UNIVERSITY OF GHANA

COLLEGE OF HEALTH SCIENCES

SCHOOL OF PHARMACY

DEPARTMENT OF PHARMACOLOGY AND TOXICOLOGY

EFFECT OF COMBINATION THERAPY OF ALPHA-LIPOIC ACID, RAMIPRIL, AND GLICLAZIDE IN A RAT MODEL OF DIABETIC CARDIOMYOPATHY

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(10804937)

A THESIS SUBMITTED TO THE SCHOOL OF GRADUATE STUDIES

IN PARTIALFULFILLMENT OF THE AWARD OF A MASTER OF

PHILOSOPHY DEGREE INPHARMACOLOGY

MARCH 2022

DECLARATION

DECLARATION BY THE CANDIDATE

I declare that this thesis is the result of my work except for the references cited, which have been duly acknowledged and have been generated by me as the result of my original research. It has never been submitted anywhere for the award of any degree.

Wonje, Quinsker Lariba **09/08/2023**

(10804937) Date

DECLARATION BY THE SUPERVISORS

We declare that we supervised the principal work and presentation of the thesis per guidelines on supervision of the thesis laid down by the University of Ghana.

Principal Supervisor Signature Date

Dr. George Johnson Dugbartey …… **14/10/2023**

Dr. Vincent Boima …… **27/09/2023**

Co-Supervisor

DEDICATION

I dedicate this work to my children Sonel, Lemuel, and Daisy

ACKNOWLEDGEMENTS

Nothing happens unless first a dream, and my dream of pursuing this course and completing this project work would not have been a reality without God, who has been my pillar in this whole journey - the tiny voice that always pushes me on every time I want to give up. I am most grateful to God for bringing me this far. Next, I am thankful to my family, especially my mother for her support and prayers. I appreciate every effort and sacrifice you made for me. You are a true definition of a virtuous woman.

I am greatly and forever indebted to my main supervisor, Dr. George Johnson Dugbartey, for his support, meticulous guidance, positive planning, and execution of this project work. You mentored and refined me into a finer graduate today and built confidence in myself in the process. The whole course became a nightmare when I started my project work. You were not only my supervisor, but you also became my counsellor. There were moments when I nearly gave up, but you gave me more than enough reasons to continue. You made time out of your busy schedules for this project, you were always just a call away. The whole course and project became meaningful to me when you started supervising me. Today, I look back at this journey and smile. There were times I thought you were too hard on me when I could not meet deadlines but here, I am today with a project work I am so proud of. Anytime I have a project meeting with you, it was a moment of knowledge impartation, inspiration, and motivation. Your patience, motivation, time consciousness, dedication, commitment, and emotional support has produced a fine graduate with improved research and writing skills. I have lost count of the many articles and other reading materials including videos about the project that you sent to

me to read and watch to increase my knowledge in the subject area. Words cannot express how indebted I am to you. Thank you very much Dr. George Johnson Dugbartey.

I also wish to express my heartfelt gratitude to my co-supervisor, Dr. Vincent Boima, for his time and dedication to this project work. Thank you for the additional contribution you made to the successful completion of this project work. I have always called you at the last minute, but you made time for this project work and provided some supplementary reading materials to help me complete this work. Thank you for your positive criticisms that enabled me to complete this project work. Thank you, very much Dr. Vincent Boima of Korle-Bu Teaching Hospital.

I wish to also thank the staff and workers at Noguchi Memorial Institute for Medical Research for their technical support during my project work, without excluding Mr. Samuel De Graft Mensah at the Oral Pathology Department of Korle-Bu Teaching Hospital for his time and patience during my histopathological analysis. I also wish to thank Mr. Olaf Tehrani for his emotional and financial support towards this project work. Thank you for being there for me. I cannot forget Dr. Evans Attito-Narh of Ridge Hospital for his fatherly role in my life. Your confidence in me made me overcomes my fears. Your words of encouragement contributed to the height I have attained so far. I am most grateful to you.

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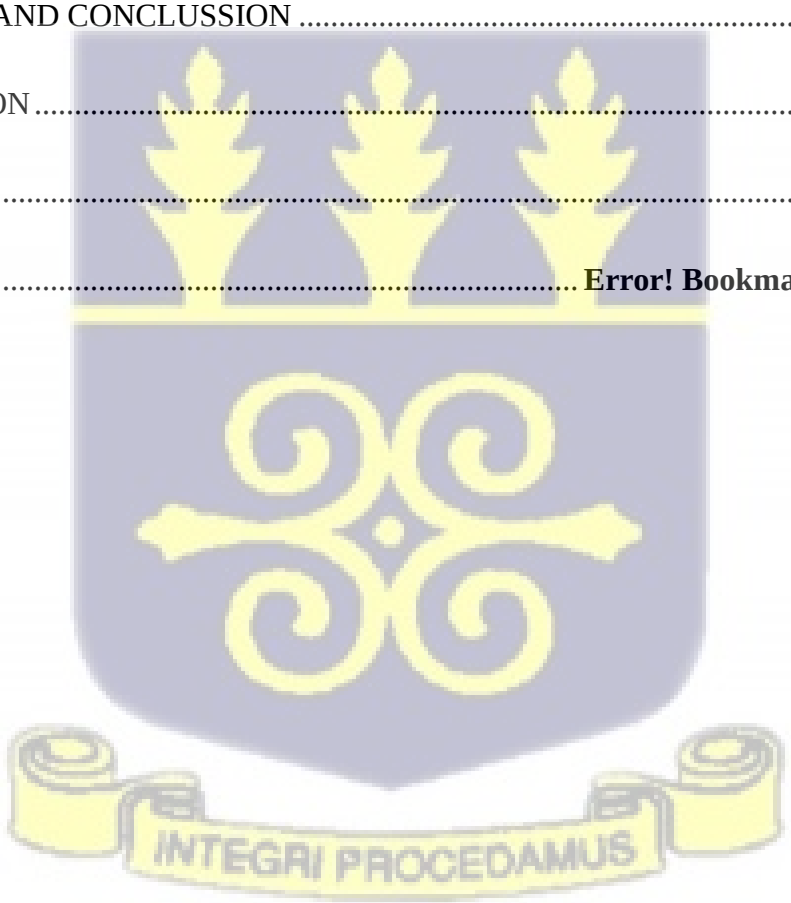

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ABSTRACT

Background

Diabetic cardiomyopathy (DCM) is a prominent long-term diabetes mellitus complication, responsible for more than 20% of diabetic patient's yearly death rate globally. Even though numerous anti-diabetic medications have improved the glycemic condition of diabetic individuals, the incidence of DCM remains high. In this research, the cardiac effects of anti-diabetic medicine supplementation with alpha-lipoic acid (ALA) are examined in experimental DCM.

