINISTRATION OF QUERCETIN AND VITAMIN E TREATMENT ON LIVER mPT PORE OFSTZ-INDUCED DIABETIC RAT

Introduction

The mitochondrion, is unarguably the most complicated, unique organelle in an organism. Its role spans from maintenance of redox homeostasis, Ca^{2+} signaling to even the eventual fate of the cell (Hou *et al.,*2014). This organelle produces vital molecules that preserve the life and determines the eventual fate of cells. In the respiratory chain, the flux of electrons through complexes 1 to IV is evident in oxidative phosphorylation but in doing this odious task, a small fraction of the electrons leak out of the highly conserved ETC. These electrons participate in the incomplete reduction of oxygen to superoxide anion which at physiological pH can be reduced to hydrogen peroxide, where superoxide dismutase is available (Sivitz and Yorek, 2010). The H_2O_2 is further converted to O_2 and H_2O where the endogenous antioxidants have not been overwhelmed (Hou *et al.,*2014). It is the ROS generation by the organelle that forms the basis for many pathological conditions including diabetes (Newsholme *et al.,*2007; Hou *et al.,*2014). Chemicals with antioxidant properties have been shown to have effects on mitochondria in diseases, however, disappointing results of some of these antioxidants when used for long-term period particularly in humans have been reported. This establish their non-performance, which may be due to their inability to access the primary site of ROS production particularly if mitochondrial is the target (Rocha *et al.,*2009). Studies have also established the exceeding beneficial roles of antioxidants on disease complications (Karunakara and Park, 2013). Due to these

conflicting findings, this experiment was aimed at investigating the role of specific antioxidants on the liver mitochondria in diabetic status.

Procedure

Low ionic strength mitochondria were isolated from STZ-induced diabetic rats that have been orally administered Q10, Q30, VitE, glibenclamide and Q10&VitE fed purina chow and water *ad libitum* for 28 days.

Mitochondrial swelling assayed as previously described in materials and methods section (page 80-82).

Results

Figure 28 shows the effect of certain treatment groups on succinate energized mitochondrial swelling. Here, the highest reversal effects of the mPT pore opening was achieved in the diabetic group treated with co-administration of Q10&VitE (89%). This indicates that it reversed diabetes-induced opening of the mPT pore noticed in the diabetic untreated group and it showed higher effect than glibenclamide (70%). Similarly, addition of Q10 reversed the opening of the mPT pore by 87%, showing higher reversal effect than glibenclamide. However, oral administration in the Q30 only reversed the mPT pore opening by 64%, indicating that this dose is not effective in keeping the intactness of the mitochondrion as other treatments. Notably, pretreatment with 30 mg/kg quercetin reversed the mPT pore opening by 77%, showing better effects than treatment with the same concentration. The extent of the reversal effect shown by vitamin E on mPT pore as presented in figure 28 on diabetic rats was 85%, indicating that oral administration of vitamin E preserves mitochondria integrity than glibenclamide.

Conclusion

This finding suggests that these antioxidants reversed the mPT pore opening in STZ-induced diabetic rats more than glibenclamide.

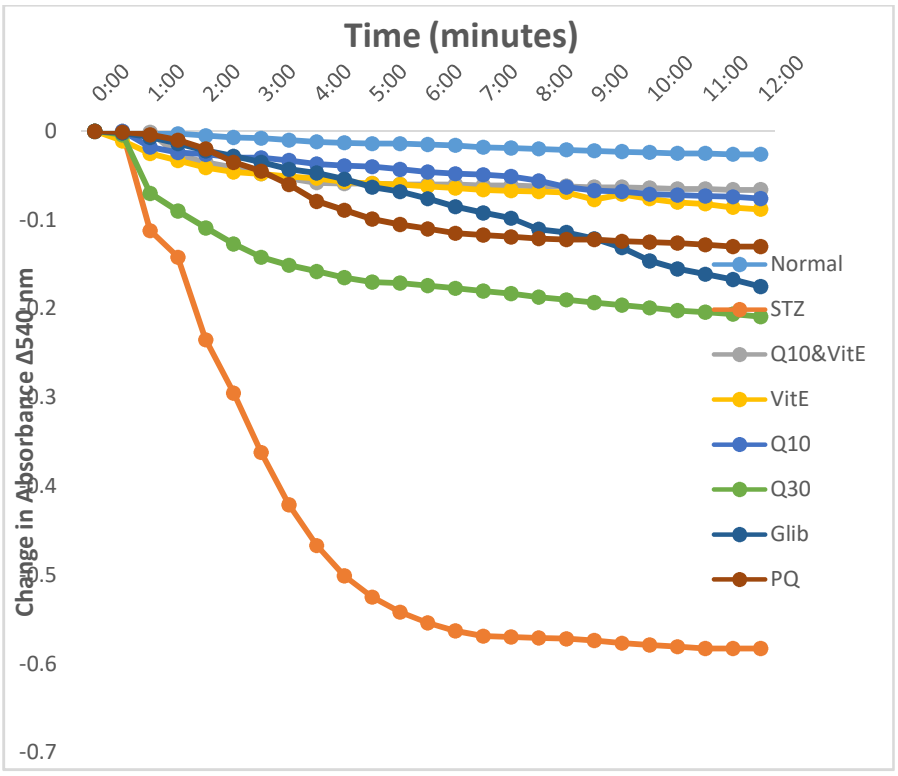


Figure 28:Effects of varying concentration of quercetin, glibenclamide, vitamin E and combined quercetin and vitamin E treatment on liver mPT pore opening ofSTZ-induced diabetic rat

Normal: Normal rat
STZ: STZ induced diabetes rats
Q10&VitE: STZ-induced diabetes rats orally administered 10 mg/kg quercetin and 10mg/kg vitamin E
Q10: STZ-induced diabetes rats orally administered 10 mg/kg quercetin
Q30: STZ-induced diabetes rats orally administered 30 mg/kg quercetin
PQ: STZ-induced diabetic rats orally pretreated with 30 mg/kg quercetin
VitE: STZ-induced diabetes rats orally administered 10 mg/kg vitamin E
Glib: STZ-induced diabetes rats orally administered 0.6 mg/kg glibenclamide

EXPERIMENT 7: ASSESSMENT OF THE EFFECT OF CO-ADMINISTRATION OF QUERCETIN AND VITAMIN E, VITAMIN E, GLIBENCLAMIDE AND VARYING CONCENTRATIONS OF QUERCETIN TREATMENT ON mPT PORE OF NORMAL RAT HEART

Introduction

The mitochondrial membrane evolved a large conductance passage called the mPT pore (Oliveira *et al.,* 2002). When the mPT pore opens, a lot of events ensue including outer mitochondrial membrane swelling, followed by depolarization of the membrane, hence uncoupling of oxidative phosphorylation, Ca^{2+} release among others. In the rat heart, the mPT pore opens when there is prolonged period of ischemia accompanied by reperfusion. The pore opens due to increased oxidative stress and Ca^{2+} overload. In the mitochondria, which can be prevented by ischaemic preconditioning. During ischaemia, there is accumulation of calcium which might be responsible for calcium overload and therefore mPT pore opening (Halestrap *et al,* 2007). Similarly, there is increase ROS concentration at reperfusion, this increase mPT pore sensitivity that would results into release of proapoptotic proteins that initiate cell death. It is assumed that if sensitization of the mPT pore is critical in reperfusion injury, the pore opening inhibition should preserve the heart from injury (Halestrap *et al,* 2007). Numerous studies have shown the protective effect of CsA and SfA on the mPT pore (Griffiths *et al,* 1993, Di Lisa *et al,* 2001, Clarke *et al,* 2002, Halestrap *et al,* 2007). Pyruvate has been shown to be the most effective inhibitor of the heart mPT pore opening, it acts by scavenging free radical, lowering pH and acting as ATP synthesis fuel (Halestrap *et al,* 2007).

Generally, antioxidants have been shown to antagonise the formation of reactive species in pathological conditions because up regulation of their concentration will boost endogenous activities, thereby this could reduce the complications that may arise from any pathological condition. However, reports have shown that these antioxidants may be ineffective for prolonged period (Sivitz and Yorek, 2010). The study determines the safety of certain antioxidants mPT pore.

Procedure

Normal healthy rats were orally given Q10&VitE, Q10, Q30, VitE, glibenclamide on group basis and fed standard diet with fresh water for 28 days. Rats were sacrificed by cervical dislocation, while the hearts were removed, cut into pieces, homogenized and then centrifuged to isolate the low ionic strength mitochondria as explained in page 78-79 under materials and methods.

Results

The data presented in Figure 29 showed that the normal rat heart mitochondria respiring on succinate in the presence of rotenone showed intactness of 76% while co-administration of Q10&VitE had intactness of 81%, thus showing higher effects than glibenclamide which had inhibition of 15%. Similarly, Q10 treatment had intactness of 79% while Q30 treatment showed inhibition of 80%. Results also showed that pretreatment with 30 mg/kg reversed the mPT pore opening by 61%. It was observed that vitamin E showed inhibitory effects of only 60%.

Conclusion

Results show that the antioxidants used in this experiment reversed the mPT pore opening, than glibenclamide on the heart.

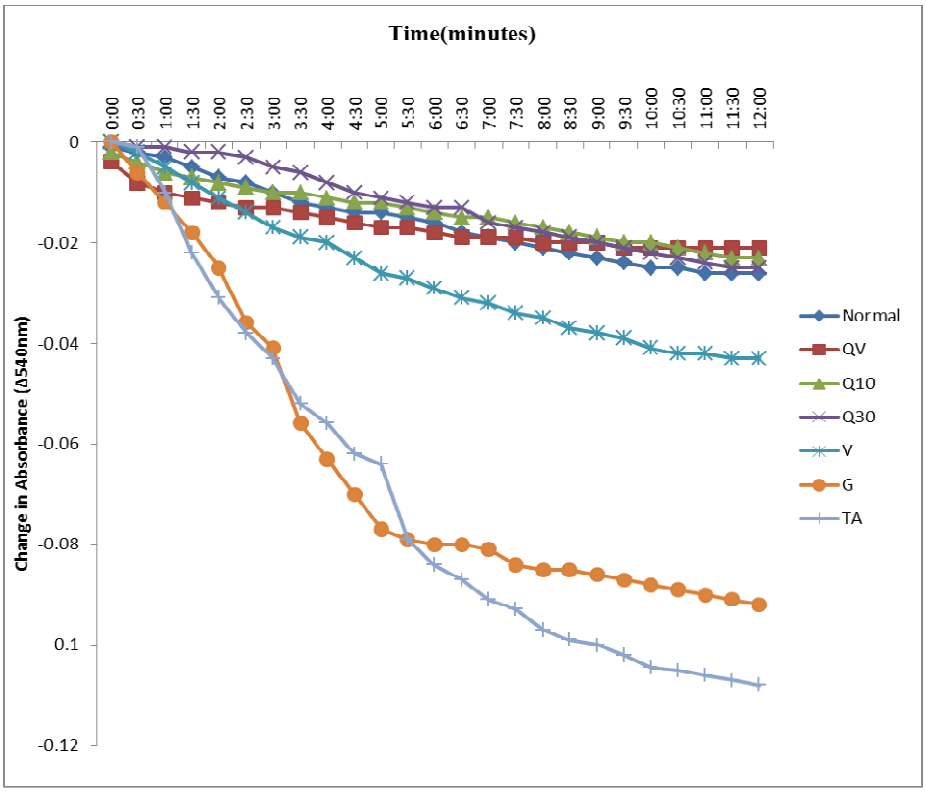


Figure29:Effect of co-administration of quercetin and vitamin E, vitamin E, glibenclamide and varying concentrations of quercetin on mitochondrial membrane permeability transition pore of normal rat heart

Normal: Normal rat
TA= Presence of calcium
Q10&VitE: rats orally administered 10 mg/kg quercetin and 10 mg/kg vitamin E
Q10: rats orally administered 10 mg/kg quercetin
Q30: rats orally administered 30 mg/kg quercetin
VitE: rats orally administered 10 mg/kg vitamin E
Glib: rats orally administered 0.6 mg/kg glibenclamide

EXPERIMENT 8:EFFECT OF VARYING DOSES OF QUERCETIN, GLIBENCLAMIDE, VITAMIN E, CO-ADMINISTRATION OF QUERCETIN AND VITAMIN E TREATMENT ON STZ-INDUCED DIABETIC RAT HEART mPT PORE

Introduction

Diabetes potentiates the risk of about 65% cardiovascular death (Qazi and Malik, 2013) and the mitochondrial has been shown to play a cardinal role as effector of cell demise (Najafi *et al.,* 2014). Tissue wastage is a peculiar feature of diabetes due to increasing apoptosis and it is hypothesized that it could be as a result of mPT pore induction. Opening of this supra-molecular structure is highly controlled and it has been found to be relevant in the initiation of critical processes in apoptosis. Reports have shown that antioxidants are beneficial in pathological conditions, they scavenge free radicals in the system thereby may be effective in inhibiting mPT opening and preventing many diseases (Morin *et al.,* 2010). Quercetin, a natural bioflavonoid that has been widely investigated and found to have numerous health benefits ranging from prevention to delay in the progression of complications in diseases was orally administered to the rat models and their effects investigated. The effects of Vitamin E was also determined on the STZ-induced diabetic rats, and the results compared with glibenclamide.

Therefore, because of the critical involvement of CVD in DM situations and paucity of sufficient information on this subject, this study investigated the cardioprotective effect of quercetin and vitamin E administration on STZ-induced diabetes and the effect of simultaneous inhibition of the mPT in DM.

Procedure

Low-ionic strength mitochondria were isolated from STZ-induced diabetic rats orally administered Q10, Q30,VitE, Q10&VitE, glibenclamide and fed with purina chow and water *ad libitum* for 28 days.

Rat heart mitochondrial swelling was monitored as described under materials and methods (page 80-82).

Results

As shown in Figure 30, induced diabetes caused significant increase in the opening of the pore by 80%, however, the inductive effect was not as high as what was observed in liver. Measurement of mitochondrial swelling in animals orally treated with Q10&VitE showed reversal of induction by 50%, while Q10 treatment showed reversal by 45%. Interestingly the data in Fig 30 showed that Q30 was more effective in inhibiting the mPT pore opening, showing inhibitory effect by 82%, while VitE inhibited the opened mPT pore in diabetic rats by 50% more than glibenclamide (43%).

Conclusion

Treatment with Q30 reversed the opening of the mPT pore in STZ-induced diabetes than other treatment groups.

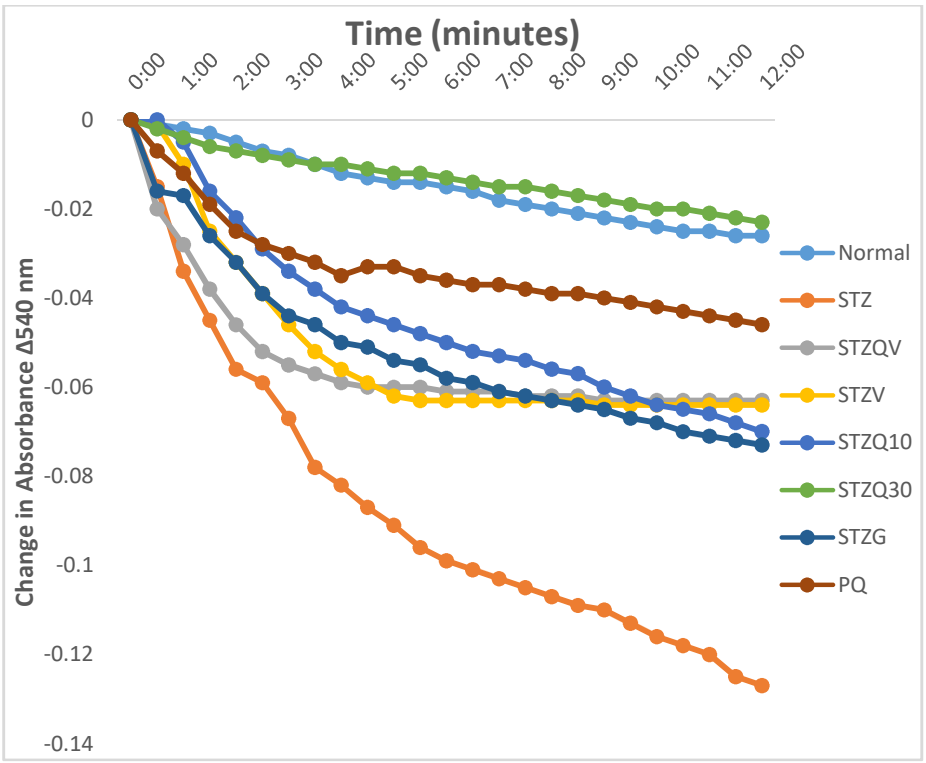


Figure 30:Assessment of the Effect of glibenclamide, vitamin E, co-administration of quercetin and vitamin E and varying concentration of quercetin on the heart mitochondrial membrane permeability transition pore of STZ-induced diabetic rat

Normal: Normal rat
STZ: STZ induced diabetes rats
Q10&VitE: STZ-induced diabetes rats orally administered 10 mg/kg quercetin and 10mg/kg vitamin E
Q10: STZ-induced diabetes rats orally administered 10 mg/kg quercetin
Q30: STZ-induced diabetes rats orally administered 30 mg/kg quercetin
PQ: STZ-induced diabetic rats orally pretreated with 30 /kg quercetin
VitE: STZ-induced diabetes rats orally administered 10 mg/kg vitamin E
Glib: STZ-induced diabetes rats orally administered 0.6 mg/kg glibenclamide

EXPERIMENT 9: EFFECT OF GLIBENCLAMIDE, VITAMIN E, CO-ADMINISTRATION OF QUERCETIN AND VITAMIN E AND VARYING CONCENTRATION OF QUERCETIN ON NORMAL RAT LIVER MITOCHONDRIAL F_0F_1 ATPase ACTIVITY

Introduction

The F_0F_1 ATPase complex resides in the mitochondria, it is a supra-molecular protein that transverses the inner mitochondrial membrane, it is a pore with the F_0-domain inside the inner mitochondria and the F_1 domain in the matrix. The F_0F_1 ATPase is capable of dissipating the electrochemical gradient produced in the respiratory chain in a controlled manner to generate ATP (Bonora *et al.,* 2014). The F_0F_1 ATPase is also called ATP synthase, it is referred to as ATP synthase because it rotates about 700 times /s to synthesize 3 ATP molecules (Bonora *et al.,*2014). In pathological condition, the complex works in reverse order, hydrolyzing ATP to ADP and Pi instead of synthesizing. Studies have shown the similarities of this protein to the mPT pore, demonstrating that it may be a requirement for the pore formation (Giorgio *et al.,*2013; Alvian *et al.,*2014; Bonora *et al.,*2014). Similarly, Dietze and colleagues explained that an intact mitochondrial coupling membrane is the criterion for the proton motive force necessary for ATP synthesis (Dietze *et al.,*2001). Therefore uncoupling of the mitochondrial membrane potential makes the ATP synthase to reverse direction and then hydrolyze ATP. To understand how mPT pore influences mATPase, an ATP hydrolysis protocol was adapted simultaneously with temperature equilibration, after which inorganic phosphate released by mitochondria was determined.Decreasing the enhanced activity of F_0F_1 ATPase may be considered pivotal to

the inhibitionof the mPT pore opening. The current study therefore undertook the task of determining the effects of certain antioxidants on mitochondrial F_0F_1 ATPase activity.

Procedure

Mitochondria were isolated from normal rat liver (that have been orally administered with Q10&VitE, Q10, Q30, VitE, glibenclamide) in 0.25 M sucrose by differential centrifugation as earlier highlighted under materials and methods (pages 93-95). The mitochondrial protein determination was estimated by the method of Lowry *et al.* (1951).

Results

The data presented in Figure31 shows that the mitochondrial F_0F_1 ATPase activity of normal rat liver was not enhanced when compared with STZ-induced rat mitochondria.

In this regard, mitochondrial F_0F_1 ATPase activity from Q10 and Q10&VitE rats did not show any significant enhancement when compared with control. Administration of Q30, VitE and Glibenclamide showed significant mitochondrial F_0F_1 ATPase enhancement when compared with control.

Conclusion

This result indicated that administration of Q10 and Q10&VitEdid not enhance mitochondrial F_0F_1 ATPase activity.