Methods

Following a 12-hour fast, a total of 44 male Sprague Dawley male rats were put into two groups: healthy control and diabetic groups, and glucose levels in blood were measured. The diabetic group was given an intraperitoneal (i.p) injection of nicotinamide and streptozotocin to induce type 2 diabetes mellitus (T2DM). The diabetic rats were given either ALA (60 mg/kg; ramipril (10 mg/kg;), gliclazide (15 mg/kg;) or all three drugs for 42 days after which they were euthanized, and samples collected for biochemical, histological and molecular analyses.

Results

T2DM induction caused pancreatic damage, high blood glucose, weight reduction, and DCM development, marked by cardiac fibrosis, vacuolation, degeneration as well as high plasma and cardiac levels of markers cardiac injury. Compared with diabetic control group, treatment with

all three drugs protected the structure of pancreatic islets, preserved normoglycemia and body weight, and prevented DCM comparable with healthy controls ($p > 0.05$). In addition, combined treatment significantly reduced plasma interleukin-1, interleukin-6, and tumor necrotic factor-alpha, and improved antioxidant and lipid profile ($p < 0.001$). Finally, treatment with all three drugs markedly downregulated TGF-β1 and its effector proteins in the heart tissue ($p < 0.001$).

Conclusion

The combined treatment provided protection against DCM via several pathways including inhibition of fibrotic pathway involving TGF-1/Smad signaling. This observation is applicable to clinical situations and could provide supplementary information on DCM pharmacological treatment in T2DM patients.

CHAPTER ONE

INTRODUCTION

1.0 Background

Diabetic cardiomyopathy (DCM) is significant long-term diabetic complications of the cardiovascular system that causes architectural and myocardial dysfunction in the absence of conventional predisposing factors such as coronary atherosclerosis, hypertension or atrial fibrillation (‘Yang F. Qin Y, 2018; G*e* Q, 2019). Its clinical presentation includes altered myocardial structure and hypertrophy, fibrosis, and abnormal diastolic and systolic pressures (Rubble et al,1972). Due to its high prevalence worldwide, DCM has emerged as global threat, which is responsible for more than 20% of global annual mortality among people with diabetes (Tillquist *et al*, 2012), with far more hospital admissions for cardiac complications than patients with non-diabetic related heart conditions. (Lee C. *et al*.,2012; Lindman *et al.*,2014).

Despite several conventional drugs for management of diabetes, DCM remains a prevalent consequence of diabetes mellitus. This implies that factors other than hyperglycaemia may be involved in the initiation and advancement of DCM. A diabetic study conducted between 1998 and 2003 identified increased production of reactive oxygen species (ROS; a toxic factor responsible for tissue damage) as an important factor that causes cardiomyocyte apoptosis in DCM (Kuethe F, 2007). Besides chronic hyperglycaemia and ROS-induced cell death, studies on interconnected pathways underlying the genesis and progression of DCM are underway. These processes have recently been revised, and they now include diminished insulin metabolic

signalling in cardiac cells of diabetic patients, high AGES (advanced glycation end-products), impaired mitochondrial function, inflammation, and other pathological pathways. (Jia *et al* 2016). Among all the identified and unidentified factors implicated in DCM, cell death resulting from excessive generation of ROS has been extensively studied to play a central role in DCM pathogenesis and progression (Ghosh *et al*.,2014). A growing body of laboratory evidence also show that oxidative stress impacts fibrotic pathway in DCM (Purnomo et al., 2013; Zhang et al., 2018; Wang *et al*., 2018). Hence, new drugs that combat hyperglycaemia, oxidative stress due to ROS, and other molecular pathways including fibrotic pathways in DCM are being investigated.

Per the preceding assertion, alpha-lipoic acid (ALA), a disulfide molecule and intrinsic ROS scavenger that serves as a key co-factor for a lot of enzymes in the mitochondria has recently been studied to be useful in diabetes and many diabetic comorbidities by improving glycemic control and lipid profile (Midaoui et al, 2003). It can also be obtained as a dietary supplement from the plant (tomatoes, potatoes, broccoli, spinach, etc.) and animal (liver) sources (Strödter *et al*, 1995).

1.1 Problem Statement

The global prevalence of diabetes is increasing, and this correspond to the increase incidents of diabetic related complications DCM currently accounts for 20% of global annual mortality in the diabetic population, with significantly higher hospitalization for other cardiovascular complications such as heart failure compared to non-diabetic clients with cardiovascular conditions. According to a recent study, people with diabetes have a 74% greater chance of

developing DCM, while those with DCM have a fourfold increased risk DCM-associated death than their counterparts who do not have DCM (Holman et al., 2021).

1.2 Justification

Alpha-lipoic acid (ALA) is a sulphur-containing antioxidant and acts as a co-factor for a lot of enzymes in the mitochondria in the subcellular pathways that involve ATP synthesis. Some studies reported lower levels of plasma ALA in diabetic individuals after which that ALA supplementation provided effective management of both T1DM and T2DM (Strodter and others 1995). While pharmacological management of DCM with conventional drugs is a common clinical practice, there is currently no study on the cardiac effect of supplementing these conventional drugs with ALA. Therefore, this project seeks to determine cardiac effect of combined ALA, gliclazide and ramipril on T2DM-induced cardiomyopathy in rats.

1.3 Research Questions

Does addition of ALA to anti-diabetic and cardioprotective medications provide extra protection against DCM?

1.4 Aim

This project investigates whether combined ALA, ramipril and gliclazide provide better cardio-protection than anti-diabetic and cardioprotective medications alone in an animal model of diabetic cardiomyopathy.

1.5 Objectives

The goals of this study are to assess the:

- anti-hyperglycemic and cardioprotective effects of ALA, gliclazide, and ramipril mono- and triple combination treatments in T2DM rats.
- underlying mechanisms of cardio protection in T2DM mice using mono- and triple combination treatments

CHAPTER TWO

LITERATURE REVIEW

2.0 DIABETIC CARDIOMYOPATHY

Diabetes cardiomyopathy is defined as myocardial dysfunction in people with diabetes who do not have other predisposing factors such as, coronary artery disease, hypertension, left ventricular disease, atrial fibrillation, or other predisposing factors (Jia *et al.*, 2018). Diabetic cardiomyopathy (DCM) is a significant complication of both T1DM and T2DM; however, because T2DM is more common than T1DM, diabetic cardiomyopathy is more frequently associated with T2DM. Animal studies reveal that reduced cardiomyocyte activity is an essential mediating mechanism for heart failure (Dillmann et al., 2020) and that this reduction in cardiomyocyte function is due to a decrease in their quantity caused by apoptosis and degeneration (Dillmann et al., 2020). Another important cause of heart failure is myocardial fibrosis.

2.1 Pathogenesis of Diabetic Cardiomyopathy

As illustrated in figure 1 below, the molecular processes underlying the pathogenesis and advancement of DCM are multifactorial and include ROS-oxidative stress, impaired insulin metabolic signalling, high free-fatty acid production, hyperglycemia, AGEs, and many more (Jia *et al*., 2018).

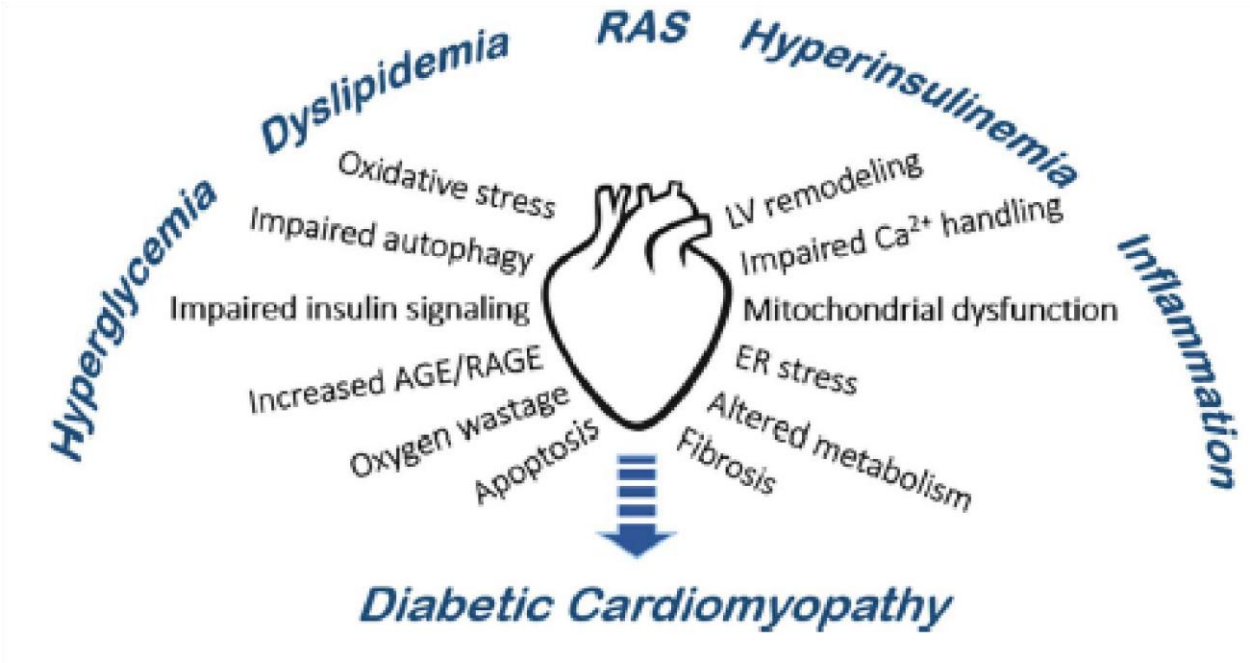

Figure 1 depicts the progression of alterations that lead to diabetic cardiomyopathy.

2.1.1 Impaired insulin metabolic signaling

The insulin signalling cascade regulates the molecular pathways involved in the recruitment of the GLUT4 (glucose transporter) to the cell membrane (Guo, 2014). These pathways include mitogen-activated protein kinases (MAPKs) and phosphoinositide 3 kinases (P13K) (Guo, 2014). GLUT 4 recruitment to the plasma membrane is often stimulated by the P13k/Akt signalling pathway, resulting in glucose absorption into cardiomyocytes (Jia et al., 2017). Excessive nutrient intake promotes chronic activation of S6k1 (serine 6 kinases 1), which leads to cardiac insulin resistance (Jia *et al*., 2018). This reduces P13K activation and protein kinase B (Akt) stimulation (Jia *et al*., 2017). In this situation, GLUT4 recruitment is diminished, resulting in decreased glucose absorption. This increases circulating glucose.

2.1.2 *Persistent hyperglycemia and oxidative stress*

Persistent hyperglycemia caused by poor glucose absorption results in a multitude of molecular and metabolic alterations in cardiomyocytes (Jia et al., 2018). Hyperglycaemia refers to fasting blood glucose levels above 7.0 mmol/L or random blood glucose readings above 9.0mmol/L. (Bornfeldt & Tabas, 2011). Surprisingly, hyperglycaemia, which leads to increased glucose metabolism, can occur for a long time before diabetes mellitus is diagnosed (Marra *et al*., 2002). Increased glucose metabolism disrupts mitochondrial oxidative balance (Wang-Soo Lee, 2017). The resulting rise in the number of free radicals causes a shift in the fragile balance between the formation of ROS and the production of antioxidants.