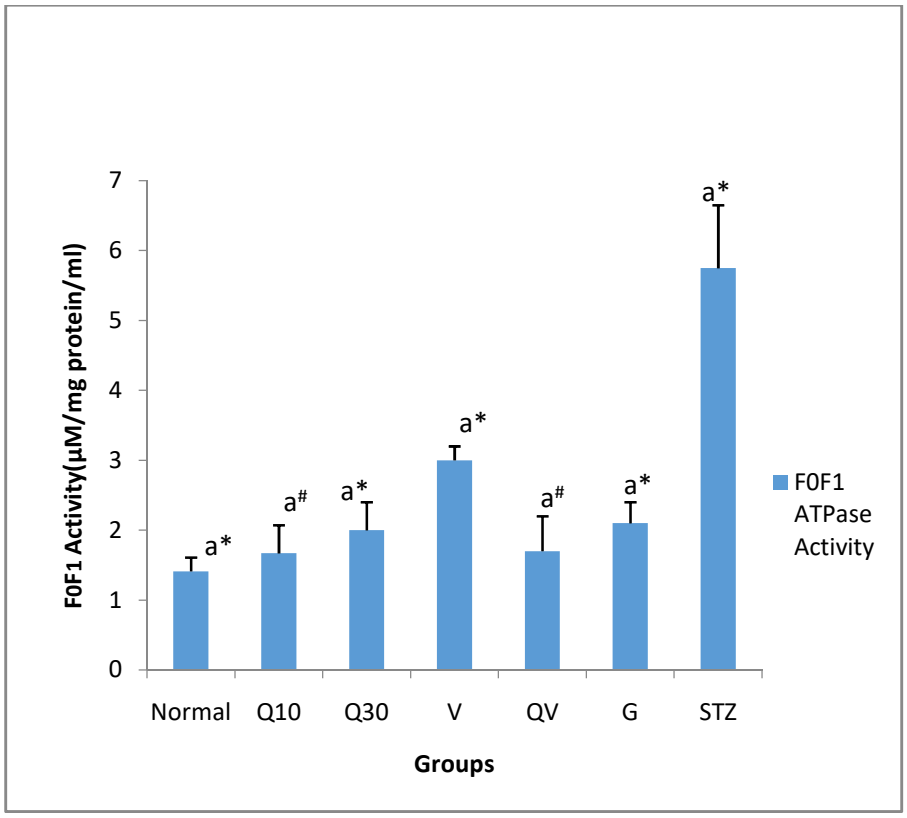


Figure 31: Effect of treatment with co-administration of quercetin and vitamin E, varying concentration of quercetin, vitamin E and glibenclamide on normal rat liver mitochondrial F_0F_1 ATPase.

Normal: Normal rat
STZ: STZ induced diabetes rats
Q10&VitE: Rats orally administered 10 mg/kg quercetin and 10mg/kg vitamin E
Q10: Rats orally administered 10 mg/kg quercetin
Q30: Rats orally administered 30 mg/kg quercetin
VitE: Rats orally administered 10 mg/kg vitamin E
Glib: Rats orally administered 0.6 mg/kg glibenclamide
a* = Statistically significantly different from normal rats
b* = Statistically significant from STZ-induced diabetic rats
a# = Not statistically significant from normal rats

EXPERIMENT 10: EFFECT OF TREATMENT WITH GLIBENCLAMIDE, VITAMIN E, CO-ADMINISTRATION OF QUERCETIN AND VITAMIN E AND VARYING CONCENTRATIONS OF QUERCETIN ON STZ-INDUCED DIABETIC RAT LIVER MITOCHONDRIAL F_0F_1 ATPase ACTIVITY

Introduction

In mammals, the mitochondrial F_0F_1 ATPase produces high amount of cellular ATP with the inhibitory factor 1 (IF_1) inhibiting the activity of the enzyme. This makes it to function in the reverse order, consuming ATP instead of synthesizing it. Therefore, IF_1 may control F_0F_1 ATPase activity physiologically and pathologically (Faccenda and Campenella, 2012). The F_0F_1 ATPase performs an indispensable role in ATP production and acts by coupling protons from the inter mitochondria membrane into the matrix with the oxidative phosphorylation of ADP. Recent studies have shown that the c subunit of the F_0F_1 ATPase may be a component of the mPT pore.

In diabetes, studies have shown the sensitization of mPT pore, if therefore, F_0F_1 ATPase is suggested as a probable component of the mPT pore (Griffiths *et al*, 1993, Di Lisa *et al*., 2001, Clarke *et al*., 2002, Halestrap *et al*., 2007), we hypothesize that investigating the effect of quercetin and vitamin E on F_0F_1 ATPase would modulate mPT pore opening probability in diabetic heart mitochondria.The study therefore is to assess whether the antioxidants would be able to mimic the role of IF_1 or not.

Procedure

Mitochondria were isolated from livers of STZ-induced diabetic rats (administered Q10&VitE, Q10, Q30, VitE and glibenclamide for 28 days) as described under materials

and methods (pages 93-95). The mitochondrial protein determination was estimated by the method of Lowry *et al.* (1951).

Results

The data presented in Figure 32 showed the activities of mitochondrial F_0F_1 ATPase at the end of 28 days after diabetes induction and treatments. Result showed that there was significant ($P<0.05$) enhancement of the activities of ATPase in untreated STZ-induced diabetic rats relative to control. Furthermore, differences were observed in levels of reduction in ATPase activities with the highest reduction of 61% shown by diabetic rats that were orally administered VitE. Similarly it was observed that co-administration of Q&VitE significantly reduced the enhanced activity of the enzyme by 55% while Q30 and Q10 reduced the enhanced activity by 48 and 45% respectively. However, it was observed that glibenclamide further increased stimulatory activity of ATPase by 20%.

Conclusion

The antioxidants considered were effective in preventing the enhancement of F_0F_1 ATPase activity whereas the standard drug (glibenclamide) caused enhancement of ATPase activity.

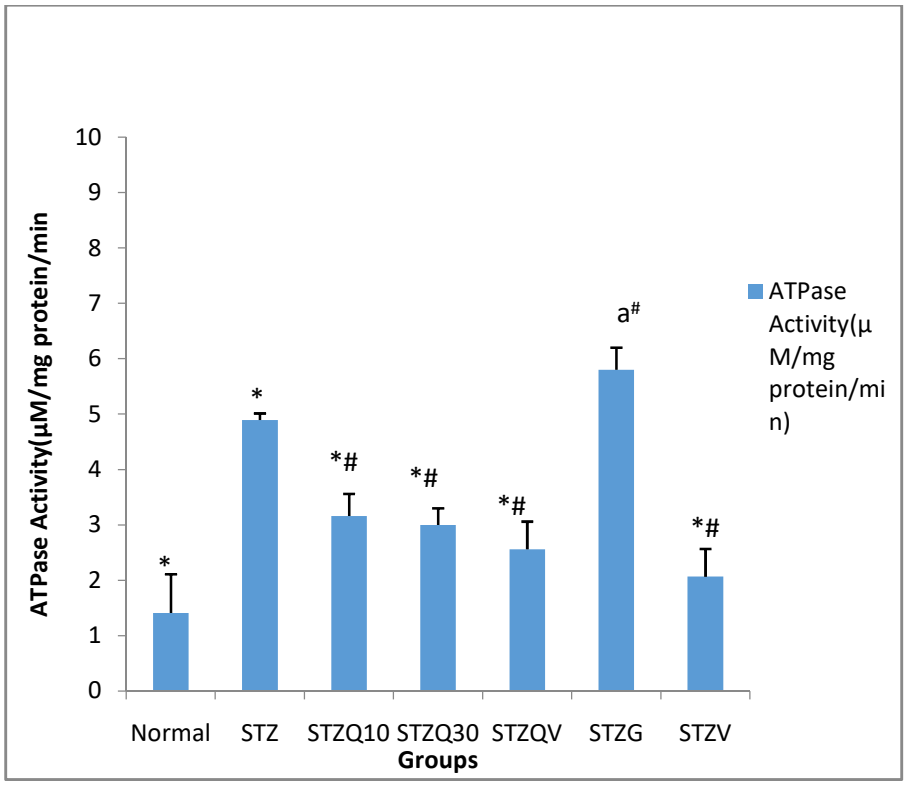


Figure 32: F_0F_1 ATPase activity of liver Mitochondrial of STZ-induced diabetic rat and treatment groups

Normal: Normal rat
STZ: STZ induced diabetes rats
Q10&VitE: STZ-induced diabetes rats orally administered 10 mg/kg quercetin and 10mg/kg vitamin E
Q10: STZ-induced diabetes rats orally administered 10 mg/kg quercetin
Q30: STZ-induced diabetes rats orally administered 30 mg/kg quercetin
VitE: STZ-induced diabetes rats orally administered 10 mg/kg vitamin E
Glib: STZ-induced diabetes rats orally administered 0.6 mg/kg glibenclamide
* = Statistically significant from STZ-induced diabetic rats
# = Statistically significantly different from normal rats
a# = Not statistically significant from STZ-induced diabetic rats

EXPERIMENT 11: DETERMINATION OF MITOCHONDRIAL F_0F_1 ATPase ACTIVITY IN NORMAL RAT HEART TREATED WITH CO-ADMINISTRATION OF QUERCETIN AND VITAMIN E, GLIBENCLAMIDE, VITAMIN E AND VARYING CONCENTRATIONS OF QUERCETIN

Introduction

The F_0F_1 ATPase complex is found in the mitochondria, this protein dissipates electrochemical gradient produced in the respiratory chain in a controlled manner to generate ATP (Bonora *et al.,*2014, Mitchell, 1961). Studies have shown the similarities of this protein to the mitochondrial mPT pore complex proving it may be a requirement for the mPT pore formation (Giorgio *et al.,*2013; Alvian *et al.,*2014; Bonora *et al.,*2014). Dietze and colleagues explained that an intact mitochondrial coupling membrane is the criterion for the proton motive force necessary for ATP synthesis, therefore uncoupling of the potential in mitochondrial makes the ATP synthase to reverse direction and hydrolyze ATP instead of synthesizing it(Dietze *et al.,*2001). To ascertain the integrity of the mPT pore, the mitochondrial ATPase activity was assessed using concentration of inorganic phosphate released as an index of enhancement. Effect of antioxidants were assessed on F_0F_1 ATPase activity.

Procedure

Normal healthy rats were and randomly divided into groups. They were orally administered Q10&VitE, Q10, Q30, VitE, glibenclamide and fed purina chow and fresh water for 28 days. Mitochondria were isolated from the rat heart in 0.25 mM sucrose by differential

centrifugation as described in materials and methods (pages 93-95). The mitochondrial protein determination was estimated by the method of Lowry *et al,* (1951).

Results

Results presented in Figure 33 showed the activities of mitochondrial ATPase in normal rat hearts and treatment groups. There was no enhancement in ATPase activity of normal rat relative to diabetic rats ($p>0.05\%$). Administration of Q10&VitE, Q10, Q30, VitE and glibenclamide showed no significant enhancement of mitochondrial ATPase activity when compared with the normal rat.

Conclusion

This show that all the antioxidants and the drug considered did not enhance ATPase activity.

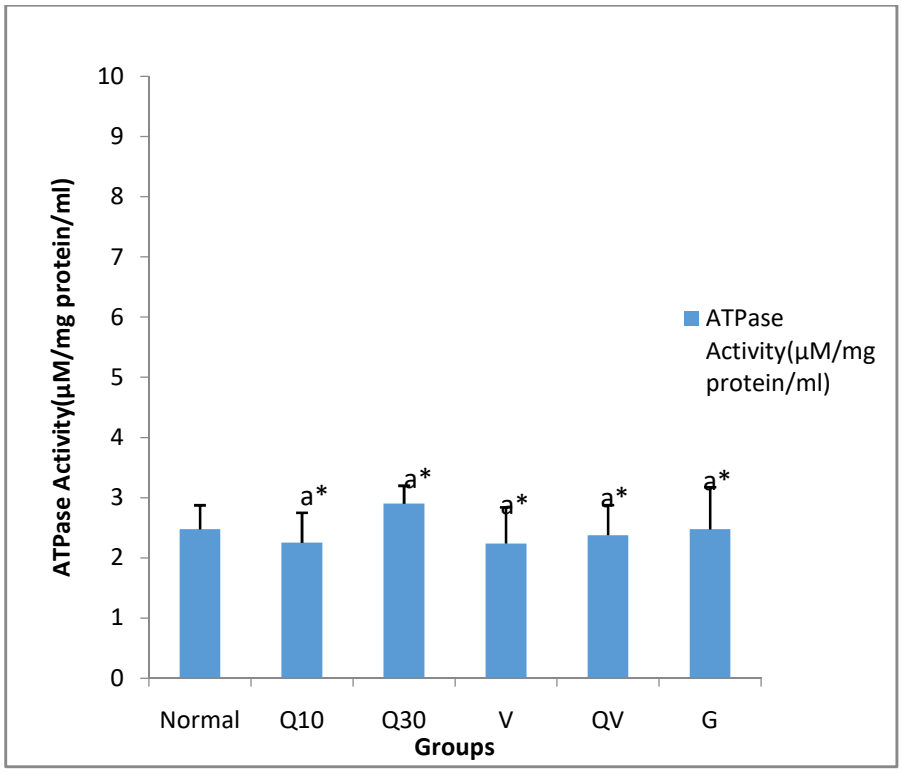


Figure 33: Activities of mitochondrial membrane bound ATPase in heart of normal rats and treatment groups.

Normal rats
Q10= Rats orally treated with 10 mg/kg quercetin.
Q30= Rats orally treated with 30 mg/kg quercetin.
VitE= Rats orally treated with 10 mg/kg vitamin E
Q10&VitE= Rats orally treated with 10 mg/kg quercetin and vitamin E.
Glib= Rats orally treated with 0.6 mg/kg glibenclamide
a* = Statistically significantly different from normal rats
$a^{\#}$ = Not statistically significantly different from normal rats

EXPERIMENT 12: DETERMINATION OF MITOCHONDRIAL F_0F_1 ATPase ACTIVITY IN STZ-INDUCED DIABETIC RAT HEART ORALLY TREATED WITH CO-ADMINISTRATION OF QUERCETIN AND VITAMIN E, GLIBENCLAMIDE, VITAMIN E AND VARYING CONCENTRATION OF QUERCETIN

Introduction

The mitfochondrial complex V is also called the F_0F_1-ATPase complex which synthesizes ATP with a concomitant reduction of oxygen to water utilizing the proton motive gradient. This enzyme has a capability to reverse role when there is cellular depletion of ATP, with the varying consequences depending on the ATP requirement of the tissue. In organs that require high amount of ATP like brain, skeletal muscle and heart, depletion in cellular ATP may enlist the cell in suicide mission by either apoptosis or necrosis as the case may be. However, in normal physiological condition there might be increase or decrease in ATP synthesis depending on the demand of the cell at that point. Studies have shown that in eukaryotes the rate of ATP utilization changes by 5-8 mM (Faccendo and Campenella, 2012) during exercise or acclimatization. The F_0F_1-ATPase can be regulated by translational control, transcriptional factor, modulation of ETC, ADP inhibition and regulatory proteins. The experiment was therefore designed is to determine if certain antioxidants would be able to reverse the enhanced activity of ATPase in diabetic condition.

Procedure

Mitochondria were isolated from heart of STZ-induced diabetic rats (that have been orally administered Q10&VitE, Q10, Q30, VitE and glibenclamide) in 0.25 mM sucrose by differential centrifugation as described in materials and methods (pages 93-95). The mitochondrial protein determination was estimated by the method of Lowry *et al,* (1951).

Results

The effects of treatment with Q10&VitE, Q10, Q30, VitE on mitochondrial F_0F_1 ATPase activity at the end of 28 days after diabetes induction are shown in Figure 34. Result showed a significant enhancement of ATPase activity in diabetic rats relative to control. Furthermore, ATPase activity was decreased by 48% in diabetic rats co-administered Q10&VitE. Similarly, ATPase activity wasdecreasedby 38% in diabetic rats treated with Q10 and VitE respectively. Treatment of diabetic rats with Q30 showed a decrease by 29% in ATPase activity. Conversely, it was observed that glibenclamide showed no reduction whatsoever, in the activity of ATPase. This trend of result is similar to what was observed in the liver mitochondrial.

Conclusion

The antioxidants reduced the enhancement of mitochondrial ATPase activities in STZ-induced diabetic rats.

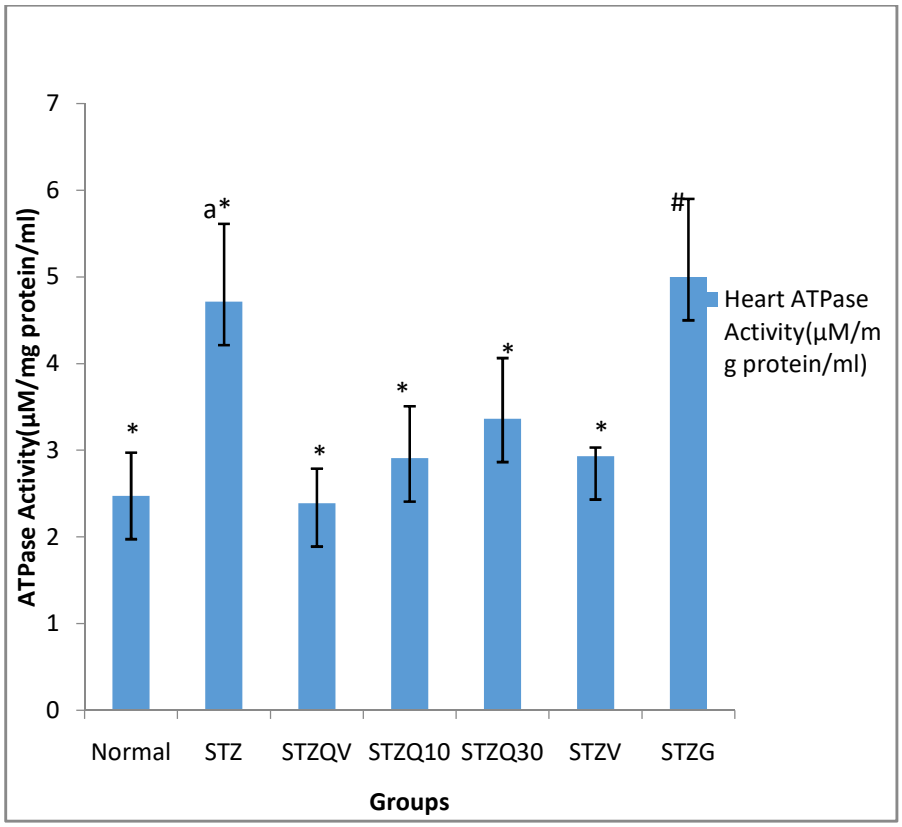


Figure 34: Activities of Mitochondrial Membrane bound ATPase in heart of STZ-induced diabetic rats and treatment groups

Normal rats
Q10= Normal rats orally treated with 10 mg/kg quercetin.
Q30= Normal rats orally treated with 30 mg/kg quercetin.
VitE= Normal rats orally treated with 10 mg/kg vitamin E
Q10&VitE= Normal rats orally treated with 10 mg/kg quercetin and vitamin E.
Glib= Normal rats orally treated with 0.6 mg/kg glibenclamide
* = Statistically significantly different from STZ-induced diabetic rats
a* = Statistically significantly different from normal rats
$^{\#}$ = Not statistically significantly different STZ-induced diabetic rats

EXPERIMENT 13: EFFECT OF CO-ADMINISTRATION OF VITAMIN E AND QUERCETIN, GLIBENCLAMIDE, VITAMIN E AND VARYING DOSES OF QUERCETIN ON NORMAL RAT LIVER MITOCHONDRIAL LIPID PEROXIDATION

Introduction

Lipid peroxidation is a common process in animal and plant, it involves formation of lipid radicals, rearrangement of double bonds, and uptake of oxygen and eventual destruction of the membrane lipids. In normal physiological condition, the level of generation of lipid peroxides is quite low hence biological membranes are protected. The mitochondrial complex 1 is very sensitive to inactivation by reactive species and some of the substrates of the complex are intermediates of lipid peroxidation and they cause impaired respiratory function in several diseases (Navarro and Boveris, 2009). The respiratory chain complex IV has been hypothesized to be subjected to protein damage due to hydroperoxyl radicals and stable aldehydes generated in lipid peroxidation (Navarro *et al.,*2010). This gave our experimental approach the drive to investigate whether antioxidants would serve as preventive therapies for mitochondria induced damage by lipid peroxidation.

Procedure

The mitochondrial Lipid Peroxidation (mLPO) was determined by TBARS assay as described in the study of Ruberto *et al.,*(2000). The aim was to measure the rate of mitochondrial lipid peroxides formed in the reaction medium. Aliquot portions of 1mg/mL liver mitochondria (normal, Q10VitE, Q10, Q30, VitE, Glib) were added to test-tubes. The solution obtained after 20% acetic acid and 0.8% (w/v) thiobarbituric acid in 1.1% SDS

were added was agitated well on a vortex mixer and boiled for 1 hour at 95^{o}C. This was cooled under a running tap and butanol (5 mL) was added (As described under materials and methods, page 89-91). The solution was then centrifuged at 3000 rpm for 10 minutes and the organic upper layer obtained. The absorbance was read at 532 nm in a spectrophotometer.