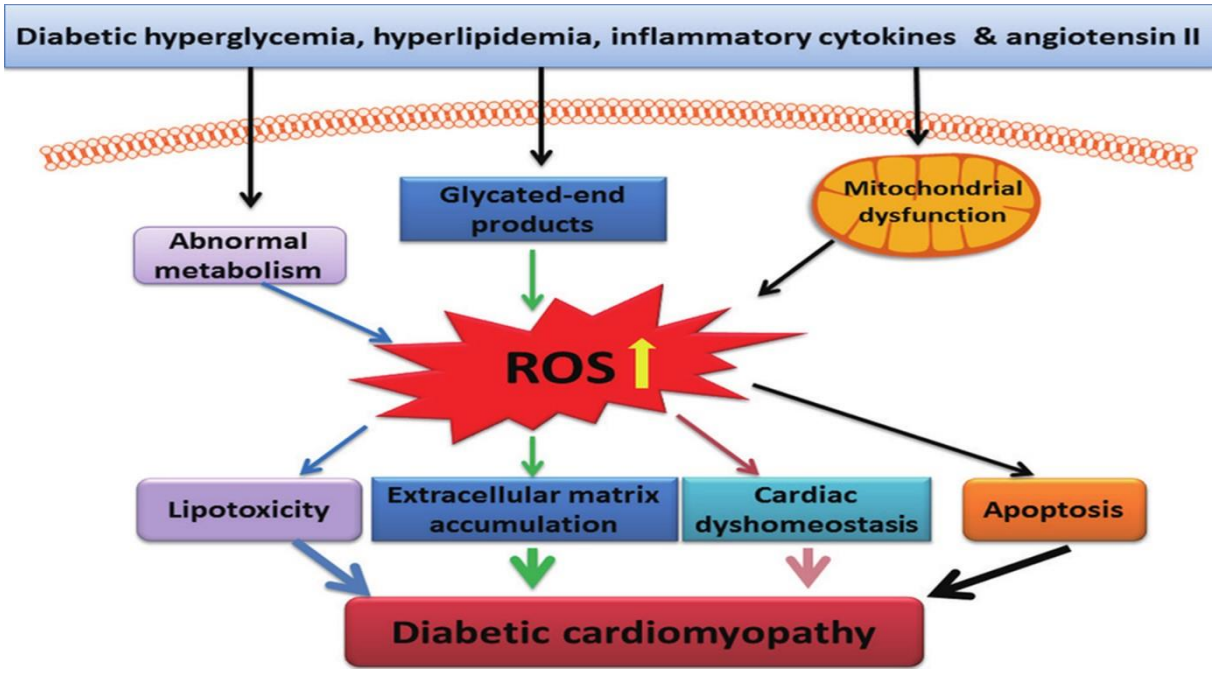

Figure 2: Molecular processes underpinning diabetes cardiomyopathy development and progression. Chen et al. (2014) (DOI: 10.4093/dmj.2014.38.5.337)

2.1.3Advanced glycation end products

Advanced glycation end-products are proteins that have been exposed to sugars, causing them to lose their functional capabilities (Stirban *et al*., 2014). AGES inhibit gene transcription via changing the proteins involved in gene transcription, as well as altering signaling between matrix and cells, resulting in cellular malfunction. AGES also activate AGE receptors (RAGE) (Stirban *et al*., 2014). RAGE and AGEs promote the signalling of nuclear factor κB (NFκB) which is a prototypical proinflammatory pathway and therefore increases the expression of proinflammatory cytokines leading to inflammation and cardiac fibrosis (Abel H. B., 2014). It also promotes apoptosis, prothrombotic activity, expression of adhesion molecules, and oxidative stress (Stirban *et al*., 2014). All these lead to cardiac stiffing and dysfunction.

2.1.4 Inappropriate renin-angiotensin-aldosterone system activation

Stretch receptor activation in the heart activates the RAAS and the sympathetic nervous system, resulting in changes in myocardial structure and remodeling, which compromises cardiac function (Sajad *et al*., 2004). RAAS inhibits cardiac insulin metabolic signaling, preventing PI3K engagement, Akt/eNOS activation, and NO generation (Jia et al, 2015). When PI3K is not engaged, the downstream component system, such as subsequent recruitment of GLUT 4 to the cell membrane, is impaired, aggravating hyperglycemia (Jia *et al*, 2015). Furthermore, decreased NO production inhibits full coronary valve relaxation and insulin-mediated capillary recruitment, both of which are required for normal myocardial energetics and the transport of insulin and glucose (Jia *et al.*, 2017). Diabetes causes RAAS upregulation.

2.2 Alpha-Lipoic Acid

Alpha-lipoic acid, a disulfide molecule and ROS scavenger that helps enzymes in the mitochondria to convert nutrients such as glucose into energy, is advantageous in diabetes and a range of other diabetic complication. Besides its biosynthesis, it is also obtained as a dietary supplement from both plant (tomatoes, spinach, etc.) and animal (liver) sources (Shay *et al*. 2009) and exerts many cellular effects including antioxidant effect (Shay et al., 2009).