Results

In Figure 35, the results showed that treatment with Q10&VitE, Q10 and Q30 showed that the levels of mLPO generated were reduced by 31, 15 and 50% respectively. Similarly, glibenclamide reduced the levels of mLPO by 20% while VitE treatment showed no effects when compared with the normal rat.

Conclusion

The antioxidants considered reduced mLPO levels, showing that they were safe on the mitochondrial.

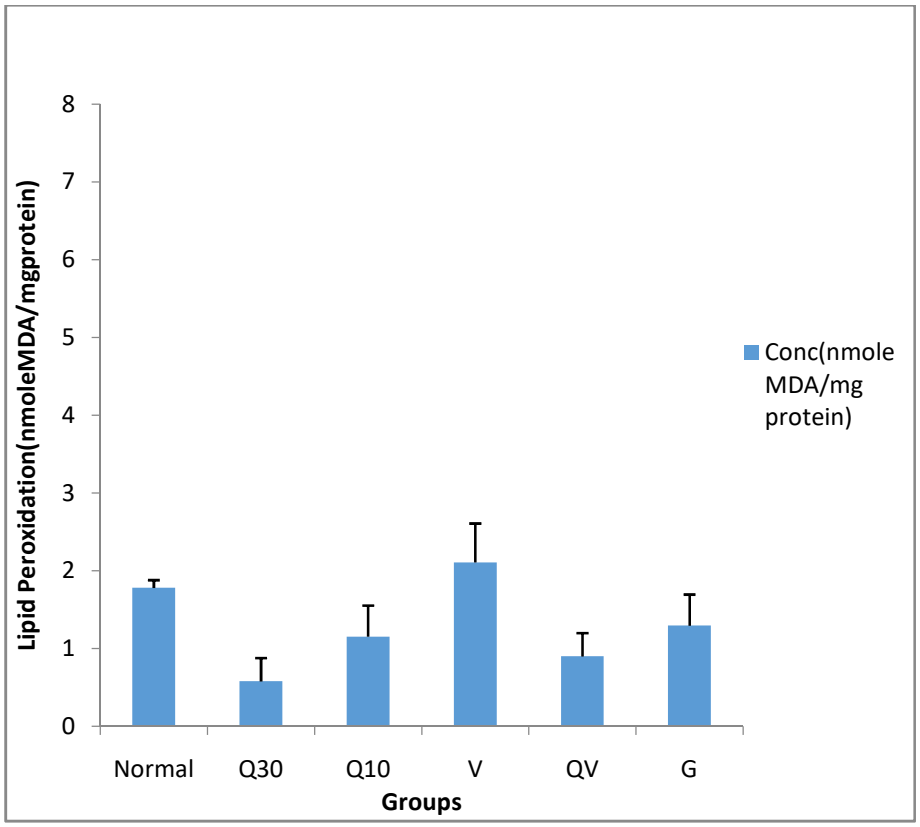


Figure 35: Effect of co-administration of vitamin E and quercetin, glibenclamide, vitamin E and varying concentration of quercetin on normal rat liver mitochondrial lipid peroxidation

Normal rats
Q10= Normal rats orally treated with 10 mg/kg quercetin.
Q30= Normal rats orally treated with 30 mg/kg quercetin.
VitE= Normal rats orally treated with 10 mg/kg vitamin E
Q10&VitE= Normal rats orally treated with 10 mg/kg quercetin and vitamin E.
Glib= Normal rats orally treated with 0.6 mg/kg glibenclamide

EXPERIMENT 14: EFFECT OF CO-ADMINISTRATION OF VITAMIN E AND QUERCETIN, GLIBENCLAMIDE, VITAMIN E AND VARYING CONCENTRATION OF QUERCETIN ON STZ-INDUCED DIABETES RAT LIVER MITOCHONDRIAL LIPID PEROXIDATION

Introduction

Lipid peroxidation is when lipid aldehydes are formed as a result of oxidants attack on the biological membrane lipids (polyunsaturated fatty acids) by reactive species. The aldehydes formed activate signaling kinases which alter the redox signaling pathway causing cytotoxicity and ultimately cell death. Several studies have shown altered cellular physiology due to formation of lipid aldehydes which range from malondialdehyde, hydroxyl-trans-2-nonenal, lipid-derived aldehydes and acrolein (Barrera, 2012; Yadav *et al.,*2015). Lipid peroxidation had been observed in several pathological conditions, the ROS are considered to be the major player in lipid peroxidation and these are formed majorly in the mitochondria. The ROS produced forms lipoperoxyl radical which yields lipid hydroperoxide and radical. Due to the instability of the former, generation of new alkoxyl and peroxyl radicals finally form secondary products which affect the permeability of the membrane hence affecting cellular structure (Barrera, 2012). Antioxidants have shown promising possibilities in reducing oxidative stress and therefore alleviating the complications in several pathologies (Yadav *et al.,*2015). The effect of selected antioxidants on lipid peroxidation levels in STZ-induced diabetic rat livers was considered in the study.

Procedure

The mLPO was determined by TBARS assay using the procedures of Ruberto *et al.,*(2000) to estimate the rate of lipid peroxides in the assay medium as described under materials and methods (page 89-91). The absorbance was read at 532 nm using a spectrophotometer.

Results

Result presented in Figure 36 showed the effect of Q10&VitE, Q10, Q30, VitE and glibenclamide on liver mLPO in STZ-induced diabetic rats. The results showed that Q10&VitE, Q10, Q30, VitE and glibenclamide inhibited mLPO by 89, 81, 78, 87 and 85% respectively.

Conclusion

Results showed that all the antioxidants considered in the study inhibited the generation of lipid peroxides in STZ-induced diabetic rats.

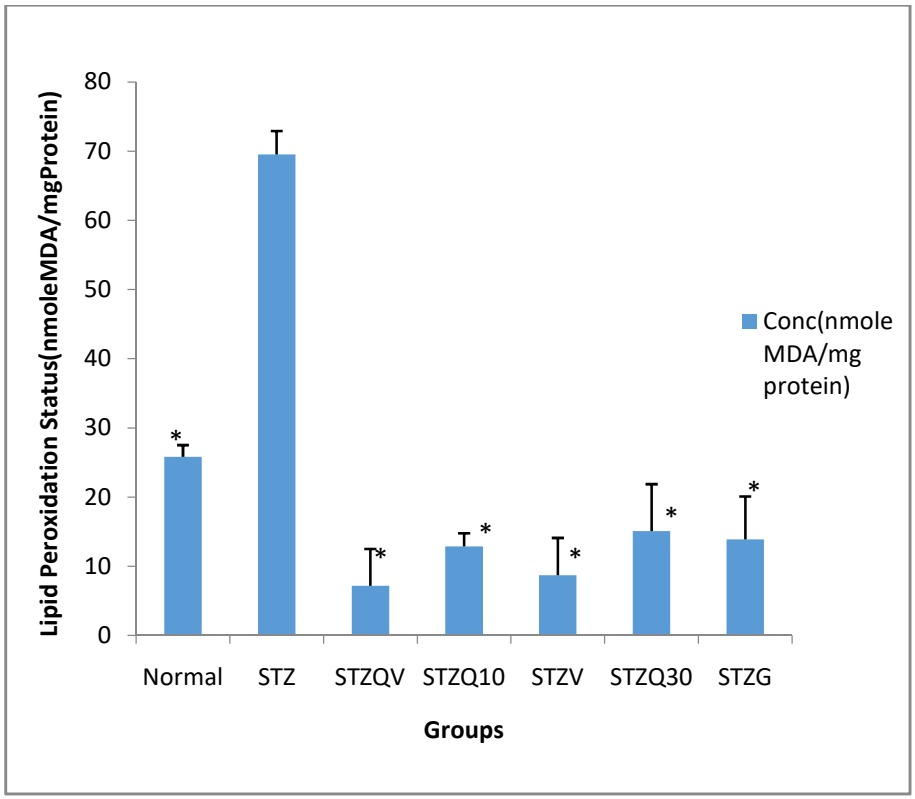


Figure 36: Effect of co-administration of vitamin E and quercetin, glibenclamide, vitamin E and varying concentration of quercetin on liver mitochondrial lipid peroxidation in STZ-induced diabetic rats

Normal: Normal rat
STZ: STZ induced diabetes rats
Q10&VitE: STZ-induced diabetes rats orally administered 10 mg/kg quercetin and 10mg/kg vitamin E
Q10: STZ-induced diabetes rats orally administered 10 mg/kg quercetin
Q30: STZ-induced diabetes rats orally administered 30 mg/kg quercetin
VitE: STZ-induced diabetes rats orally administered 10 mg/kg vitamin E
Glib: STZ-induced diabetes rats orally administered 0.6 mg/kg glibenclamide
*= Statistically significant from STZ-induced diabetic rat

EXPERIMENT 15: EFFECT OF CO-ADMINISTRATION OF VITAMIN E AND QUERCETIN, GLIBENCLAMIDE, VITAMIN E AND VARYING CONCENTRATION OF QUERCETIN ON NORMAL RAT HEART MITOCHONDRIAL LIPID PEROXIDATION

Introduction

Antioxidants are able to reduce lipid peroxidation by preventing oxidative stress to cell structures. Numerous antioxidants and bioflavonoids have been shown to display anti-lipid peroxides role including vitamin E (Navarro *et al.,*2005). The effect of certain antioxidants compared with glibenclamide on STZ-induced diabetic rat heart was examined.

Procedure

The mLPO of the heart was determined by TBARS assay using the procedures of Ruberto *et al.,*(2000) to estimate the level of lipid peroxidation in the assay medium as described under materials and methods (page 89-91). The absorbance was read at 532 nm using a spectrophotometer.

Results

The data presented in Figure 37 showed that co-administration of Q10&VitE, Q10, Q30, VitEshowed no significant difference from control (5, 9, 3 and 0.3% respectively). In contrast, glibenclamide treatment showed a significant difference in the level of lipid peroxides (31%), relative to control.

Conclusion

The results showed the antioxidants did not interact with the lipid peroxide levels in the mitochondria.

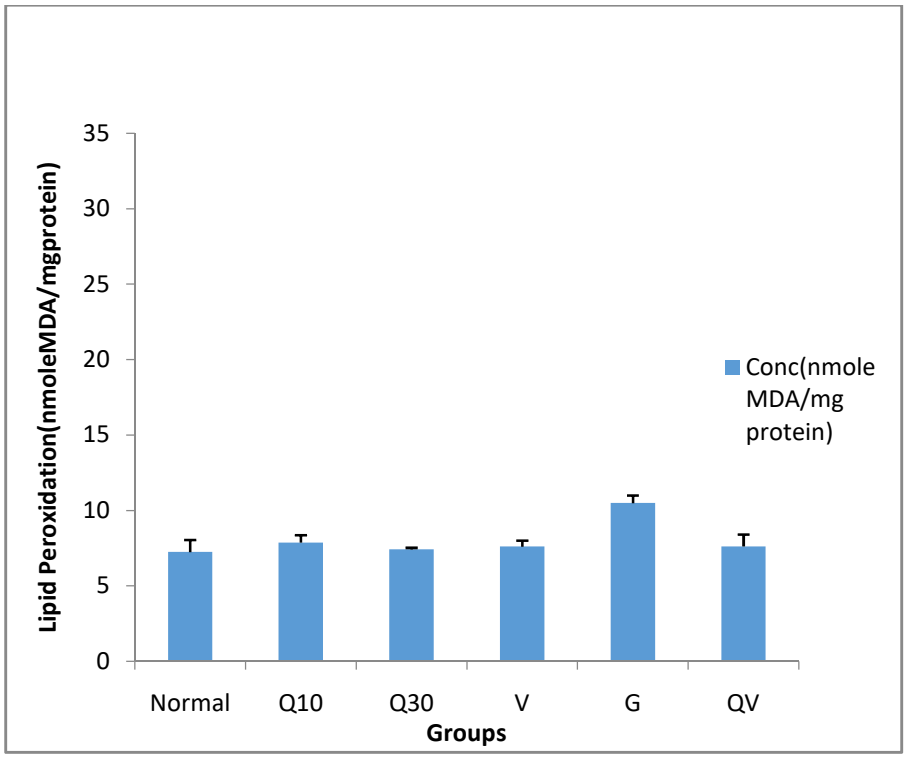


Figure 37: Effect of co-administration of vitamin E and quercetin, glibenclamide, vitamin E and varying concentration of quercetin on normal rat heart mitochondrial lipid peroxidation

Normal: Normal rat
Q10= Rats orally treated with 10 mg/kg quercetin.
Q30= Rats orally treated with 30 mg/kg quercetin.
VitE= Rats orally treated with 10 mg/kg vitamin E
Q10&VitE= Rats orally treated with 10 mg/kg quercetin and vitamin E.
Glib= Rats orally treated with 0.6 mg/kg glibenclamide

EXPERIMENT 16: EFFECT OF CO-ADMINISTRATION OF VITAMIN E AND QUERCETIN, GLIBENCLAMIDE, VITAMIN E AND VARYING CONCENTRATION OF QUERCETIN ON MITOCHONDRIAL LIPID PEROXIDATION OF STZ-INDUCED DIABETIC RAT HEART

Introduction

Excessive generation of ROS in pathological conditions damage biomolecules like proteins (by causing denaturation, loss of function), nucleic acids and this cellular membrane destruction due to lipid peroxidation (Chance *et al.,*1979). The major constituent of the cell membrane are the phospholipids whose fatty acid constituents on carbon 1 and 2 are oxidatively broken down causing documented hepato-, neuro- and nephro-toxicity (Boveris *et al.,*2008). The cardiolipin found in the mitochondrial makes up 18% of the total phospholipids whose fatty acyl groups are unsaturated therefore oxidation compromises the integrity of the membrane initiating outer mitochondrial opening which permits Cyt C exit from the inner membrane causing cell death (Navarro and Boveris, 2009). The goal of the study was to examine the effect of certain antioxidants on lipid peroxidation levels in STZ-induced diabetic rat hearts.

Procedure

The mLPO was determined by TBARS assay using the procedures of Ruberto *et al.,*(2000) to estimate the level of lipid peroxides in the assay medium as described under materials and methods (page 89-91). The absorbance was read at 532 nm using a spectrophotometer.

Results

Figure 38 showed the lipid peroxide levels in the heart mitochondrial of STZ-induced diabetic rat and treatment groups. Result showed that there was an increase in the lipid

peroxide levels in STZ-induced diabetic rat heart relative to control (P>0.05). Treatment with Q10&VitE showed inhibition of 78% when compared with STZ-induced diabetic rats (P>0.05). While Q10 showed higher inhibition of 87%, Q30, VitE and glibenclamide showed inhibition of 73, 62, 59% respectively.

Conclusion

The antioxidants inhibited diabetes-induced lipid peroxidation more effectively than glibenclamide with Q10 having the highest effect. This findings is consistent with the results observed in normal rats.

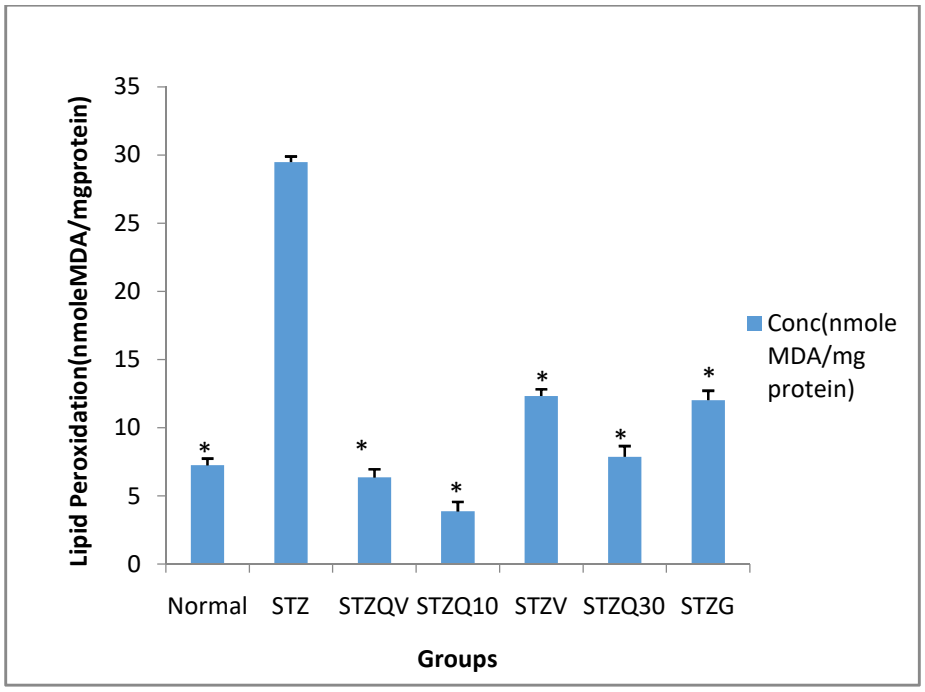


Figure 38: Effect of co-administration of vitamin E and quercetin, glibenclamide, vitamin E and varying concentration of quercetin on heart mitochondrial lipid peroxidation in STZ-induced diabetic rats

Normal: Normal rat
STZ: STZ induced diabetes rats
Q10&VitE: STZ-induced diabetes rats orally administered 10 mg/kg each of quercetin and vitamin E
Q10: STZ-induced diabetes rats orally administered 10 mg/kg quercetin
Q30: STZ-induced diabetes rats orally administered 30 mg/kg quercetin
VitE: STZ-induced diabetes rats orally administered 10 mg/kg vitamin E
Glib: STZ-induced diabetes rats orally administered 0.6 mg/kg glibenclamide
* Statistically significant from STZ-induced diabetic rats

EXPERIMENT 17: DETERMINATION OF CYTOCHROME C RELEASE, CASPASES 3 AND 9 ACTIVATION IN STZ-INDUCED DIABETIC RAT LIVER AND TREATMENT WITH CO-ADMINISTRATION OF QUERCETIN AND VITAMIN E, VARYING CONCENTRATION OF QUERCETIN, VITAMIN E AND GLIBENCLAMIDE

Introduction

There is uncontrolled cell loss due to dysregulated apoptosis in pathologies. Apoptosis is also a normal physiological event in tissue development, homeostasis and regulation of the immune system among others. Robust evidences show the mitochondria regulate apoptosis (Eleftheriadis *et al.,*2016, Phaneuf and Leeuwenburgh, 2002). So many death-inducing signals trigger the mitochondria to release Cyt C (Phaneuf and Leeuwenburgh, 2001), which is a soluble protein that performs a unique role in the respiratory chain by transferring electrons form complex III to complex IV. The location of this heme protein is very crucial because when it exits the mitochondria, apoptosis ensues. During the process of apoptosis, the heme protein trans locates from the mitochondria to the cytosol where it binds Apaf-1and forms a wheel-like structure called apoptosome. The apoptosome activates procaspase-9 through its caspase recruitment domain, when this gets to work, the executioner caspases are activated and they include caspases-3, 6 and 7 (Eleftheriadis *et al.,*2016). Determining the presence of Cyt C in the extracellular space is a valid marker for mitochondrial impairment. Therefore the experiment was to investigate if the opening of the mPT pore is accompanied by Cyt C release in the liver of STZ-induced diabetic and possible decrease by certain antioxidants.

Procedure

Rats were sacrificed by cervical dislocation and the livers obtained were trimmed to remove excess tissues and placed in 10% phosphate-buffer formalin for proper fixation. Before sectioning, the tissue was placed in a cryomedia and these were dehydrated with alcohol wash of different concentrations on slides. They were then cleared using detergent like xylene (As described under materials and methods, page 74).

Results

Results from Figure 39, 40 and 42 showed that there was increased Cyt C release, caspases 3 and 9 activation in STZ-induced diabetic rats relative to normal rat liver section showing a statistically significant difference ($p<0.05$) as examined by Fiji analytical package. Treatment with Q10&VitE decreased Cyt C release showing a statistical difference relative to STZ-induced diabetic rats ($p<0.05$). Interestingly, VitE showed the most impressive result, where it decreased Cyt C release to as much as what was observed in normal rat, showing no statistically significant difference ($p>0.05$). Treatment with Q10 and Q30 decreased Cyt C release with a statistically significant difference relative to STZ-induced diabetic rats. However, it was shown from Figure39 that glibenclamide showed an increase in Cyt C release in a significantly significant manner ($P>0.05$) relative to STZ-induced diabetic rats.

Results depicted in Figure 40 showed that the normal rat liver showed reduced caspase 9 activation, while the STZ-induced diabetic rats showed increased caspase 9 activation when compared with control($p>0.05$). Treatment with Q10&VitE, Q10, Q30 and VitE had no effect on caspase 9 activation with respect to STZ-induced diabetic rats. However,

glibenclamide increased caspase 9 activation with respect to STZ-induced diabetes ($P>0.05$).