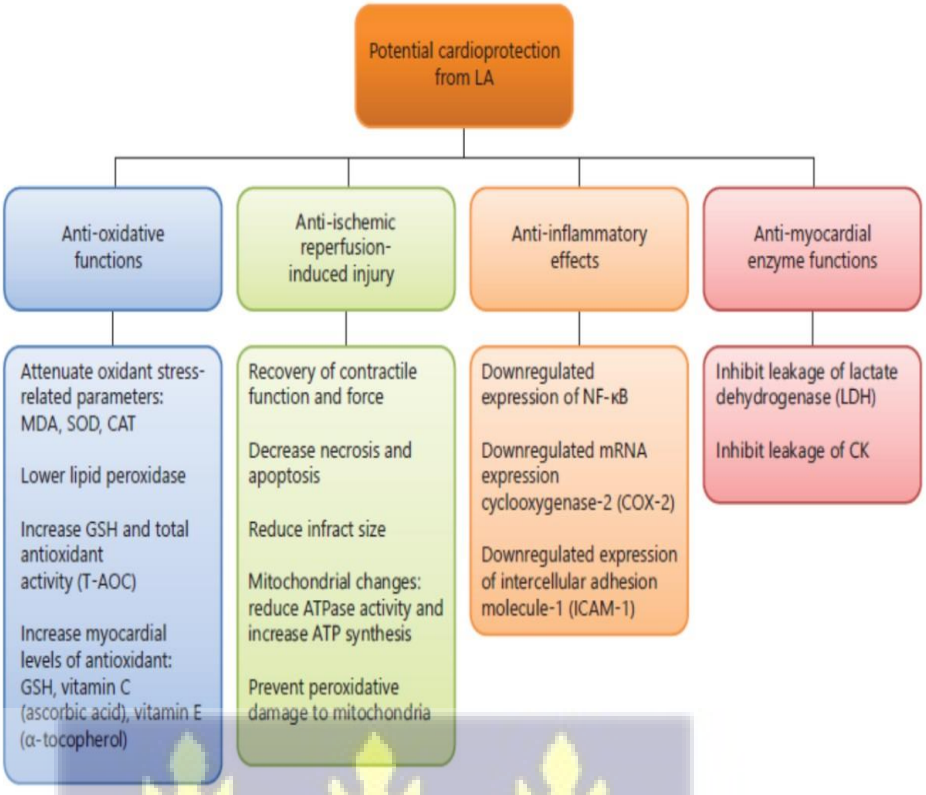

Figure3. A diagram depicting the cardioprotective properties of alpha-lipoic acid. Zhang and McCullough (2016) (DOI: 10.1159/000448666)

2.2.1 Biological Functions and Therapeutic Potentials of Alpha-lipoic Acid

Several investigations have indicated that the unbound form of ALA has a variety of biological functions in tissues including the heart. Among these biological tasks is the neutralization of reactive oxygen species (ROS). ALA acts directly as an antioxidant by scavenging ROS, lowering all the negative consequences associated with elevated ROS activity. It also potentiates other antioxidants such as vitamin C, glutathione, and others, which is why it raises antioxidant levels in the body (Golbidi et al., 2011). ALA is unique in that it is both lipophilic and hydrophilic, allowing it to act as an antioxidant both within and outside the cell (Hagena M).

Apart from its biological roles, ALA has a high therapeutic potential, since it is favorable for absorption of muscle glucose, tolerance of glucose throughout the body, and insulin resistance

(Petersen *et al*., 2009). Furthermore, the antioxidant capacity of ALA decreased inflammation caused by`oxidative stress in diabetic peripheral neuropathy (Rochette et al., 2015), indicating that it may be effective in both the prevention and treatment of diabetic neuropathy. ALA supplementation enhanced the redox status of plasma and endothelium-dependent NO-mediated vasodilation, reducing oxidative endothelial damage caused by stress (Petersen et al., 2009).

2.3 Ramipril

Ramipril is a long-acting prodrug that inhibits angiotensin-converting enzyme (ACE). It interrupts the renin-angiotensin-aldosterone pathway like an ACE inhibitor (RAAS). In vivo, it is hydrolyzed to ramiprilat, the active medication (Perry, 2002). Ramiprilat binds to ACE and inhibits the enzyme's activity as well as the formation of angiotensin II from angiotensin I. ACE inhibitors such as ramipril, are used to treat and manage a variety of illnesses, including hypertension, heart failure, and post-myocardial infarction, by blocking angiotensin II production.

2.3 Gliclazide

Gliclazide is a sulphonylurea that is used to treat type 2 diabetes. It has an intermediate half-life of approximately 11 hours. The revised Dutch type 2 recommendations expressly recommend gliclazide as the preferable second-line medication rather than sulphonylureas in general (Singh, 2016). The World Health Organization (WHO) has also placed gliclazide in its 2013 Model List of Essential Medicines, citing its safety in older people (Singh, 2016). Gliclazide promotes insulin secretion by cells of the beta `sulfonylurea receptor, and possibly

an action on the transport of intracellular calcium. It mainly improves the abnormal release of insulin in T2DM, Campbell (1991). Gliclazide improves insulin signaling by acting directly on PI3K insulin-resistant skeletal muscle. The PI3K

CHAPTER THREE

MATERIALS AND METHODS

3.0 Drugs and chemicals

Nicotinamide Nanjing Yasnt Bio-Tech Co. Ltd (China), ramipril (Teva Pharmaceuticals, United Kingdom). TGF 1 key vaccine (inventory number SC7892, Santa Cruz Biotechnology, Inc.), phospho Smad2 (inventory no. Ab53100, Abcam, Canada), phospho Smad3 (inventory no. Ab52903, Abcam), β-actin (invention no. Ab8227, Abcam, Canada), Streptozotocin (USA), gliclazide (China), horseradish peroxidase (HRP) conjugated secondary antibodies (feline. No. Ab13168, Abcam; feline. Nos. Sc 2350 and sc 2371, Santa Cruz Biotechnology, Inc.).

3.1 Ethical statement

The study was approved by Animal Ethics Board of University of Ghana and performed at Noguchi Memorial Institute for Medical Research (with Animal Welfare affirmation number A7604-01) while observing standard laboratory practice.

3.2 Animal handling

A total of 44 male Sprague-Dawley rats weighing 150–200 g and 6–8 weeks of age were purchased from the Department of Animal Experimentation, Noguchi Memorial Institute for Medical Research, University of Ghana, Legon, Accra, and kept under 23±2°C and 45–55 percent room temperature and relative humidity. The rats Were fed with standard rat cow (Agricare, Kumasi) and tap water ad libitum and they were allowed to acclimatize for seven days.

3.3 Animal experimental protocol

3.3.1 Establishment of T2DM animal model

After 12 hours of starving the rats, the level of glucose in the blood was measured with a glucometer, and rats were randomly divided into healthy control group (n = 7) and diabetic group (n = 35). T2DM was established in the diabetic group by injecting nicotinamide 110 mg/kg intraperitoneally followed by 55 mg/kg streptozotocin (STZ). Three days after establishment of T2DM, the animals were starved again for 12 hours after which blood glucose level was measured. Animals with blood glucose level equal to or more than 13.9 mmol/L were included as T2DM in the study while those below the threshold value were excluded (Brahmanaidu *et al.*, 2017).

3.3.2 Treatment of diabetic animals

After T2DM was confirmed on the third day using the threshold glucose value, the diabetic rats were administered either gliclazide 15 mg/kg (DM+GLC group; n = 7), ALA 60 mg/kg (DM+ALA group; n = 7), ramipril 10 mg/kg (DM+RAM group; n = 7) or all three drugs (DM+ALA+GLC+RAM; n =7). Diabetic rats which received neither of these three drugs were given distilled water as diabetic control group (DM Untreated group; n = 9). Clinical parameters such as body weight and glycosylated hemoglobin (HbA1c) levels were measured every 7 days, (day 7,14, 21, 28, 35, and 42) at Tema General Hospital in Ghana using an automated biochemical analyser (Nycocarrd Reader, Axis Shield, Oslo, Norway).