In Figure 41, there was increased caspase 3 activation in STZ-induced diabetic rats when compared with the normal control as determined statistically using Fiji statistical software ($p>0.05$). Treatment with Q10&VitE, Q10, Q30 and glibenclamide decreased caspase 3 activation caused by diabetes significantly ($p>0.05$).

Conclusion

Diabetic condition induced Cyt C release, caspase 3 and 9 activation. All the antioxidants considered in the study decreased Cyt C release, with the most outstanding result by VitE, however, glibenclamide did not decrease Cyt C release.

Treatment with Q10&VitE decreased caspase 9 activation in STZ-induced diabetic rats with no decrease by glibenclamide. Caspase 3 activity was decreased by all the antioxidants considered, and here, glibenclamide, showed a decrease in caspase 3 activity.

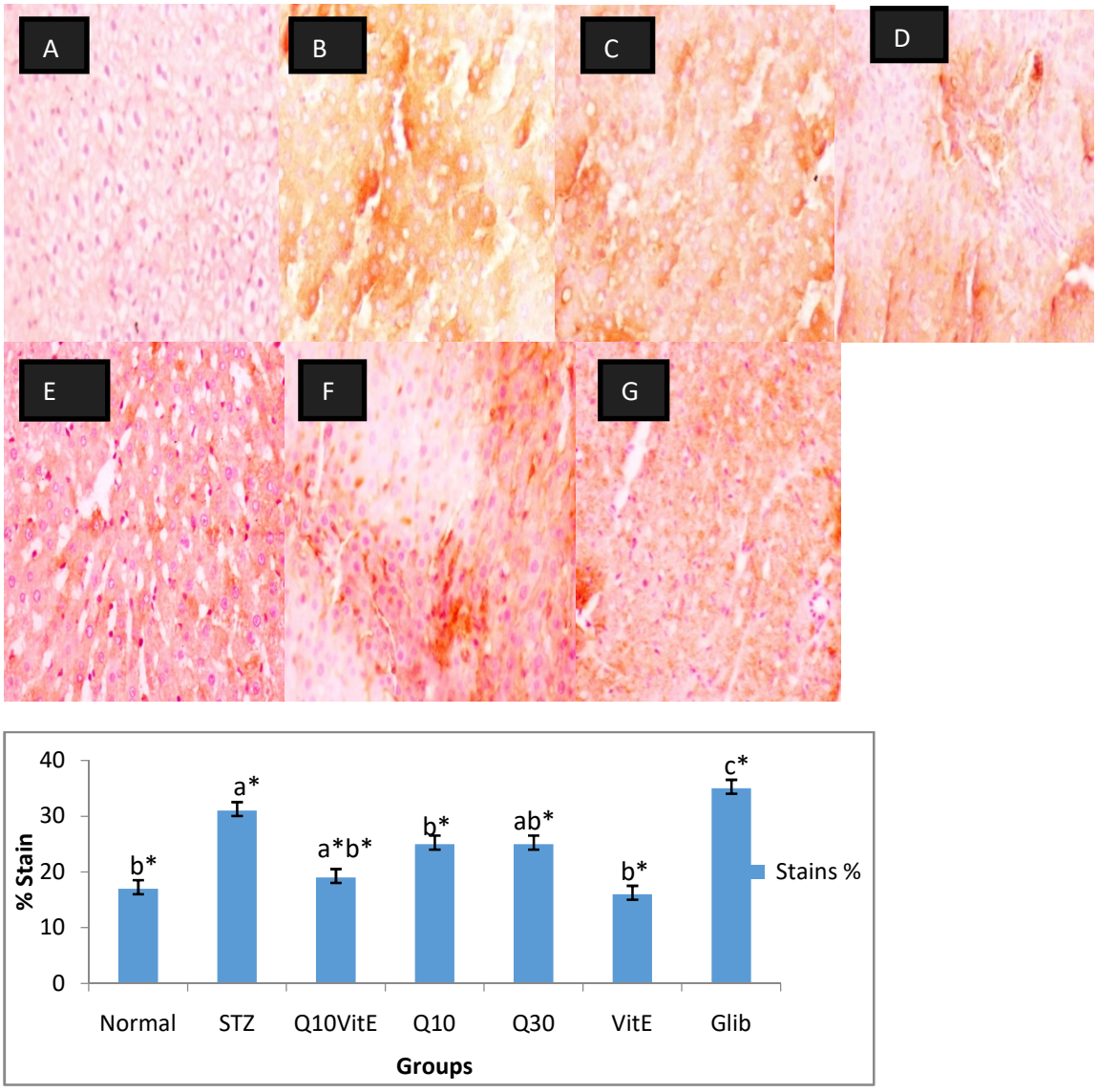


Figure 39: Cytochrome Crelease in the liver of STZ-induced diabetic rat and treatment with co-administration of quercetin and vitamin E, varying concentration of quercetin, vitamin E and glibenclamide

A= Normal Liver showed cytochrome c expression
B= STZ-induced diabetes showed increased cytochrome c expression
C= Glibenclamide treated STZ-induced diabetes showed increased cytochrome c expression
D=Q10 treated STZ-induced diabetes showed decreased cytochrome c expression
E=Q30 treated STZ-induced diabetes showed decreased cytochrome c expression
F=Q10&VitE treated STZ-induced diabetes showed decreased cytochrome c expression
G=VitE treated STZ-induced diabetes showed decreased cytochrome c expression
a*= Statistical significance from normal b*= Statistical significance from STZ
c*= Statistical significantly higher than STZ

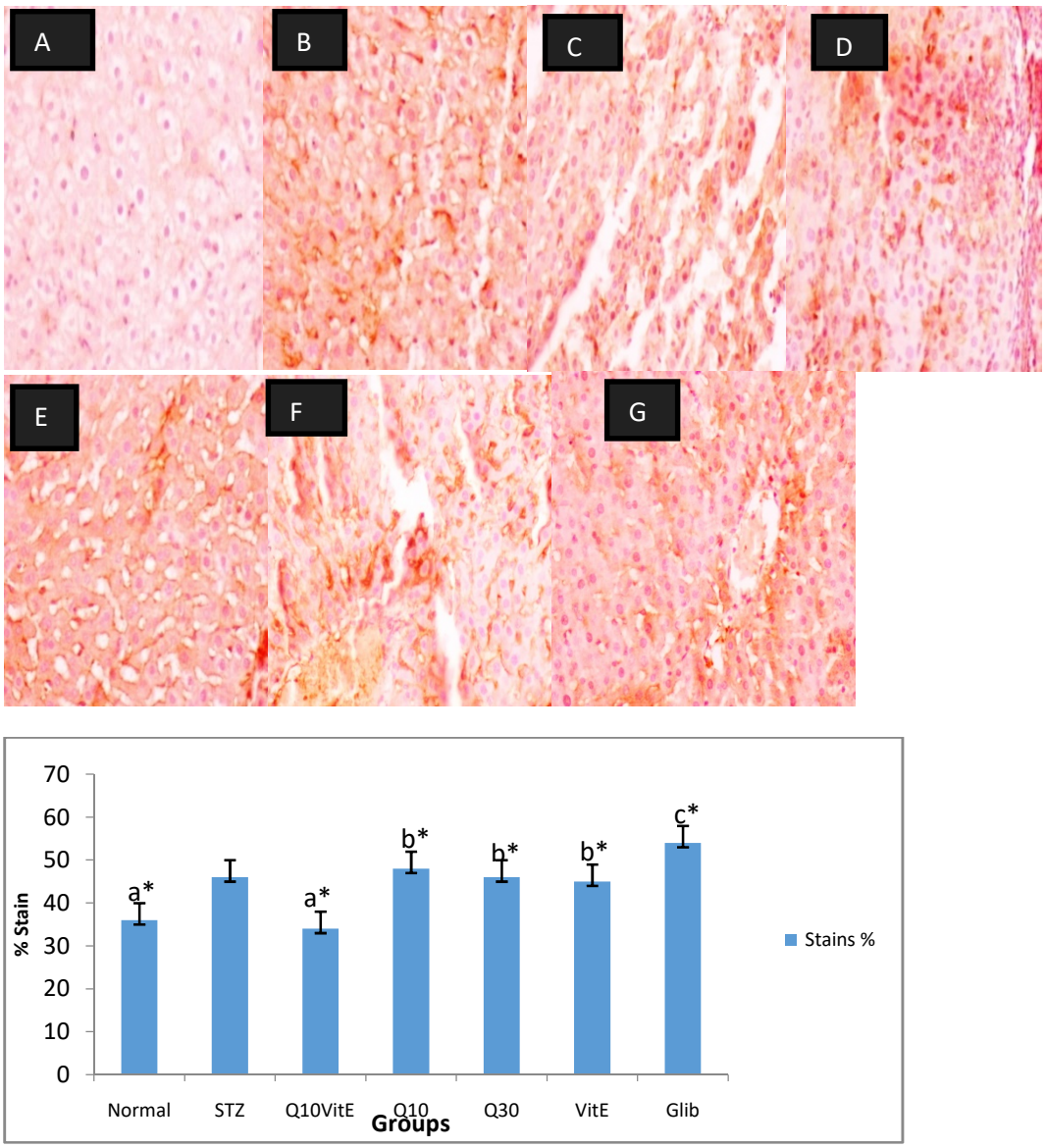


Figure 40: Caspase 9 activation in the liver of STZ-induced diabetic rat and treatment with co-administration of quercetin and vitamin E, varying concentration of quercetin, vitamin E and glibenclamide

A=Normal liver showed caspase 9 activation
B= STZ showed caspase 9 activation
C=Glibenclamide treated STZ-induced diabetes showed reduced caspase 9 activation
D= Q10 treated STZ-induced diabetes showed reduced caspase 9 activation
E= Q30 treated STZ-induced diabetes showed reduced caspase 9 activation
F=Q10&VitE treated STZ-induced diabetes showed reduced caspase 9 activation
a*= Statistical significantly lower than STZ-induced diabetic rats
b*=No statistical difference from diabetic rats
c*= Statistical significantly higher than STZ-induced diabetic rats

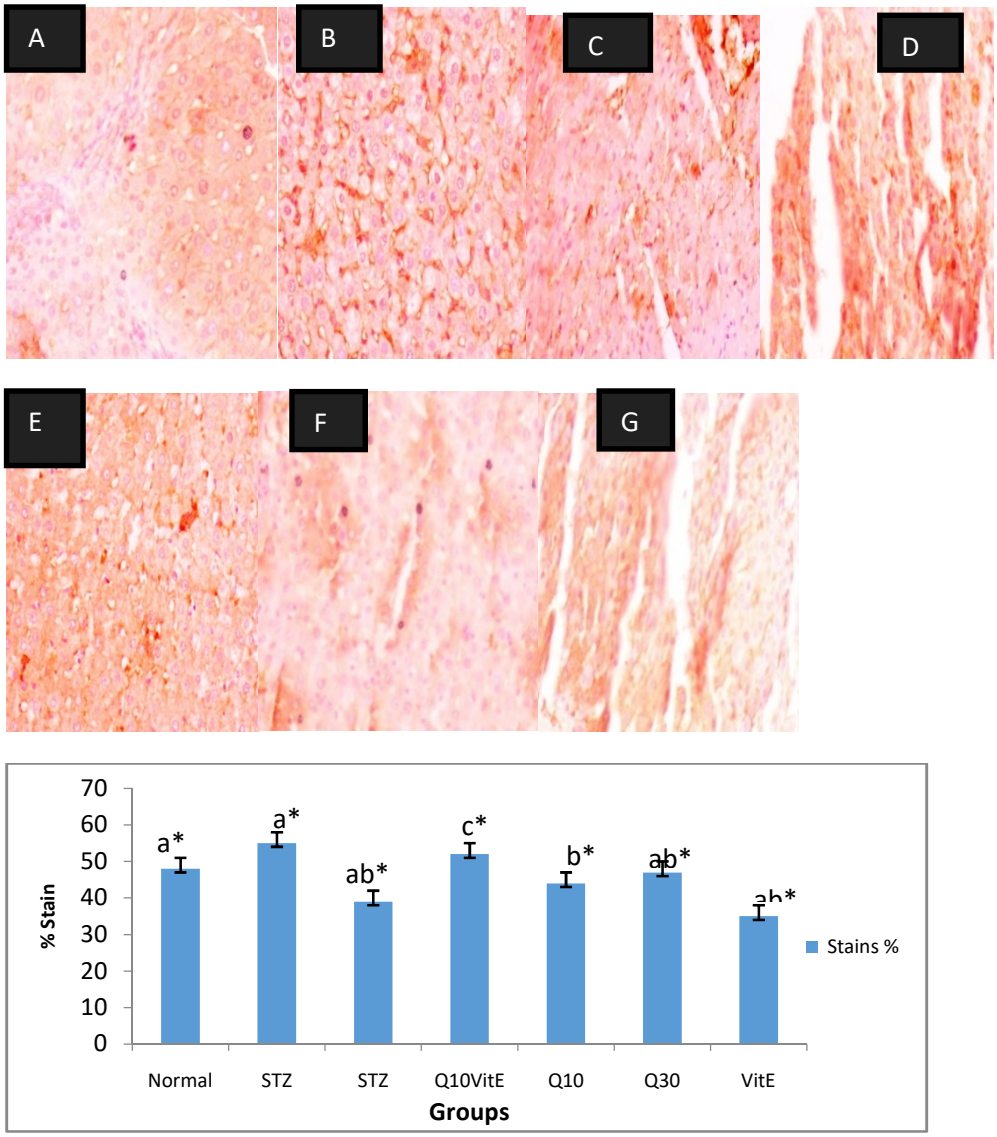


Fig 41 : Expression of caspase 3 in the liver of STZ-induced diabetic rat and treatment with co-administration of quercetin and vitamin E, varying concentration of quercetin, vitamin E and glibenclamide

A=Normal rat liver showed caspase 3 expression
B= STZ-induced diabetes showed increased caspase 3 activation
C=Glibenclamide treated STZ-induced diabetes showed decreased caspase 3 activation

D= Q10 treated STZ-induced diabetes showed decreased caspase 3 activation
E= Q30 treated STZ-induced diabetes showed decreased caspase 3 activation
F= Q10&VitE treated STZ-induced diabetes showed decreased caspase 3 activation
G=VitE treated STZ-induced diabetes showed decreased caspase 3 activation
a*= Statistically Significantly different from normal rat
b*= Statistically significantly different from STZ-induced diabetic rats
c*= Statistically significantly the same with normal and STZ-induced diabetic rats

EXPERIMENT 18: ASSESSMENT OF CYTOCHROME C RELEASE, CASPASES 3 AND 9 ACTIVATION IN STZ-INDUCED DIABETIC RAT HEART AND TREATMENT WITH CO-ADMINISTRATION OF QUERCETIN AND VITAMIN E, VARYING CONCENTRATION OF QUERCETIN, VITAMIN E AND GLIBENCLAMIDE

Introduction

Cytochrome C is a protein that is released subsequent to apoptosis, literatures have established that the protein can be released when the mPT pore opens and also independent of the mPT pore permeabilization since apoptosis inducing factor, another apoptotic protease is released to initiate apoptosis. The study was to investigate the extent of release of Cyt C in STZ-induced diabetes and effect of certain antioxidants.

Caspases are inactive molecules that form dimers and cleaves when activated, they are primary drivers of apoptotic process carving the path-way to death in cells. The initiator caspase 9 is activated by binding of Cyt C to Apaf-1 with the participation of dATP, these recruits and activates pro-caspase 9 which consequently activates caspase 3. This disable homeostatic and repair enzymes causing programmed cell death. Caspases activate endonuclease which causes internal DNA breaks, destruction of lamin (nuclear matrix), dismantle the actin resulting in DNA ladder, condense chromatin condensation and complete cell death. The study was designed to determine how Cyt C release induce activation of caspase 3 and 9 in diabetes.

Procedure

STZ-induced diabetic rats were sacrificed by cervical dislocation, heart section was removed and properly fixed. After placement in paraffin wax, sectioning was carried out using a microtome at the range of 4-40 μm. Fixation was done according to the methods described in chapter three. Stained cells were then viewed by microscopy.

Results

Result from Figure 42 showed that the extent of Cyt C release in STZ-induced diabetic rats was significant when compared with normal rats ($p>0.05$). In treatment with Q10&VitE, Q10, Q30, VitE and glibenclamide, there was reduction in the levels of Cyt C release ($p>0.05$).

In Figure 43, result showed that there was an increase in caspase 9 activation in diabetic rat relative to the normal control ($p>0.05$). Treatment with Q10&VitE, Q30 and VitE decreased caspase 9 activation. However Q10 treated rats did not show decrease in caspase 9 activation relative to STZ-induced diabetic rats ($p>0.05$).

Result in Figure 44, showed that caspase 3 activation following oral administration of various treatments to STZ-induced diabetic rats for 28 days. Whereas the extent of cleaved caspase 3 activation in STZ-induced diabetic rats was increased, treatment with Q10&VitE, Q10, Q30, VitE showed decreased caspase 3 activation relative to STZ-induced diabetic rats. However, glibenclamide showed no decrease in caspase 3 activation relative to diabetic rats ($p>0.05$).

Conclusion

All the antioxidants considered reduced the level of Cyt C release in STZ-induced diabetic rats, whereas in caspase 9 activation, all the antioxidants considered decreased caspase 9 activation comparing favorably with glibenclamide. In contrast, Q10 that did not show changes in the level of caspase 9 activation relative to STZ-induced diabetic rats ($p>0.05$). In caspase 3 activity, treatment with Q10&VitE, Q10, Q30 and VitE showed more effect in decreasing the activation than glibenclamide.

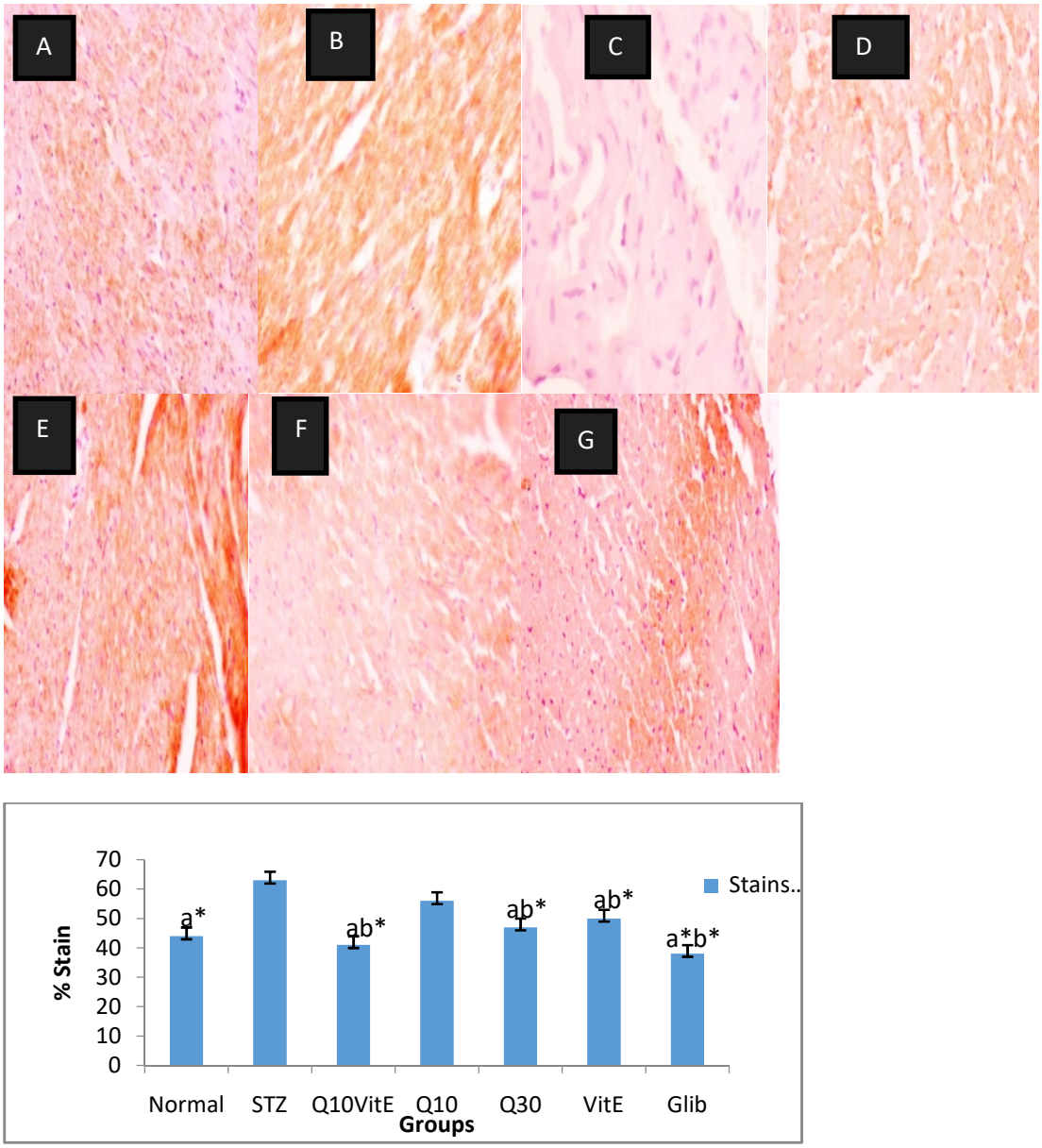


Figure 42: Cytochrome C release in STZ-induced diabetic rat heart and treatment with co-administration of quercetin and vitamin E, varying concentration of quercetin, vitamin E and glibenclamide

A=Normal rat liver showed cytochrome C release
B= STZ-induced diabetes showed cytochrome C release
C=Glibenclamide treated STZ-induced diabetes showed cytochrome C release
D= Q10 treated STZ-induced diabetes showed decreased cytochrome C release
E= Q30 treated STZ-induced diabetes showed decreased cytochrome C release
F= Q10&VitE treated STZ-induced diabetes showed decreased cytochrome C release
G=VitE treated STZ-induced diabetes showed decreased cytochrome C release
a*= Statistically significantly different from normal rat
b*= Statistically significantly different from STZ-induced diabetic rats

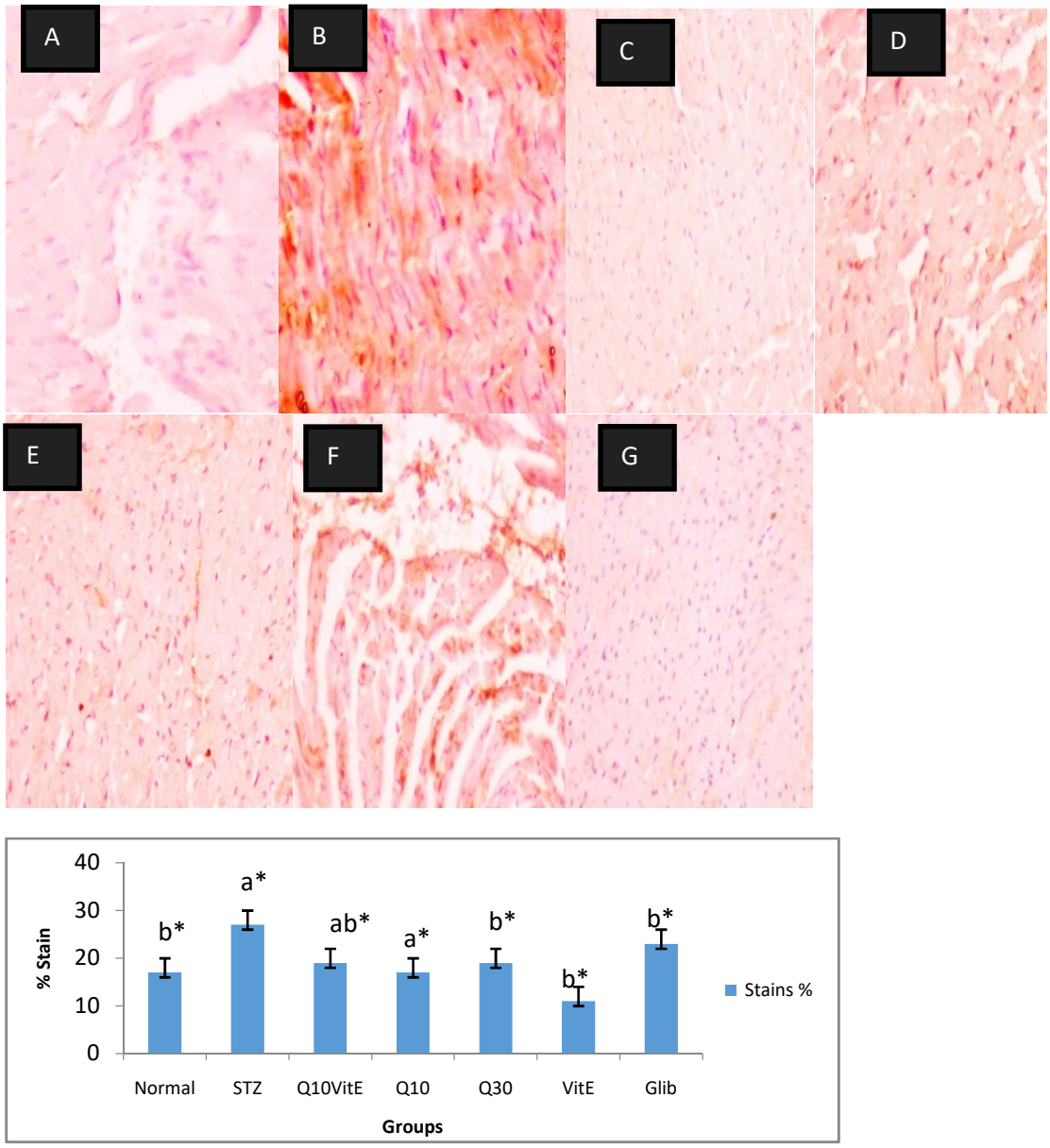


Figure 43: Caspases 9 activation in STZ-induced diabetic rat heart and treatment with co-administration of quercetin and vitamin E, varying concentration of quercetin, vitamin E and glibenclamide

A=Normal rat heart showed expression of caspase 9
B= STZ-induced diabetes showed increased caspase 9 activation
C=Glibenclamide treated STZ-induced diabetes showed decreased caspase 9 activation
D=Q10 treated STZ-induced diabetes showed increased caspase 9 activation
E= Q30 treated STZ-induced diabetes showed decreased caspase 9 activation
F= Q10&VitE treated STZ-induced diabetes showed decreased caspase 9 activation
G=VitE shows no expression
a*= Statistically different from normal rat b*= Statistically different from diabetic rat

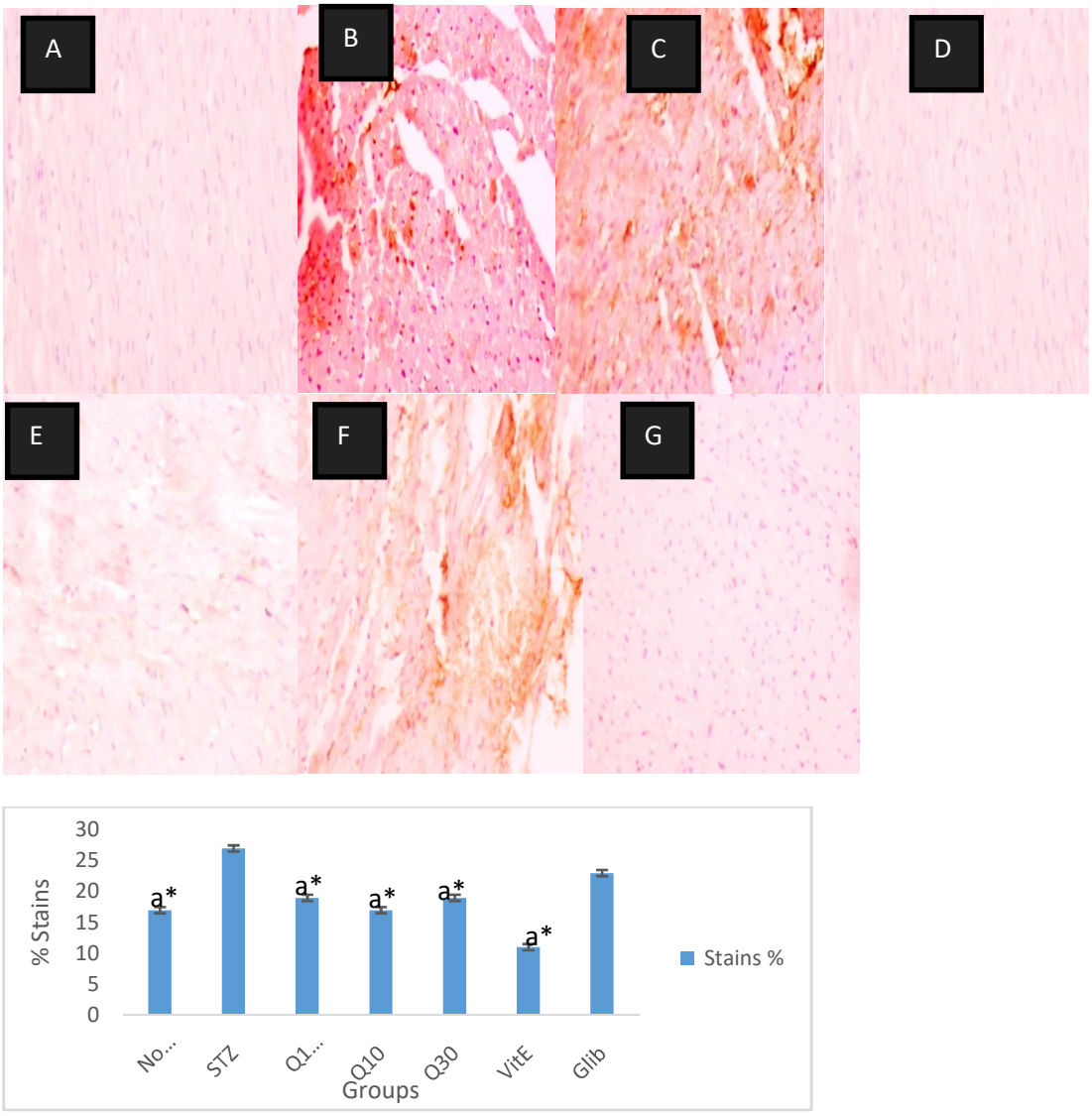


Figure 44: Caspases 3 activation in STZ-induced diabetic rat heart and treatment with co-administration of quercetin and vitamin E, varying concentration of quercetin, vitamin E and glibenclamide

A=Normal rat heart showed expression of caspase 3
B= STZ-induced diabetes showed increased caspase 9 activation
C=Glibenclamide treated STZ-induced diabetes showed expression of caspase 3
D=Q10 treated STZ-induced diabetes showed expression of caspase 3
E= Q30 treated STZ-induced diabetes showed decreased caspase 3 activation
F= Q10&VitE treated STZ-induced diabetes showed decreased caspase 3 activation
G=VitE shows no decreased caspase 3 activation
a*= Statistically different from normal rat b*= Statistically different from diabetic rat

EXPERIMENT 19: ASSESSMENT OF SPERM VIABILITY, CYTOCHROME C RELEASE, CASPASE 3 AND 9ACTIVATION IN STZ-INDUCED DIABETIC RAT TESTES AND TREATMENT WITH CO-ADMINISTRATION OF QUERCETIN AND VITAMIN E, VARYING CONCENTRATION OF QUERCETIN, VITAMIN E AND GLIBENCLAMIDE

Introduction

Studies have shown the prevalence of infertility in male patients with DM, resulting in poor quality of life (Condorelli, *et al.,* 2018). Diabetes infulences sperm DNA repair gene, it causes mitochondrial respiratory chain dysfunction and excessive DNA fragmentation (Condorelli, *et al.,* 2018). It has been shown that the plasma membrane of the spermatozoa is sensitive to plasmatic insulin level, therefore, decrease in its level through resistance/deficiency would probably decrease spermatogenesis. This could potentially cause infertility, however, literatures have shown that these facts are still controversial (Petroianu *et al.,* 2009). Excessive apoptosis has also been implicated in the testes of diabetic patients (Condorelli *et al.,* 2018), with the increase in the level of Cyt C release. Under normal physiological condition, apoptosis in the testes prevents multiplicity of spermatozoa with damaged DNA, regulating spermatogenesis (Liu *et al.,*2006). However, excessive Cyt C release in the testes will lead to decreased fertility, since viable spermatozoa would be destroyed and eventually causing cell death(Liu *et al.,*2006). The aim of the study therefore was to determine if treatment with certain antioxidants would influence sperm quality and inhibit germ cell apoptosis in STZ-induced diabetic rats.

Procedure

Rats were sacrificed by cervical dislocation, testes were removed and properly fixed in bouin's reagent. Sectioning was carried out using a microtome at the range of 4-40 µm. The slices were mounted on slides, then dehydrated using alcohol washes of increasing concentration and cleared using xylene as detergent. Cells were harvested and treated with 100 µL digitonin (50 µg/ml in PBS with 100 mM KCl) for 5 minutes on ice (until >95% were permeabilized as assessed by trypan blue exclusion). Fixation was done according to the methods described in chapter three (page 74). Stained cells were then viewed by microscopy.

Results

The effects of treatment of diabetic rats with antioxidants and glibenclamideon sperm analysis ispresented in Figure 45 and 46. The results obtained showed that there was a significant difference between the sperm motility and count of STZ-induced diabetic rat testes, when compared with the control, however, treatment of diabetic rats with Q10&VitE, Q10, Q30, VitE and glibenclamide, showed an increase in the sperm motility and count, comparing positively with the normal rats.

Result in Figure 47 showed that there was increased Cyt C releasein STZ-induced diabetic rat testes relative to normal control rats. Treatment with Q10&VitE, Q10, Q30, VitE and glibenclamide showed decreased Cyt C release relative to STZ-induced diabetic rats ($p>0.05$). In Figure 48, result showed that treatment with Q10&VitE, Q30, VitE showed decreased caspase 9 activation ($p>0.05$). However, Q10 and glibenclamide treatment did not show any decrease in caspase 9 activation relative to STZ-induced diabetic rats

(p>0.05). InFigure 49, there was increased caspase 3 activation in STZ-induced diabetic rat testes. Treatment with Q10&VitE, Q30 and VitE (p>0.05) showed a distinct decrease in this activity, however, Q10 and glibenclamide treatment did not decrease caspase 3 activation in STZ-induced diabetic rats (P>0.05).

Conclusion

Treatment with all the antioxidants and glibenclamide increased the sperm output in diabetic rats. Similarly, all the treatment groups decreased Cyt C release in STZ-induced diabetic rat, while caspase 9 activation was decreased by Q10&VitE, Q30 and VitE treatment. In caspase 3 activation, Q10&VitE, Q30 and VitE treatment decreased STZ-induced caspase 3 activation than glibenclamide.

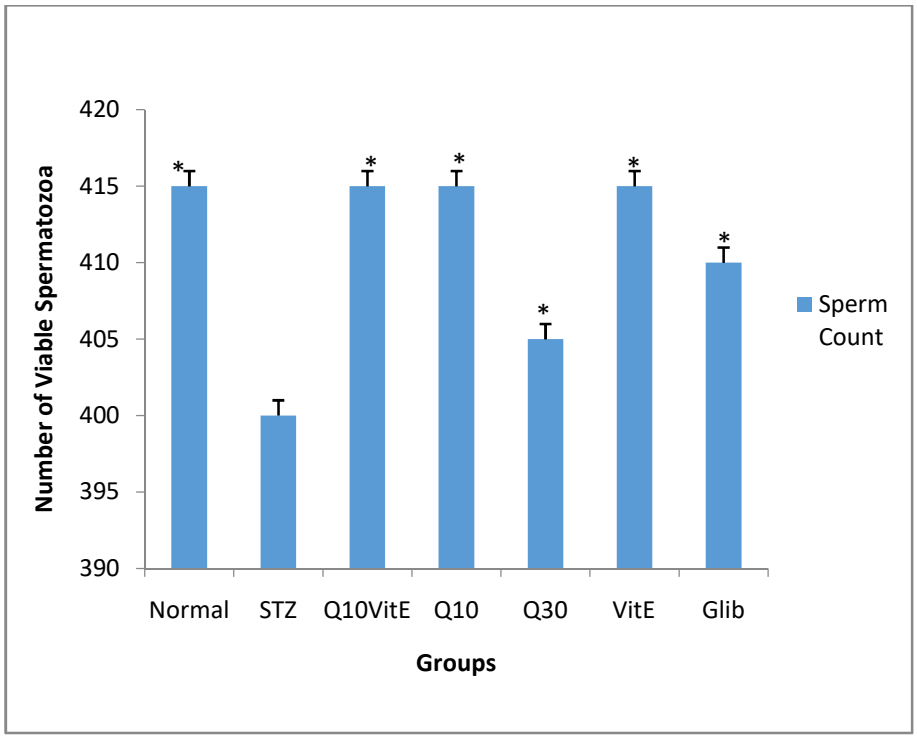


Figure 45: Sperm count in STZ-induced diabetic rats and treatment with varying doses of quercetin, co-administration of quercetin and vitamin E, glibenclamide and vitamin E

Normal rat
STZ= STZ-induced diabetic rats with reduced viable spermatozoa
Q10&VitE=STZ-induced diabetic rats treated with co-administration of quercetin and vitamin E
Glib= STZ-induced diabetic rats treated with glibenclamide
Q10= STZ-induced diabetic rats treated with 10 mg/kg quercetin
Q30= STZ-induced diabetic rats treated with 30 mg/kg quercetin
VitE= STZ-induced diabetic rats treated with 10 mg/kg vitamin E
*= Statistically different from normal rat

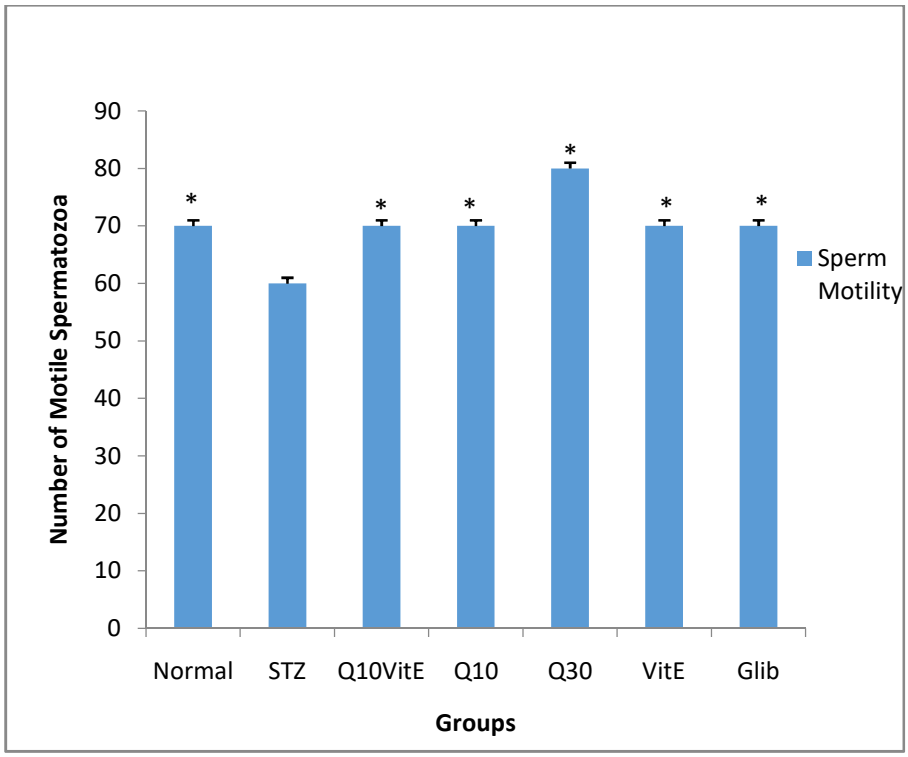


Figure 46: Sperm motility in STZ-induced diabetic rats and treatment with varying doses of quercetin, co-administration of quercetin and vitamin E, glibenclamide and vitamin E

Normal rat
STZ= STZ-induced diabetic rats with reduced viable spermatozoa
Q10&VitE=STZ-induced diabetic rats treated with co-administration of quercetin and vitamin E
Glib= STZ-induced diabetic rats treated with glibenclamide
Q10= STZ-induced diabetic rats treated with 10 mg/kg quercetin
Q30= STZ-induced diabetic rats treated with 30 mg/kg quercetin
VitE= STZ-induced diabetic rats treated with 10 mg/kg vitamin E
*= Statistically different from normal rat