3.3.3 Sacrifice of experimental animals

All rats were sacrificed by intraperitoneal injection of ketamine: xylazine after 42 days of medication. At least 8 mL of blood was taken via cardiac puncture from each rat. Heart and pancreas collected and weighed before being stored in paraformaldehyde and -80 °C freezer for histological and molecular investigations respectively.

3.4 Preparation of plasma for analysis of biochemical parameters

Blood samples during euthanasia were stored in EDTA tubes, the samples were then centrifuged at 3000 rpm for 15 minutes at 4 °C to obtain plasma. The plasma samples were centrifuged and kept at -20 °C in Eppendorf tubes. An automated biochemical analyser was used to assess total cholesterol, triglycerides, and HDL levels in the blood per manufacturer's instructions. Mediators of inflammation in plasma such as TNF-α, IL-1β, and IL-6 were evaluated by ELISA using a DuoSet Kit as described by Bortolon et al (2021). Markers of cardiac injury in plasma (cardiac troponin I [cTnI], brain natriuretic peptide [BNP] and creatine kinase-myocardial band [CK-MB]) were also measured.

3.5 Tissue processing and histological examination

Heart and pancreatic tissue samples fixed in paraformaldehyde were used for histological examination. In summary, the paraformaldehyde-fixed tissue samples were dehydrated by placing them in ethanol in an increasing order after which the samples were dipped in xylene, and then placed in molten paraffin wax to form a tissue block. Next, the paraffin-embedded tissues samples on glass slides were cut at 4µm thickness with a microtome, and dried in an oven. The samples were then dipped in xylene, water and in ethanol followed by immersion in distilled water. Finally, periodic acid Schiff (PAS) reagent was used to stain the tissues. The stained tissue sections were examined under a light microscope and pictures were taken at 400x magnification.

3.6 Determination of antioxidant status

3.6.1 Assessment of tissue glutathione and superoxide dismutase activity

Heart tissue samples stored at -80°C were used for evaluation of the level of glutathione and superoxide dismutase with the help of a test kit from Nanjing Kaiji Bio, Nanjing, China, and as previously reported (Xu et al,2013).

3.6.2 Malondialdehyde measurement

The plasma level of malondialdehyde (MDA, lipid peroxidation product) in cardiac muscle was evaluated using TBARS method (Dugbartey *et al*., 2015). To do this, a volume of 100 mL

PBS was mixed with heart tissue and blended after which it was spun at 10000 rpm for 300 seconds. Thereafter, the supernatant was taken. MDA standard was added followed by incubation for 300 seconds. A volume of 125 mL of TBA catalyst was included and heated at 95 °C for 60 minutes and then cooled at room temperature for 5 minutes. The mixture was centrifuged at 15,000 rpm for 5 minutes at 4 °C. The supernatant was used to measure MDA level spectrophotometrically.

3.8 Western Blotting

RIPA lysis buffer solution and phenylmethylsulphonyl were used to homogenize heart tissue samples that were stored at -80 °C. After centrifugation at 12,500g for 10 minutes, supernatant was removed. The amount of protein concentration of heart tissues was determined using Bicinchoninic (BCA) protein test kit, and the proteins were run through direct electrophoresis. Thereafter, the proteins were electronically transmitted for 50 minutes at 10 12 V in the PVDF membrane (EMD Millipore, Billerica, MA, USA). PVDF membranes are then treated with TGF-β1, Smad2, Smad3 and β-actin primary antibodies.

3.9 Statistical analysis

Results are shown as standard deviation (SD). Prism software was used for statistical analysis, which included one-way variance analysis (ANOVA) and Tukey posthoc test (Prism 8, GraphPad Software, Inc., San Diego, CA, USA). A p value of less than 0.05 indicates a statistically significant difference between groups.

CHAPTER FOUR

RESULTS

4.0 Impact of treatment with ALA, gliclazide and ramipril on clinical parameters

We measured body weight and evaluated glycated hemoglobin (HbA1c) and pancreas histology in both diabetic and non-diabetic rats to ascertain how triple combination treatment influenced body weight and glycemia, as well as the pancreas. T2DM induction resulted in severe loss of weight and damage to pancreatic islets (Fig. 4A, 4B and 4C). Except for the untreated diabetic (DM untreated) and ramipril-treated (DM+RAM) groups, none of the groups had a change in body weight (Fig. 4A).

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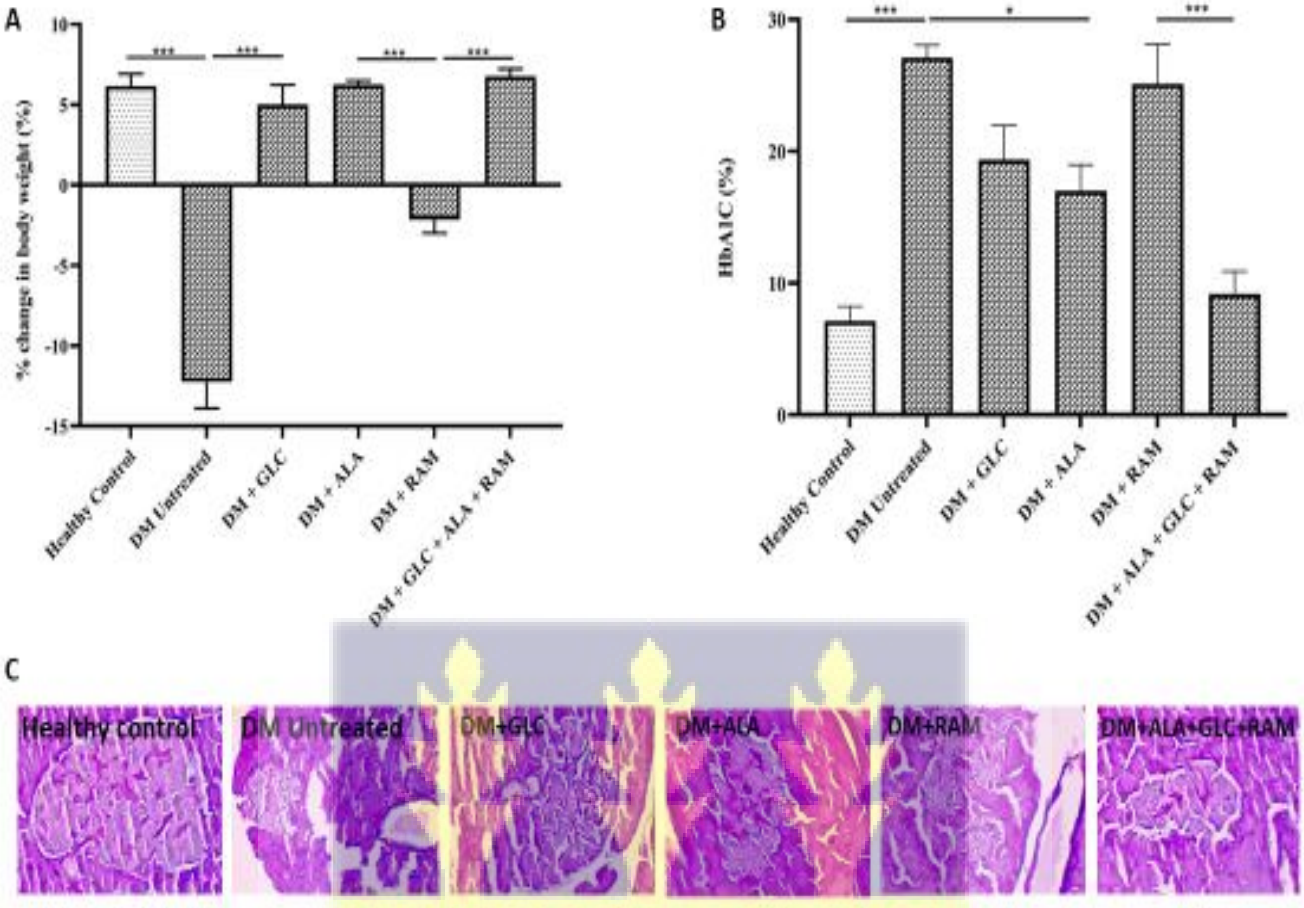

Figure 4. Changes in clinical parameters and pancreas structure. *(A) Change in body weight, (B) glycemic status, and (C) images of pancreas taken at 400x magnification*

4.1 Impact of treatment with ALA, gliclazide and ramipril on diabetic cardiomyopathy

Induction of T2DM followed by no treatment resulted in substantial myocardial fibre degeneration and vacuolation, high relative heart weight and cardiac damage marker5C). While

monotherapies provided some cardioprotection, a higher protection was seen in the group that received all three drugs (ALA, gliclazide and ramipril) (Fig. 5A-5G)

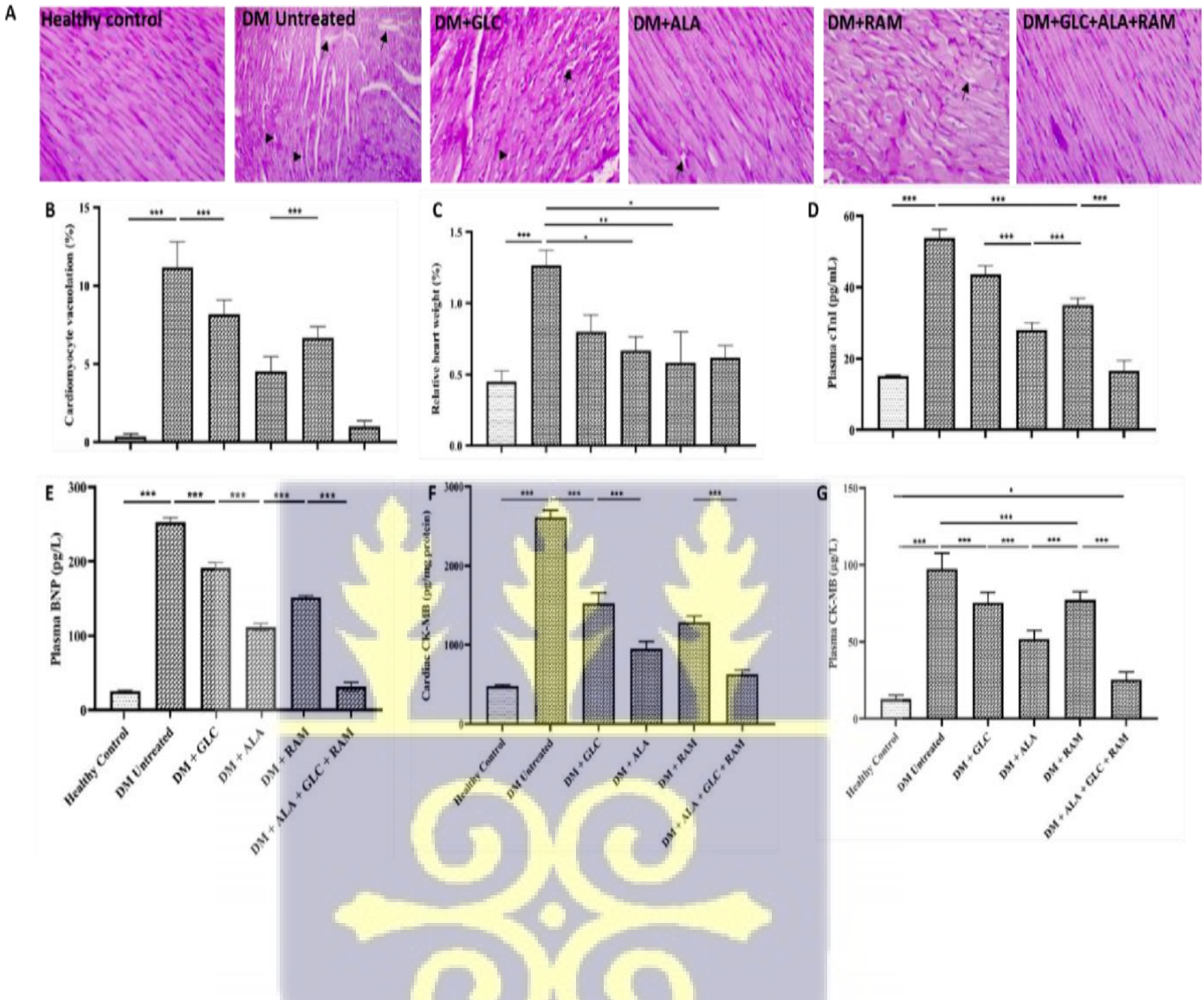

Figure 1. Myocardial structure and markers of cardiac injury. (A) Myocardial structure, and evaluation of (B) cardiomyocyte vacuolation, (C) heart weight/body weight ratio, (D) cardiac troponin I, (E) BNP, (F) cardiac CK-MB and (G) plasma CK-MB. Arrows and arrow heads in histological images point to cardiomyocyte vacuolation and myocardial degeneration respectively.