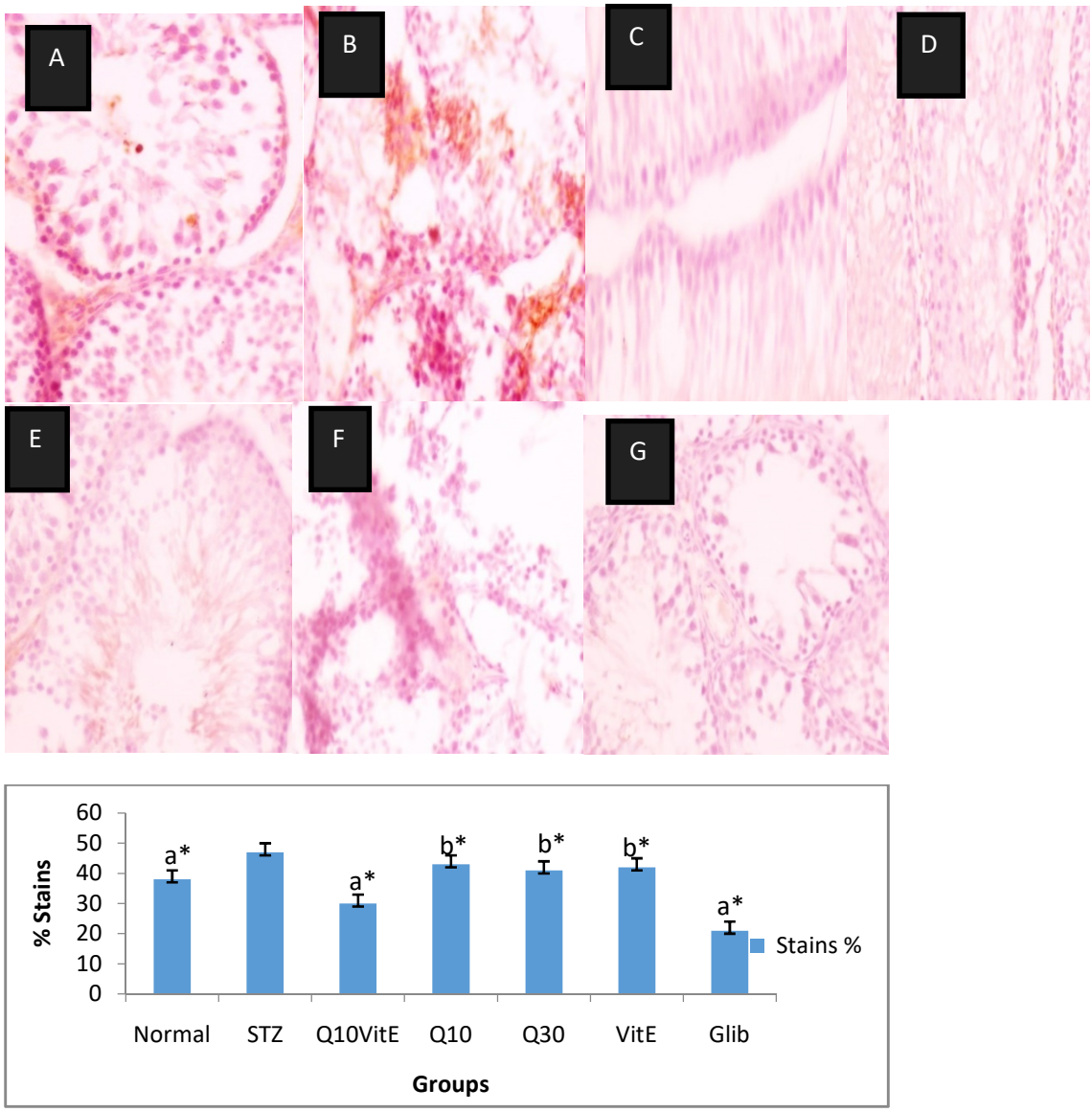
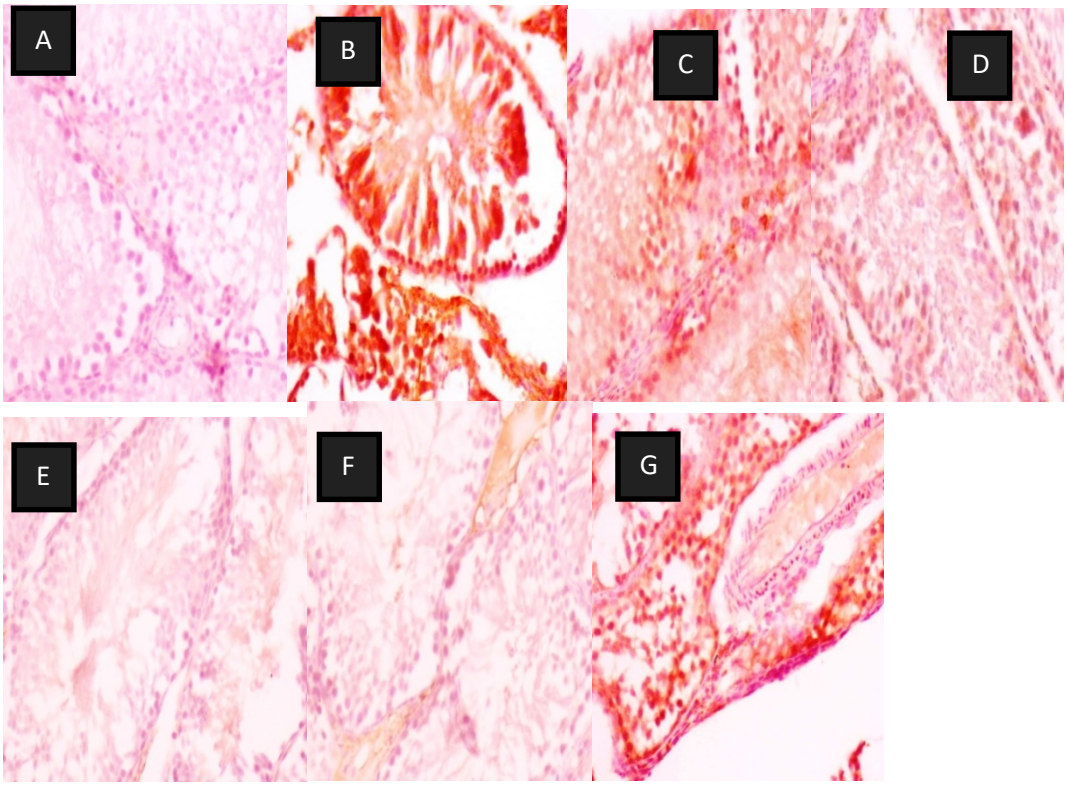


Figure 47: Cytochrome C release in STZ-induced diabetic rat testes and treatment with co-administration of quercetin and vitamin E, varying concentration of quercetin, vitamin E and glibenclamide

A=Normal rat testes showed release of Cyt C
B= STZ-induced diabetes showed increased Cyt C release
C=Glibenclamide treated STZ-induced diabetes showed decreased Cyt C release
D=Q10 treated STZ-induced diabetes showeCyt C release
E= Q30 treated STZ-induced diabetes showed decreased Cyt C release
F= Q10&VitE treated STZ-induced diabetes showed decreased Cyt C release
G=VitE shows decreased Cyt C release
a*= Statistically different from normal rat b*= Statistically different from diabetic rat



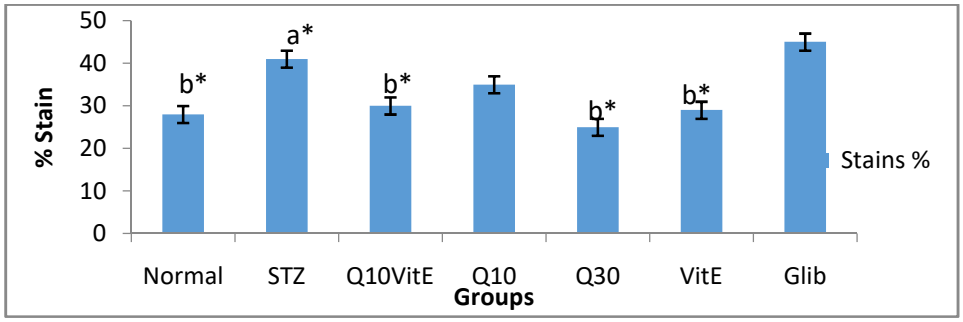
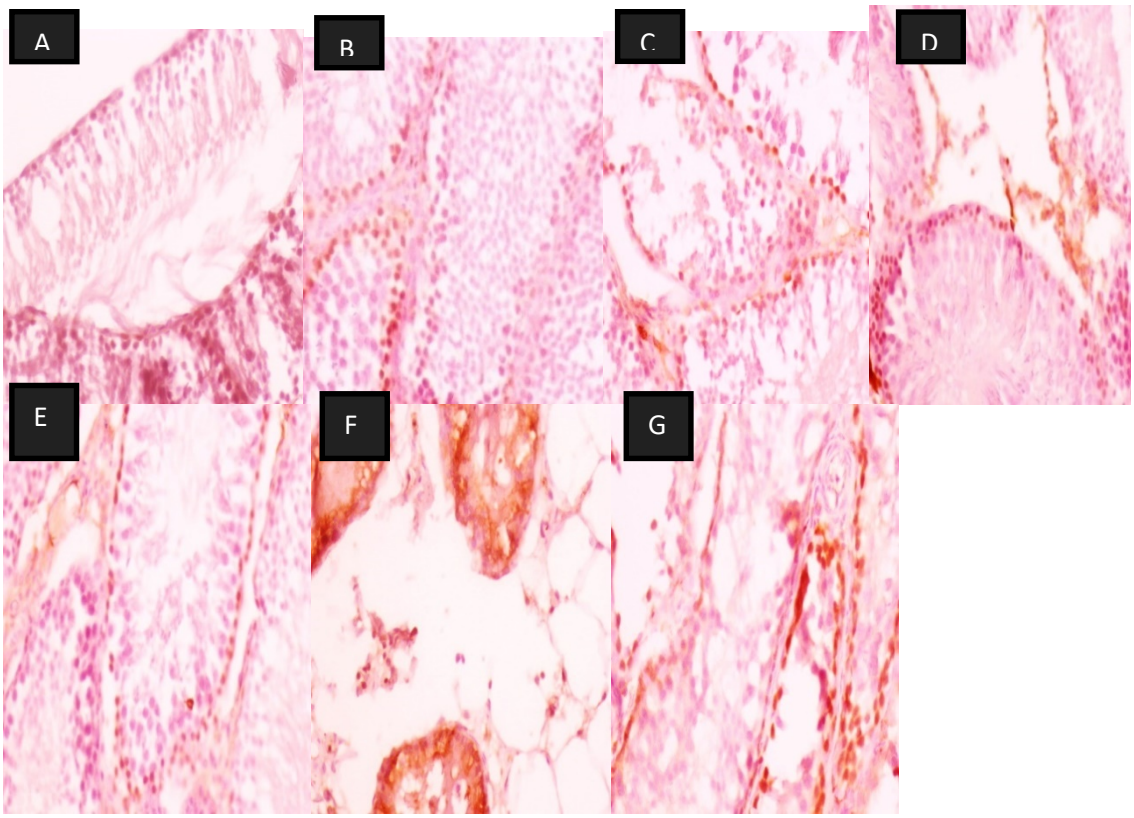


Fig 48: Caspase 9 activation in STZ-induced diabetic rat testes and treatment with co-administration of quercetin and vitamin E, varying concentration of quercetin, vitamin E and glibenclamide

A=Normal rat testes showed expression of caspase 9
B= STZ-induced diabetes showed increased caspase 9 activation
C=Glibenclamide treated STZ-induced diabetes showed decreased caspase 9 activation
D=Q10 treated STZ-induced diabetes showed caspase 9 activation
E= Q30 treated STZ-induced diabetes showed decreased caspase 9 activation
F= Q10&VitE treated STZ-induced diabetes showed decreased caspase 9 activation
G=VitE showed caspase 9 activation
a*= Statistically different from normal rat b*= Statistically different from diabetic rat



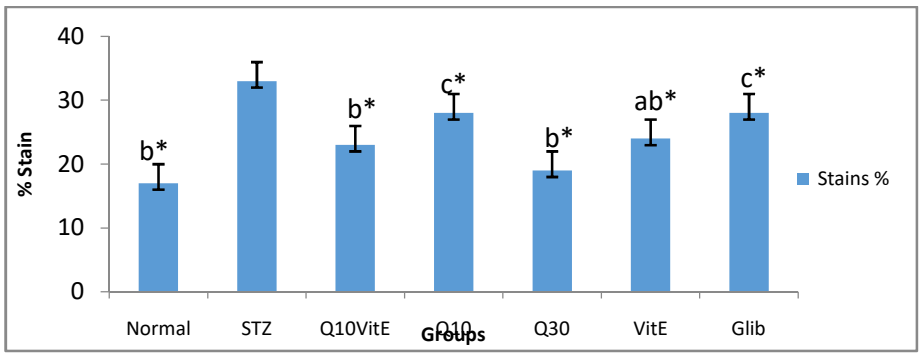


Figure 49: Caspase 3 activation in the testes of STZ-induced diabetic rats and treatment with co-administration of quercetin and vitamin E, vitamin E, varying concentration of quercetin and glibenclamide

A=Normal rat testes showed expression of caspase 9
B= STZ-induced diabetes showed increased caspase 9 activation
C=Glibenclamide treated STZ-induced diabetes showed caspase 9 activation
D=Q10 treated STZ-induced diabetes showed caspase 9 activation
E= Q30 treated STZ-induced diabetes showed decreased caspase 9 activation
F= Q10&VitE treated STZ-induced diabetes showed decreased caspase 9 activation
G=VitE showed decreased caspase 9 activation
a*= Statistically different from normal rat b*= Statistically different from diabetic rat

EXPERIMENT 20: HISTOPATHOLOGICAL ASSESSMENT OF STZ-INDUCED DIABETIC RAT LIVER, HEART, TESTES AND TREATMENT WITH CO-ADMINISTRATION OF QUERCETIN AND VITAMIN E, VARYING CONCENTRATION OF QUERCETIN, VITAMIN E AND GLIBENCLAMIDE

Introduction

Histological examination is a standard for which cellular injuries are monitored, it is a yardstick through which experiment and clinical algorithms are matched and results validated (Brunt and Tiniakos, 2010).

Procedure

Induced diabetic rats and the treated ones were sacrificed by cervical dislocation, liver specimen obtained were fixed in 10% formalin, and subsequently put in paraffin before cutting with microtome. Dehydration by varying concentration of alcohol wash was carried oute and clearing by xylene detergent. These were then placed in paraffin wax, after deparaffinization and dehydration, the paraffin blocks were stained with hematoxylin, washed and embedded in acid alcohol to remove the excess stain and then counter stained by 10% eosin before microscopic examination.

Results

The result presented in Figure 50 showed no visible lesions in normal rat liver sections while rats exposed to STZ showed presence of lesions. Diabetic rats treated with glibenclamide showed congestion of vessels and Q10 treatment showed no visible lesions. Furthermore, diabetic rats treated with Q30 showed very mild periportal infiltration by inflammatory cells. Similarly, Q10&VitE treatment showed mild to moderate disseminated

periportal infiltration by inflammatory cells, while VitE treatment showed mild disseminated steatosis.

In Figure 51, congestion of coronary vessels was observed in normal heart while haemophargic lesions and congestion of coronary vessels were seen in STZ-induced diabetic rats. Treatment with Q10&VitE and glibenclamide showed focal area of inflammation involving the myocardium and pericardium. While treatment with Q10, Q30and VitE showed areas of mild inflammation and haemophagic lesions respectively. Results presented in Figure 52 showed that there was focal area of congested vessels in normal rats seminiferous tubules whose epithelium show maturation arrest with absence of terminally differentiated cells was observed in STZ-induced diabetic rats. Treatment with glibenclamide and VitE showed no visible lesions while Q10 animals showed seminiferous tubules which consisted of spermatocytes, spermatids, spermatozoa and sertoli cells. Treatment with Q30 showed active cell division and maturation of the germ cells as evidenced in abundance of terminally differentiated cells. Conclusively, treatment with Q10&VitE showed that the leydig cells were normal and the attached epididymis has abundant spermatozoa.

Conclusion

The results showed that the lesions presented by diabetic conditions iliver, heart and testes have been reduced in all the treatment groups. In the testes, it was observed that antioxidants treatment improved the integrity of the testes and completely abolished the observed lesions. It was also noted that glibenclamide was only able to abrogate the presence of the lesions without improving the integrity of the testes. Findings form the study, presented the antioxidants as preferable options to glibenclamide.

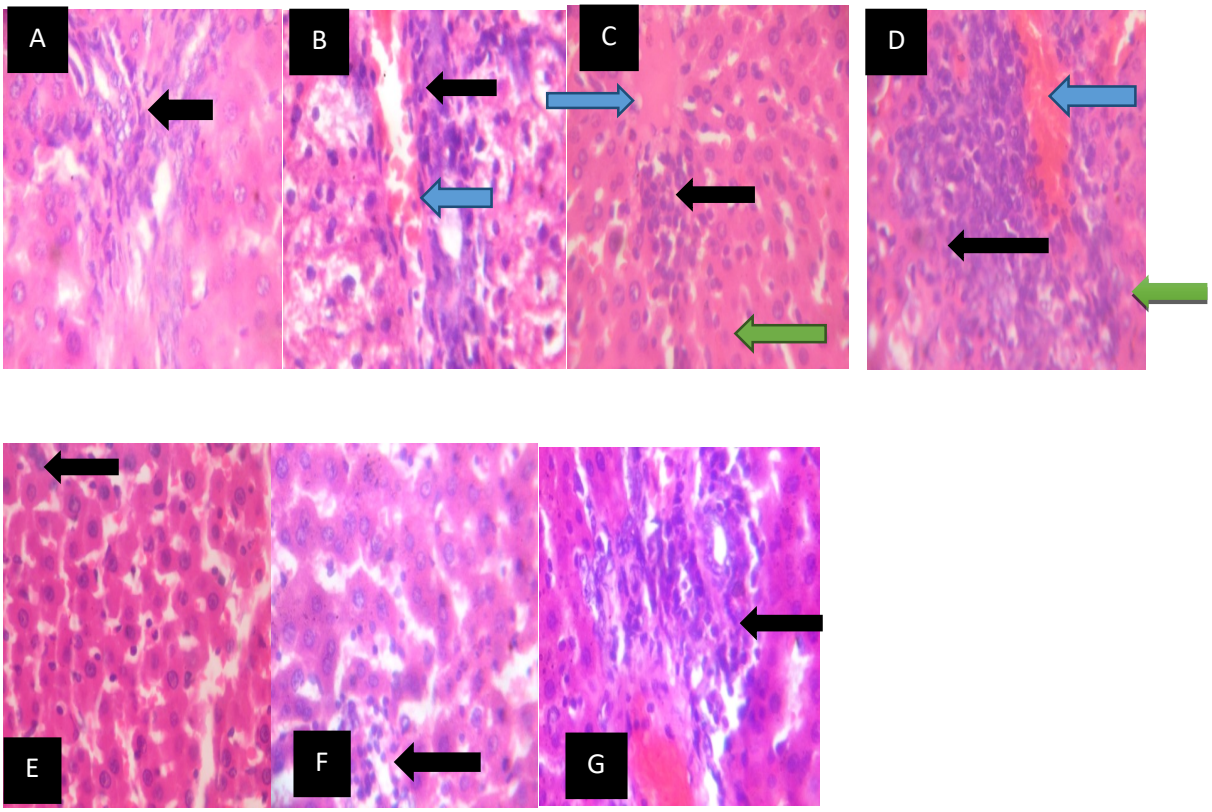


Figure 50:Histopathological assessment of STZ-induced diabetic rat liver and treatment with co-administration of quercetin and vitamin E, varying concentration of quercetin, vitamin E and glibenclamide

Black arrow showed periportal infiltration by inflammatory cells, blue arrow showed congestion of vessels and sinusoid, green arrow showed marked disseminated microvesicular steatosis and black arrow showed focal area of mild periportal infiltration by inflammatory cells.

A=Normal rat liver showed no visible lesion
B= STZ-induced diabetes showed lesions
C=Glibenclamide treated STZ-induced diabetes showed congestion of vessel and sinusoid expression decreased caspase 9 activation
D=Q10 treated STZ-induced diabetes showed no lesions
E= Q30 treated STZ-induced diabetes showed very mild periportal infiltration by inflammatory cells
F= Q10&VitE treated STZ-induced diabetes showed shows mild to moderate disseminated periportal infiltration by inflammatory cells
G=VitE showed shows mild disseminated steatosis.

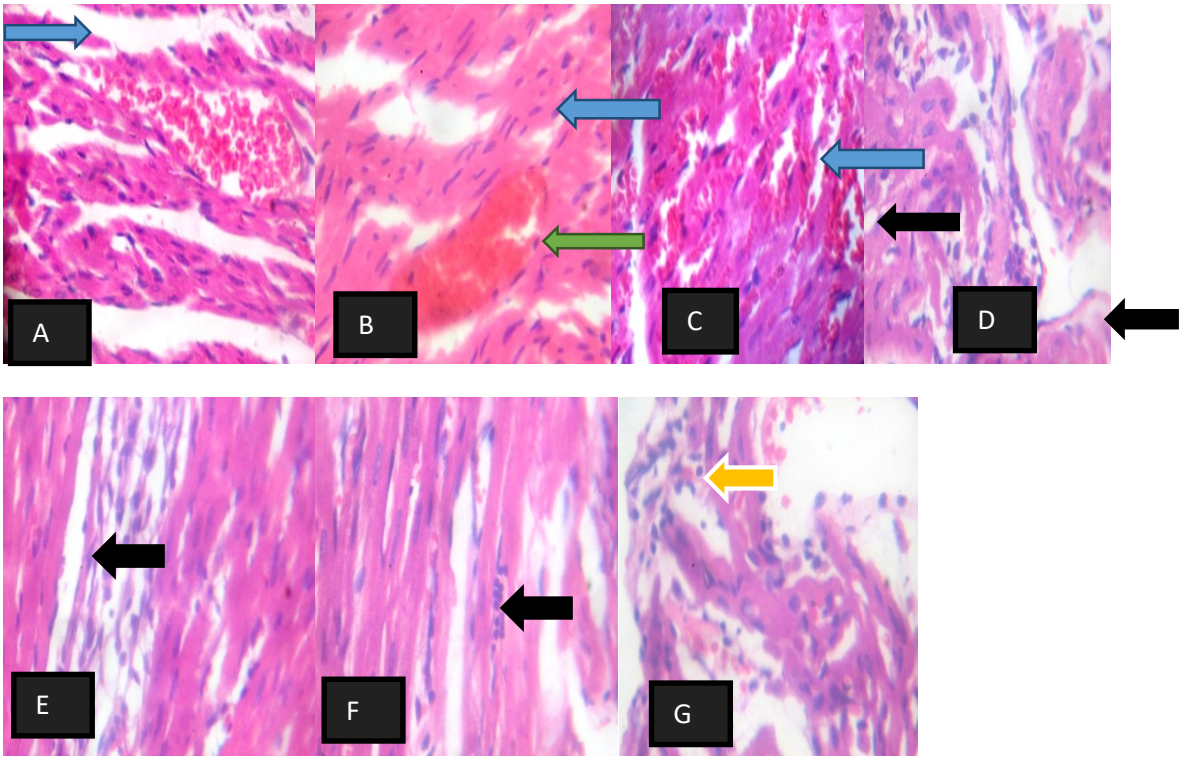


Figure 51: Histological assessment of STZ-induced diabetes rat heart and treatment with co-administration of quercetin, varying concentration of quercetin, vitamin E and glibenclamide

Blue arrow showed congestion of coronary vessels, green arrow showed haemorrhagic lesion, black arrow showed focal area of inflammation and orange arrow showed multi-focal areas of moderate inflammation involving the myocardium and pericardium

A=Normal heart showed no lesions B= STZ-induced diabetic rats showed haemophargic lesions and congestion of coronary vessels
C= Glibenclamide treated STZ-induced diabetes showed focal area of inflammation

D= Q10 treated STZ-induced diabetes showed mild inflammation E= Q30 treated STZ-induced diabetes showed haemophargic lesion

F= Q10&VitE treated STZ-induced diabetes showed multi-focal area of moderate inflammation
G=VitE treated STZ-induced diabetes showed haemophargic lesion.

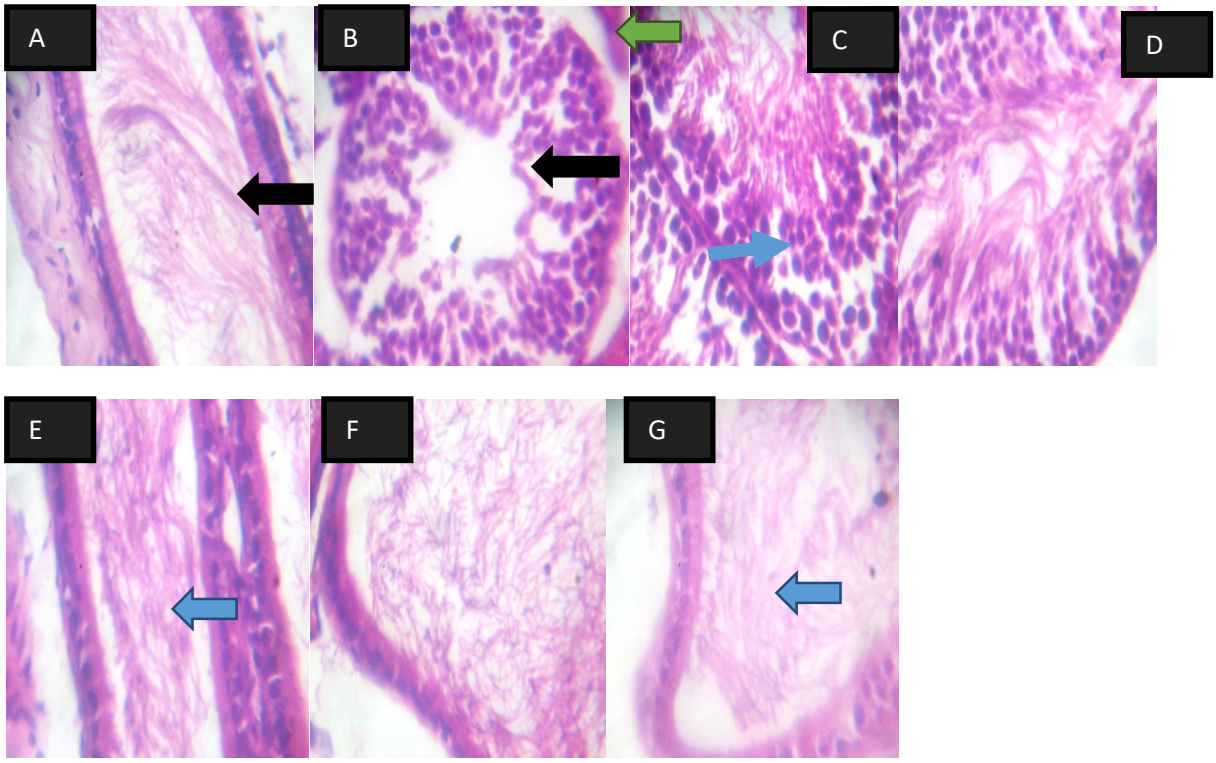


Figure 52: Histological assessment of STZ-induced diabetes rat testes and treatment with co-administration of quercetin, varying concentration of quercetin, vitamin E and glibenclamide

Normal control plates showed that the seminiferous epithelium consists of spermotogonia, spermatocytes, spermatids, spermatozoa and sertoli cells, while the interstitia have the leydig cells. There is active cell division and maturation of the germ cells and the leydig cells are normal(blue arrow). STZ seminiferous epithelium show maturation arrest with absence of terminally differentiared cells (black arrows), there is a focal area of congested vessels (green arrows).

A=Normal rat testes showed no visible lesions
B= STZ-induced diabetic rats showed the absence of terminally differentiated cell with attached epididymis having no spermatozoa.
C=Glibenclamide treated diabetes showed no visible lesions
D=Q10 treated diabetic rats showed seminiferous tubules with spermotogonia, spermatocytes, spermatids, spermatozoa and sertoli cells.
G=VitE treated diabetic rats showed no visible lesions
E= Q30 treated diabetic rats showed active cell division
F=Q10&VitE showed normal leydig with the attached epididymis having abundant spermatozoa

DISCUSSION

Diabetes-induced complication is a major cause of morbidity and mortality. It is also a cause of cellular stress-induced apoptosis (Wu *et al.,*2016).Studies have shown that about 382 million people worldwide are suffering from the disease and the number is expected to escalate to about 592 million in 2035 (IDF, 2013; Ojeabu *et al.,* 2017). Due to the growing burden of the disease, there is huge implication on the world socio-economic status, particularly in Africa with a known staggering economy. Reports have shown that diabetes would affect about 23.9 million people in Africa by 2030 (Whiting *et al.,* 2011; Ojeabu *et al.,* 2017). Sadly, Nigeria has the highest-burden and occurrence of diabetes in Africa, with about 2 million cases undiagnosed (Oputa *et al.,* 2015). Of all cases of diabetes reported in Nigeria, T2DM has about 95% occurrence with the prevalence more in men than women (Oputa *et al.,* 2015). Studies have shown that despite available therapies for T2DM, recommended targets are not obtained in patients, in fact, they are left at higher risk of complications (Maurico *et al.,* 2017; Goldenberg and Steen, 2018). Survey report in Canada has shown that only 50% of the studied population of patients with T2DM given anti-diabetic drugs had controlled glycated Hb target (Goldenberg and Steen, 2018). Furthermore, studies by Kasznicki and Drzewiski (2014) have shown that therapies like sulfonyurea, gliptins or glitazones increase the risk of heart failure, hence should be assessed with care. Report from the United States showed that treatment of diabetic patients with drugs met the glycated Hb level target by <7% between 2011-2014 (Goldenberg and Steen, 2018). Consequently, there is a current paradigm shift in the approach of diabetes treatment to targeting the molecular features of the disease with antioxidants, since synthetic drugs have not been able to completely alter the course of the

disease (Siddiqui *et al.,* 2013). Dietary antioxidants that may reduce the initiation of radicals that causes/maintains pancreatic damage responsible for all kinds of diabetes are been investigated. Diabetes is a metabolic assembly of disorders characterised by hyperglycemia, hyperlipidemia, cardiomyopathies, nephropathies, testicular dysfunction, and oxidative stress among other complications. A peculiar feature of all these complications is the upregulation of apoptosis. Apoptosis is a normal physiological event that aids the development and well-being of organisms (Bernardi and Di Lisa*,* 2015). Upregulation of this process has been shown in numerous diseases including diabetes. In diabetes, destruction of pancreas by apoptosis causes elevation of blood glucose among other complications (Gong *et al.,* 2011). The mitochondrion performs a central function in the apoptotic process, and hence directly important in excessive apoptosis associated with T2DM and the attendant possible tissue wastage.While antioxidants, like quercetin and vitamin E, have been shown to be useful in the delay of progression in diabetes-induced complications,however, the influence of these agents on T2DM-associated mitochondrial-mediated apoptotic process is unknown.

Diverse mechanisms have been proposed and proven to be accountable for different antioxidants role in diabetes (El-Abhar and Schaalan, 2014; Mishra *et al.,*2015), however, more search for molecules that are able to better regulate the progression of complications from the disease is on the rise. Studies have shown the relationship between diabetes and apoptosis (Krijnen *et al.,*2009); and approaches aimed at intercepting the conserved apoptotic pathway is currently being adjudged as an innovative blue-print in developing molecular based anti-diabetic agents (Tudzarova and Osman, 2015).

Several studies have reported detailed results on the antioxidant, anti-inflammatory, anti-cardiotoxic, anti-hepatotoxic properties of quercetin and vitamin E in various disease prototypes including diabetes (Aggarwal *et al.,*2010; Keklikoglu and Akinci, 2013; Mahmoud *et al.,* 2013). However, the molecular-mechanisms accountable for the cardio-, hepato- and testiculo-protective effects in diabetes have not been well-deciphered. Decreased glucose utilization due to insulin resistance, as well as diminished glucose transporter content, is a risk factor in the heart of diabetic patients; in this study, it was shown that blood glucose level, a key marker in diabetes pathophysiology was elevated probably due to pancreatic β-cell apoptosis. This findings is in consonance with the studies of Behl *et al.* (2015). Reports have shown that elevated blood glucose result in generation of reactive species by well-defined mechanism (Giacco and Brownlee, 2010; Mapanga and Essop, 2015). The study confirmed the non-inferiority of Q10, Q10&VitE treatment to glibenclamide in reducing blood glucose level. Similarly, in the study, we observed that the body weight reduction in diabetic rats treated with Q10&VitE clearly showed similarity with those treated with glibenclamide. This could have acted by increasing insulin production attributable in part to mild regeneration of the pancreas as reported by Kim and colleagues (Kim *et al.,*2008) and or suppression of gluconeogenesis thereby reversing diabetes-induced tissue wastage. Several reports have shown increased triglyceride and cholesterol in diabetes dyslipidemia which could cause cardiovascular diseases (Bitzur *et al.,* 2009; Daniel, 2011). This may make the heart cells susceptible to toxicity. Several studies have shown that 65% of people with DM have associated CVD and may die of the disease but the mechanism of action is yet to be elucidated (Qazi and Malik, 2013; Low Wang *et al.,* 2016). However, the involvement of mitochondria in this event has been

implicated (Nafaji *et al.,* 2014). Mitochondrion is an important organelle in the decision of life or death of an organism; disruption in the integrity of this organelle has been identified as the origin of the complications arising from diabetes. The altered mitochondrial function leads to mPT pore opening, a crucial occurrence in cell death activation programs. This makes the pore a possible innovative target that would be able to influence the process of diseases if well manipulated (Bernardi and Lisa, 2015).

Compelling evidences have shown that antioxidants are important agents in attenuating diabetes-induced cytotoxicity; they have the unique capability of protecting the mitochondria from damage in pathological conditions. Generally antioxidants demonstrate protective capabilities in the mitochondria, however they are dose dependent and there is lack of scientific proof to validate the rationale for safety dose regimen *in vivo*. This may explain in part their failure sometimes in crossing the clinical trial hurdle (Nunez-Cordoba and Martinez-Gonzalez, 2011). This background information, stimulated further investigations on the role of quercetin and vitamin E as possible anti-diabetogenic agent with specific interest on their inhibitory role in mitochondria-mediated apoptosis, a hallmark of all kinds of diabetes.

One of the commonly used methods in measuring mPT involves the addition of Ca^{2+} to isolated mitochondria, in the study, the mitochondrial mPT pore of mitochondria isolated from the hearts of normal rats were assessed by measuring the loss of absorbance of the mitochondrial suspension by spectrophotometry (Javadov *et al.,*2009, Lapidus and Sokolove, 1993). Employing this method, it has been shown that the mitochondria suspension preparations used in the study were susceptible to induction due to

Ca^{2+}challenge and like the normal intact mitochondria (Sokolove and Lapidus, 1993), the pore opening was reversed by spermine, a known standard inhibitor of the pore opening (Lapidus and Sokolove, 1993). This observation confirmed that the mitochondria were intact and not uncoupled and therefore suitable for further use.

In the diabetic rat heart mitochondria, there was swelling of the isolated mitochondrial that may be due to ROS generation by hyperglycemia. The process of this event occurs due to glucose toxicity that translated to depletion of glutathione/NADPH in polyol pathway, a process that converts glucose to sorbitol. Similarly, hyperglycemia-induced activation of PKC pathway/increase hexosamine flux among others may be the source of ROS in glucose toxicity (Giacco and Brownlee, 2010). Since mitochondria have been shown to play crucial roles in human health; triggering the release of cell death effectors which are fundamental for cell survival, it would not be surprising that the mPT pore performs a pivotal regulation in intrinsic apoptosis. Therefore the events leading to the induction of the pore in diabetes may possibly increase the Bax/Bcl 2 ratio thereby favouring the assemblage of the pore components like cyclophillin D, VDAC or ANT (Elrod *et al.,* 2013). For the first time, this study showed that treatment with Q10&VitE reversed the triggered mPT pore opening than the varying concentration of quercetin and vitamin E considered in the study, while administration of glibenclamide exhibited the least reversal effect. These effects by the antioxidants suggest that they may have replenished the endogenous antioxidant stores thereby decreasing the possibility of oxidative stress. Similarly, the antioxidants may have interacted with the main pore components by inhibiting ROS, pH, P*i* or Ca^{2+} thereby prevented their inductive activity. Thus the mPT

pore opening reversal effects of the antioxidants may be the reason for their cardio-protective role.

Studies have shown the relationship between the mPT pore and the mitochondrial ATPase, proving that it may be a requirement for the pore formation (Alvian *et al.,*2014, Bonora *et al.,*2014, Giorgio *et al.,*2013) Dietze and colleagues explained that an intact mitochondrial coupling membrane is the criterion for the proton-motive force required by ATP synthesis (Dietze *et al.,*2001), therefore the direction of ATP synthase is reversed when there is mitochondrial-membrane potential uncoupling. ATP synthase performs a bifunctional role of hydrolyzing and synthesizing ATP, thereby modulating the concentration of cytosoli P*i* concentration, a known inducer of the pore opening. In this study, to ascertain the participatory role of the mATPase activity in mPT pore opening, effects of antioxidants and glibenclamide were investigated on ATPase activity. The enzyme was proven for the first time in this study to be elevated in diabetes, suggesting excessive ATP hydrolysis and consequent mitochondrial dysfunction. The data gathered in the study showed that co-administration of quercetin and vitamin E reduced the enhanced mATPase activity better than varying concentration of quercetin and vitamin E. In this regard, the levels of ATPase activity shown by the antioxidant-treated groups were similar to that observed in normal rat. These reports support our previous findings that the antioxidants were able to reverse the opening of the heart mPT pore observed in STZ-induced diabetes. This report is also consistent with the suggestions of earlier researchers that said that the mPT pore opening may potentiates the uncoupling of oxidative phosphorylation (Bonora *et al.,* 2013; Bernardi and Di Lisa, 2015). Taken together, the assumption that mATPase is required for mPT formation (Alvian *et al.,*2014, Bonora *et al.,*2013, Giogio *et al.,*2013, Pucci *et al.,*2008,

Shchepina *et al.,*2002) can be upheld carving a new pathway for its molecular relevance in pathophysiology. Furthermore in the study, treatment with quercetin and vitamin E reduced the activity of ATPase more than the reference drug, this showed that treatment could maintain normal ATPase activity in diabetes. We therefore hypothesised, that the antioxidants may possibly have directly interacted with the enzyme complex mimicking the inhibitory activities of ADP, IF1, Mg^{2+},oligomycin and/or CsA which dissociate Cyp D from the complex. This is similar to the suggestion of Bonora and colleagues (2014), who proposed that oligomycin inhibits the activity of the enzyme by binding to F_0 subunit thereby inhibiting mPT pore opening (Bonora *et al.,*2014). Rasola and Bernardi also proposed that the lateral stalk of ATPase has Cyp D bound to it and the influence is ameliorated by CsA binding (Rasola and Bernardi, 2014). The study showed that attenuation of cytosolic P*i* concentration by treatment groups to almost non-diabetic levels caused reversal of the mPT pore opening, attributable to ischemia reperfusion injury in the heart. It has been shown that cardiac injury occurs in diabetic condition in both animal and human (Sloan *et al.,* 2012). The study further showed that any procedure that would inhibit the mPT pore opening in diabetic heart may at least in part attenuate cardiac injury (Sloan *et al.,* 2012). Another school of thought also stated that prevention of ATP depletion during ischemia reperfusion injury may cause inhibition of ATP synthase activity (Murphy and Steenbergen, 2008b). Premise on this background, we may conclude that the antioxidants prevented ATP depletion in diabetes-induced ischemia reperfusion injury than glibenclamide. This outcome is consistent with the result obtained in rat heart mPT pore opening. This suggests that co-administration of quercetin and vitamin E, vitamin E and varying concentration of quercetin are better alternatives to glibenclamide. While this is the

first finding to show that mATPase is hydrolysed in diabetes with the antioxidants reversing the effect and concluding that this validates mPT pore status, the investigators understand that the protein components that form the pore are still being debated (Sloan *et al.,* 2012).

Taken together, it is likely that modulating the activity of ATPase may be important not just in oxidative phosphorylation, but also in alleviating the complications arising from the mitochondrial pore opening of the diabetic rat heart.

The mitochondria are considered as location of ROS generation and this is a major player in peroxidation of lipid involved in several pathological conditions. The ROS produced forms lipoperoxyl radical which yields hydroperoxide and radical of lipids that are quite unstable therefore produce new peroxyl and alkoxyl radicals which finally form secondary products that increases the fluidity of membrane by reacting with the phospholipid bilayers of the membrane. This affect the permeability of the membrane hence alters the cellular structure (Barrera, 2012). With the knowledge of ROS as volatile oxidants that are the basis of many pathological conditions, it became apparent to determine if antioxidants that have shown promising possibilities in reducing oxidative stress would be able to ameliorate and inhibit mitochondrial lipid peroxidation as a probable mechanism of alleviating complications promoting mitochondria dysfunction (Yadav *et al.,* 2016).

The heart has been shown to accumulate lipid peroxide during oxidative stress because there is no cardiomyocytic turnover (Foglia and Poss, 2016). Studies have also revealed that mitochondria are prime target and source of lipid peroxides predominant in diseases like ischemia reperfusion injury, heart failure and diabetes (Foglia and Poss, 2016). This

could be the reason while mitochondrial cardiolipin that makes up 18% of the total phospholipids have their unsaturated fatty acyl groups oxidized by lipid peroxidation. This event compromises the integrity of the mitochondrial membrane, causes opening of the mitochondrial inner membrane, swelling and burst of the outer membrane to enable efflux of cytochrome c and this eventually causes cell death (Navarro and Boveris, 2009). Our findings in the study showed that treatment with Q10&VitE, Q10 and 30 administered scavenged free radicals capable of causing mitochondrial lipid peroxidation, with higher effects from Q10. This event inhibited the alteration of the membrane phospholipid contents by ROS necessary for Cyt C efflux, a point of no return for cell death. Similarly vitamin E and glibenclamide also inhibited the generation of lipid peroxides in STZ-induced diabetic rats, however, not as prominent as Q10 and 30 or Q10&VitE.

Studies have shown that prolonged mPT pore opening due to various stress stimuli can activate the well-conserved factors of the intrinsic apoptotic pathway, such as Cyt C, Apaf-1, caspases 3 and 9 (Huttemann *et al.,* 2011). The Cyt C release is a critical event in apoptosis (Huttemann *et al.,* 2011). It initiates the rapid recruitment of Apaf-1 and apoptosome, which recruits procaspase 9 and this subsequently activate procaspase 3. Activation of this intrinsic apoptotic factors lead to increased DNA fragmentation and eventual cell death (Bressenot *et al.,* 2009). Despite current mPT concept evolution, elaborate evidences have shown that it's uncoupling is accountable for Cyt C release which strategically initiates cell demise via apoptosis (Suh *et al.,* 2013). The mPT pore finalizes the fate of a cell; hence its activation stimulates apoptosis. In the study, it was shown that diabetes-induced mPT pore opening was reversed by all treatment groups, we therefore hypothesised that these treatment may influence Cyt C release. Since it is a fact that Cyt C

release from the mitochondrial is an irreversible point in the apoptotic process, decreased Cyt C levels observed in diabetic rats administered the antioxidants may explain the reversal of diabetes-induced mPT pore opening. This result showed that the increase Cyt C release following STZ-induced diabetes induction was decreased by treatment with Q10&VitE, Q30, VitE and glibenclamide. Reports by Sloan and colleagues (2012) have shown that in the diabetic heart, enhanced mPT pore opening is due to reperfusion injury because redox change occurs when the ROS burden is high (Sloan *et al.,* 2012). In this study, diabetic heart mitochondrial pore was opened, this caused increased mLPO as assessed by mutagenic MDA level thereby causing reperfusion injury evidenced by Cyt C release. Apoptogenic Cyt C release is a decisive point for caspase activation in intrinsic apoptosis.

Caspases are primary drivers of apoptotic process carving the pathway of ruin in cells by disabling homeostatic and repair enzymes mechanisms responsible for programmed cell death. These caspases play initiation and executional roles, the executioners are activated by the initiators whose activity are turned on by uncontrolled release of apoptogenic mediators like Cyt C. In this study, treatment showed that Q10&VitE, Q30 and glibenclamide reduced caspase 9 activation in diabetic rat hearts. Interestingly, treatment with VitE showed better amelioration in the activity of caspase 9 than the other antioxidants. However, treatment with Q10 showed no decrease in the activation of caspase 9. It was observed that treatment of the diabetic rats with antioxidants considered in the study decreased the caspase 3 activation but glibenclamide did not reduce caspase 3 activation. This supports our hypothesis that the antioxidants act by overwhelming the endogenous ROS that makes the mitochondria liable to insults. This is in consonance with

the reports of earlier investigators (Li *et al.,* 2008; Morin *et al.,* 2010). Considering therefore the role of Cyt C release in intrinsic apoptosis and activation of the initiator and execution caspases, the inhibition of these factors by antioxidants may partly explains the molecular basis of their cardio-protective effect. This study mat therefore have succeeded in addressing an unmet need in T2DM.

Evidence from studies have confirmed the relationship between diabetes and heart failure (Kasznicki and Drzewoski, 2014), however the relevance of this coexistence is not well understood, since the cause is multifactorial. Histology results of the heart have shown that haemophargic lesions and congestions of coronary vessels in diabetic rats treated with quercetin and vitamin E were reduced to inflammation of the myocardium and pericardium. This reduction in the severity of the lesions may be as a result of the antioxidants ability to regulate glucose toxicity as earlier reported in the study. This fact is supported by the unified hypothesis that alteration in the glucose level influences molecular changes that cause susceptibility to diabetic cardiomyopathy as suggested by Williams and colleagues (2017).

In summary, the study has presented evidence that the mPT pore opening and oxidative phosphorylation uncoupling in diabetic heart is due to reperfusion injury. The diabetic heart mitochondria showed increased mLPO which translated to apoptogenic Cyt C release and subsequent caspases 3 and 9 activation. Shift in mPT pore status in diabetic heart mitochondria was reversed either by quercetin or vitamin E, suggesting that decreased ROS burden in the diabetic heart alters the intracellular redox environment and inhibit mPT pore opening. In the heart sections, injury was significantly decreased, suggesting either direct

or indirect inhibition of mPT pore opening as a pharmacological relevance in reducing the challenge of ischemic heart disease in diabetes.

While mitochondria-mediated apoptosis may be inhibited by antioxidants in diabetic rat heart as supported by experimental data, efficacy of these same antioxidants on the hepatocytes was investigated and their roles in delaying progression of complications in diabetes determined. The study showed that hepatic enzymes (AST and ALT) were elevated in diabetic group and treatment with Q10&VitE, Q10 and 30, VitE and glibenclamide showed apparent reduction in these activities. The serum enzymes are indicators of hepatic damage, our findings have shown probable alleviation of hepatotoxicity which is in accordance with the previous studies of Jeong *et al.,* (2012). The antioxidants appeared to show more sensitivity in decreasing plasma enzyme activities in diabetes than glibenclamide.

Protective effect of quercetin via diverse mechanisms has been shown in a number of organs, this study determined the effect of diabetes on the mLPO. Lipid peroxidation is a well-established pathway for oxidative stress and ROS generation and it became important to determine if the mPT is a possible mechanism facilitating the organelle's dysfunction. Lipid peroxidation can effectively generate ROS that induce mitochondrial damage by Ca^{2+} dependent pathway. This study measured the level of MDA used as the marker for oxidative stress and it was observed that the level of lipid peroxides were higher in diabetic rats than the normal control. This is supported by the studies of Khaki *et al.,* (2010). Studies by Morin *et al.,* (2010) have shown that lipid peroxidation increases the fluidity of

the mitochondrial membrane, makes the pore liable to opening and loosens Cyt C binding from the inner mitochondrial membrane. This potentiates its dissociation and escape from the inner membrane through the outer membrane to generate a cascade of event that may culminate in apoptosis (Morin *et al.,* 2010). In the study, we noted that the extent of mLPO was decreased by quercetin and vitamin E treatment, with an outstanding result. This effect completely alleviated the effect of diabetes on lipid peroxidation level and it explains that mitochondrial membrane is protected in diabetic condition from radicals that would induce organelle's damage and cause dysfunction. This event could partly explain the increase in liver enzyme activity, which may be a pointer to a recovery process. Similarly, all the other treatments showed decreased lipid peroxidation levels in diabetic condition. If the mitochondrial fluidity is affected in diabetes, as a result of increased lipid peroxidation observed in the study, we hypothesised that the liver mPT pore might not be protected in diabetic condition.

Quercetin and vitamin E have shown outstanding potentials in attenuating stress stimuli-induced hepatotoxicity as well as oxidative stress via diverse mechanism (Khaki *et al.,* 2010). The study investigated the inductive reversal role of quercetin and vitamin E in rat liver mPT pore of diabetic rats. Elaborate findings by researchers have shown that administration of quercetin and vitamin E showed increased antioxidants enzyme in the liver which have the unique characteristic of decreasing the ROS availability (Khaki *et al.,* 2010). In the present study, Ca^{2+}-induced swelling of the isolated normal rat liver mitochondrial was inhibited by spermine, a standard inhibitor of the mPT pore, this is consistent with the earlier study of Lapidus and Sokolove, (1993). There was no observed change in the absorbances recorded in the absence of Ca^{2+}, this showed that the isolated rat

liver mitochondrial were intact thereby retained their structural integrity and suitable for further use. In the diabetic rat liver mitochondrial, there was permeability of the mitochondrial-membrane which allows a non-specific pore formation. This is consistent with the earlier reports of Ajayi and colleagues (Ajayi *et al.,* 2016). Treatment with quercetin and vitamin E potently reversed the mPT pore opening in diabetic rats than glibenclamide and other antioxidants considered in the study. This demonstrated that direct inhibition of liver mPT pore permeabilization could constitute relevant objective in hepato-protection in diabetic rats. The co-administration of quercetin and vitamin E may have directly/indirectly interfered with factors that regulate apoptotic modulators like Bax and Bcl-2 that interact directly with the mPT pore. This may have been achieved by directly keeping Bax bound to Ku autoantigen or its derived peptides in the cytosol as suggested by Kroemer *et al.,* (2007) and Sawada *et al.,* (2003). The antioxidants may also have acted by altering the concentration of the mPT pore inducers like pH, Ca^{2+}, ROS thereby inhibiting the pore opening. Studies have shown that pretreatment with quercetin before induction of diabetes prevents the complete degeneration of the pancreas, but administration of the same concentration of quercetin to already diabetic rats produced less pronounced effect, while other researchers were completely at variance with that finding. These contradictions were observed by the studies of Dai and colleagues (Coskum *et al.,* 2005; Dai *et al.,* 2005; Mahesh and Manem, 2004). Similarly, this study showed that there was greater swelling amplitude in mPT pore opening of diabetic rat liver treated with quercetin than the pore treated with quercetin after 28 days than the pore of the animals pre-treated with the same concentration of quercetin after diabetes induction. This showed that administration of Q30 prior to diabetic induction was more therapeutic than after diabetes induction, confirming

the discrepancies suggested by Dai *et al.,*(2005). An impressive observation in this study was that the swelling amplitude in the liver cells was more than the heart cells. This showed that the liver cells were more susceptible to injury than the heart cells. This fact has also been shown by the detailed study of Guicciardi and colleagues (201

3) that stated that liver cells are more liable to apoptosis than the heart cells when exposed to the same kind of insult (Guicciardi *et al.,* 2013). Therefore the targeting of liver mPT pore by antioxidants may be a promising pharmacological approach in DM, since many studies have shown that almost any procedure that inhibit mPT pore opening have the potential to positively impact cyto-protection therapy (Sloan *et al.,* 2012).

Sequel to the confirmation that the liver mPT pore is permeabilized in diabetic rats, we assumed that this event may impact on the mATPase activity, since it is being proposed as an interface between the ATPase dimer or tetramer (Bonora *et al.,* 2013; Giogio *et al.,* 2013; Alvian *et al.,* 2014) or that the pore arises from the c-ring of the F_0 sector (Bernardi and Di Lisa, 2006; Giorgio *et al*., 2013). In the study, we examined the effect of mATPase hydrolysis as a proof of mPT pore opening in diabetic rats. We found out that isolated liver mitochondrial added to suspension of ATP stimulated the hydrolysis of the energy compound. This showed that there might have been uncoupling of the electron flux that establishes electrochemical gradient in the inner mitochondrial membrane necessary for oxidative phosphorylation. This event would lead to metabolic impairment as a result of decreased ATP production as previously suggested by the findings of Martel and colleagues (Martel *et al.,* 2012). Furthermore, the study showed that treatment with all the antioxidants inhibited ATP hydrolysis, however, treatment with glibenclamide did not impact on the mATPase activity. This finding showed the ability of the

antioxidantsdirectly/indirectly translate the enzyme from an energy dissipating to energy conserving component and this is of great relevance in cell survival. Given the mATPase activity preservation, it is possible to suggest that the antioxidants might have mimicked the role of CsA, which dissociates Cyp D from binding to the ATP synthase thereby partially inhibiting its activity as suggested by Rasola and Bernardi (2014) and Giorgio *et al.,* (2009). The Cyp D, a proposed mPT pore component interacts with the subunit b, d and OSCP of the lateral stalk of ATP synthase, therefore disabling its role will mean alleviating its participatory role in increased ATP synthesis. The antioxidants treatment impact on diabetes by creating a survival mechanism for the hepatocytes, thereby reducing diabetes – induced tissue wastage.

Cytochrome C becomes a key player in apoptosis when released from the mitochondrion and this adventure would only be possible if the mitochondrial membrane opens. The event facilitates apoptosis by initiation of caspase 3 via caspase 9 activation (Mcllwain *et al.,* 2013; Jiang and Wang, 2000). Under normal physiological condition, the apoptogenic protein is localized in the mitochondrial matrix (Mcllwain *et al.,* 2013; Bressenot *et al.,* 2009; Jiang and Wang, 2000). Upon translocation from the mitochondria, it binds to Apaf-1, this recruits caspase 9, then activates caspase 3and subsequently causes cleavage of DNA strand by poly-ADP-ribose polymerase leading to apoptosis (Bressenot *et al.,* 2009). In the study we realized that quercetin and vitamin E administration decreased Cyt C release in rat liver. This result lay credence to the fact that any molecule that decreases Cyt C release would have the potential to promote cell survival.

Following excessive Cyt C release as the hallmark of mitochondrial-mediated apoptosis, the extent of caspase 9 activation was assessed and found to be heightened in diabetic rats,

this initiated caspase 3 activation. Intervention by quercetin and vitamin E decreased caspase 9 activation comparing favorably with glibenclamide. Caspase 9-induced caspase 3 activation occurrence in diabetic rats, is an event that causes excessive apoptosis and seals the fate of hepatocytes for death. Treatment with quercetin and vitamin E reduced this, attenuating the effect of excessive apoptosis. Our goal is to uncover new molecules directed to the mitochondria that will selectively target the disease condition without showing hepato-toxicity and the present study was instructive in presenting co-administration of quercetin and vitamin E as a promising alternative to glibenclamide in the complications associated with liver cells apoptosis. Considering the role of these downstream proteins in mitochondrial-mediated apoptosis, we may conclude that this partly explain the molecular basis of their hepato-protective effect.

Histopathological examinations in the liver tissues of diabetic rats showed visible lesions while quercetin and vitamin E, showed very mild periportal infiltration by inflammatory cells. Similarly, treatment with glibenclamide reduced the lesions to congestion of vessels, while Q10 gave the most outstanding result with no visible lesions. These suggest that the lesions incurred by diabetes were totally abrogated by treatment with Q10 in the liver. Our findings is in consonance with the recovery of the liver enzymes by antioxidants treatment, since they are markers of hepatocellular damage.

Research have indicated that diabetes adversely affect the testes by causing decrease in leydig cells number resulting in down-regulation of androgen biosynthesis responsible for decreased libido/spermatogenesis (Orth *et al.,*1979; Bucholtz *et al.,*2000; Ballester *et al.,*2004; Bahey *et al.,*2014). Studies have also shown that these testicular dysfunctions may be due to lack/reduced insulin availability (Bucholtz *et al.,*2000). Up till recent times,

there is no unified model to elucidate the mechanism through which insulin affect the testicular function, this has limited the possibility of pharmacological intervention for the treatment of male-infertility associated with diabetes. Our results showed that diabetes decreased the LH level which is responsible for decreased leydig cells number as revealed by histology. Insulin has been shown to modulate the level of LH, therefore diabetes-induced insulin decrease resulted in LH decrease, even as shown in this study. This subsequently affect the sperm count and motility which can lead to infertility, giving credence to the previous findings of Ballester and colleagues (2004). In this study, we were interested in combining the therapy of quercetin and vitamin E to investigate if this would improve the level of insulin in diabetic condition. Results showed increased plasmatic insulin level, which impact on the synthesis of LH. Furthermore, this increased the leydig and sertoli cell numbers, improved maturation of germ cells with the attached epididymis having abundant spermatozoa shown histologically. This proved that treatment of diabetic rats with quercetin and vitamin E may elicit the upregulation of insulin and LH, which improve spermatozoa numbers responsible for fertility.Ballester and colleagues (2004) have hypothesised that insulin also has a direct effect on FSH (Ballester *et al.*, 2004), we therefore, investigated the level of FSH in diabetic rat plasma. The result showed a positive correlation between FSH and insulin levels, suggesting that insulin may have directly/indirectly stimulated FSH activity. Several studies have postulated that increase in FSH and testosterone level inhibit progression of cell death (Olanlokun *et al.,* 2018), we therefore hypothesized that any treatment that would increase insulin/FSH and testosterone level might be able to decrease excessive apoptosis observed in diabetic rats. Since the level of FSH modulates spermatogenesis, it may be that diabetes alters spermatogenesis by

FSH-mediated mechanism as suggested by our findings and supported by previous investigators (Chartterjee *et al.,*2012). Furthermore, the level of testosterone was decreased in diabetes, treatment with quercetin and vitamin E improved the concentration of the hormone. This event may be responsible for the increased viable sperm count and motility. These parameters are critical for fertilization in corpulation (Vasan, 2011; Olanlokun *et al.,* 2018). Given the reference range of more than 40% sperm motility required for fertilization in humans, the values obtained in the study by the treatment groups may be sufficient for effective fertilization. Histology results showed diminished sperm cell proliferation in diabetic rats which may have caused prostatic atrophy agreeing with Bahey *et al.* (2014), but treatment resulted in increase in secretory epithelial cells number as revealed by histology. This finding is in consonance with the previous work of some researchers (Rashid and Sil, 2015; Khaki *et al.,*2010). This study therefore showed the testiculo-protective potential of quercetin and vitamin E in diabetic rats.

Researches have speculated that certain sex hormones like FSH, testosterone, estrogen, progesterone have been implicated in programme cell death (Fedotcheva *et al.,* 2008; Oludele *et al.,* 2018), we therefore investigated the effect of these hormones on some apoptotic indices in the testes. The level of Cyt C release in the testes of diabetic rat was found to be increased and this was markedly reduced by treatment with co-administration of quercetin and vitamin E. Since Cyt C causes apoptosis, we suggest that the alterations in its level induced by diabetes may have a potential effect on caspase 9 activation. In this study, there was an increase in activation of caspase 9 in diabetic rats; however, treatment with Q10&VitE, Q30, VitE reduced the apoptotic index but treatment with Q10 and glibenclamide did not show any marked reduction in the levels of caspase 9 activation.

Furthermore in the study, the executioner caspase 3 whose activity was triggered by caspase 9 was shown to be activated in diabetic rats and this fact is supported by the previous findings of Kaneto *et al.,*(2005), Rashid and Sil, (2015) among others. The elevation in caspase 3 activation is an indicator of excessive apoptosis probably responsible for atrophy in the testes of diabetic rats. Intervention was achieved by treatment with Q10&VitE, Q30, VitE in the study. The study showed that the antioxidants protected the testes from excessive apoptosis.

In summary according to the findings in this study, there was alteration in the structureof the mPT pore in diabetic rats and the antioxidants have provided insight into the preservation of the pore's functional integrity of proteins necessary for apoptosis. Based on the current findings in the study, we have demonstrated improved biological function of the mPT pore by the antioxidants, confirming cardiovascular and hepatocellular safety in diabetic condition, while addressing an unmet need in diabetes treatment. We have also confirmed the potency of quercetin and vitamin E treatment in restoring sexual functions in diabetes. We therefore propose a model treatment of diabetes with the antioxidants considered in the study, especially on co-administration of quercetin and vitamin E. This treatment hasshown proof that assemblage and inhibition of the mPT pore opening was possible in diabetes. This ameliorates the increased cytosoli P*i* and Cyt C release that induces pore opening. The decrease Cyt C release from the inner-mitochondrial membrane prevented the ignition of a battery of highly specialized procaspase- 9 which in pathophysiology activates executioner procaspase-3 that commits the cell to death by apoptosis. This finding confirms the synergestic role of co-administration than single therapy in the treatment of diseases and this would be of tremendous pharmacological

relevance in diabetes especially if the molecules are compounded with substances that will enable them reach the target organ before metabolism to enhance better activity.

Early diagnosis of diabetes and treatment with quercetin and vitamin E, most especially with the co-administration of both quercetin and vitamin E may serve therapeutic role. Precaution should be taken in the use of glibenclamide has it may be cyto-toxic. In view of this study, co-administration therefore merit more investigations on its potential for the management of diabetes and may therefore be considered as more effective therapeutic alternative to glibenclamide in the progression and complications arising from diabetes. It is therefore a target for the management of the disease.

CONTRIBUTIONS TO KNOWLEDGE

- Co-administration of quercetin and vitamin demonstrated more hypoglycemic activity than glibenclamide.
- The antioxidants are cardio and hepatoprotective in mitochondrial-mediated apoptosis in STZ-induced diabetic rats.
- The study showed that the synergestic relationship in quercetin and vitamin E treatment increased the sperm quality/fertility in STZ-induced diabetic rats.
- Although the antioxidants considered in the study should be subjected to clinical trials, we propose that glibenclamide did not reduce heart mitochondrial F_1F_0 ATPase, therefore its use should be with caution.

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