4.2 Impact of treatment with ALA, gliclazide and ramipril on lipid profile and inflammation.

We observed impaired lipid profile in untreated diabetic rats (Fig. 6A-6C). This was characterized by significantly higher triglyceride and total cholesterol levels while high-density lipoprotein was markedly reduced (Fig. 6A-6C;). Pro-inflammatory cytokines followed a similar pattern as plasma triglyceride and total cholesterol (Fig. 6D-6F). Although single therapy (either ALA, gliclazide or ramipril) of T2DM tried to restore these anomalies, lipid profile and inflammatory level in rats that received all three drugs (ALA, gliclazide and ramipril) were restored near healthy control values (Fig. 6A-6F; $p > 0.05$), (Fig. 6C).

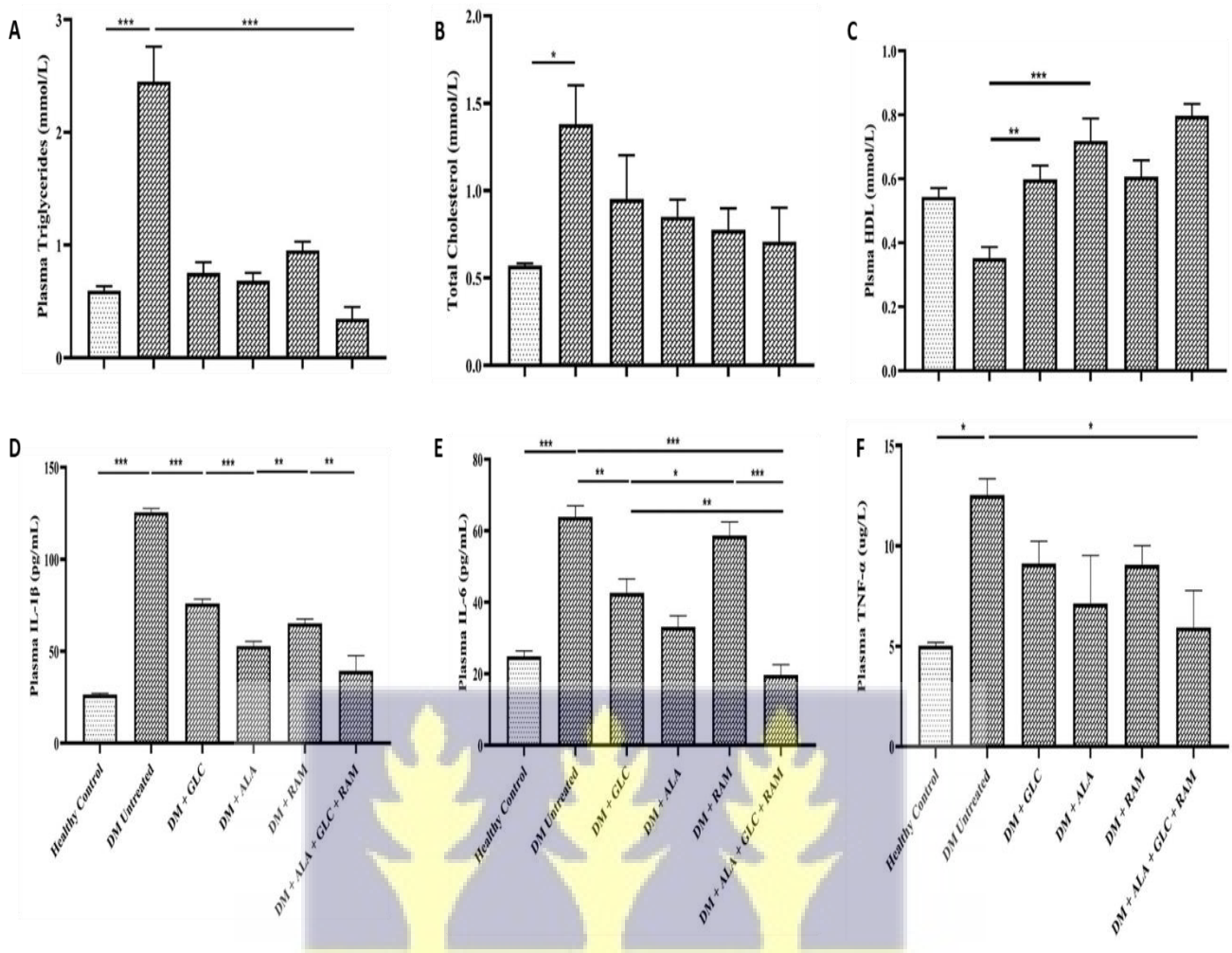

Figure 6. Lipid profile and inflammation. (A) Triglyceride, (B) total cholesterol, (C) plasma high-density liprotein, (D) IL-1β, (E) IL-6 and (F) TNF-α.

4.3 Impact of treatment with ALA, gliclazide and ramipril on antioxidant status in the heart and plasma

We also observed significantly higher production of ROS, indicated by higher MDA level, and markedly lower levels of glutathione and superoxide dismutase antioxidants in untreated diabetic rats (Fig. 7A-7D). Individual treatments with either ALA, gliclazide or ramipril reduced ROS production and raised glutathione and superoxide dismutase levels. However**,** treatment of T2DM rats with all three drugs restored antioxidant status (Fig. 7A-7D).

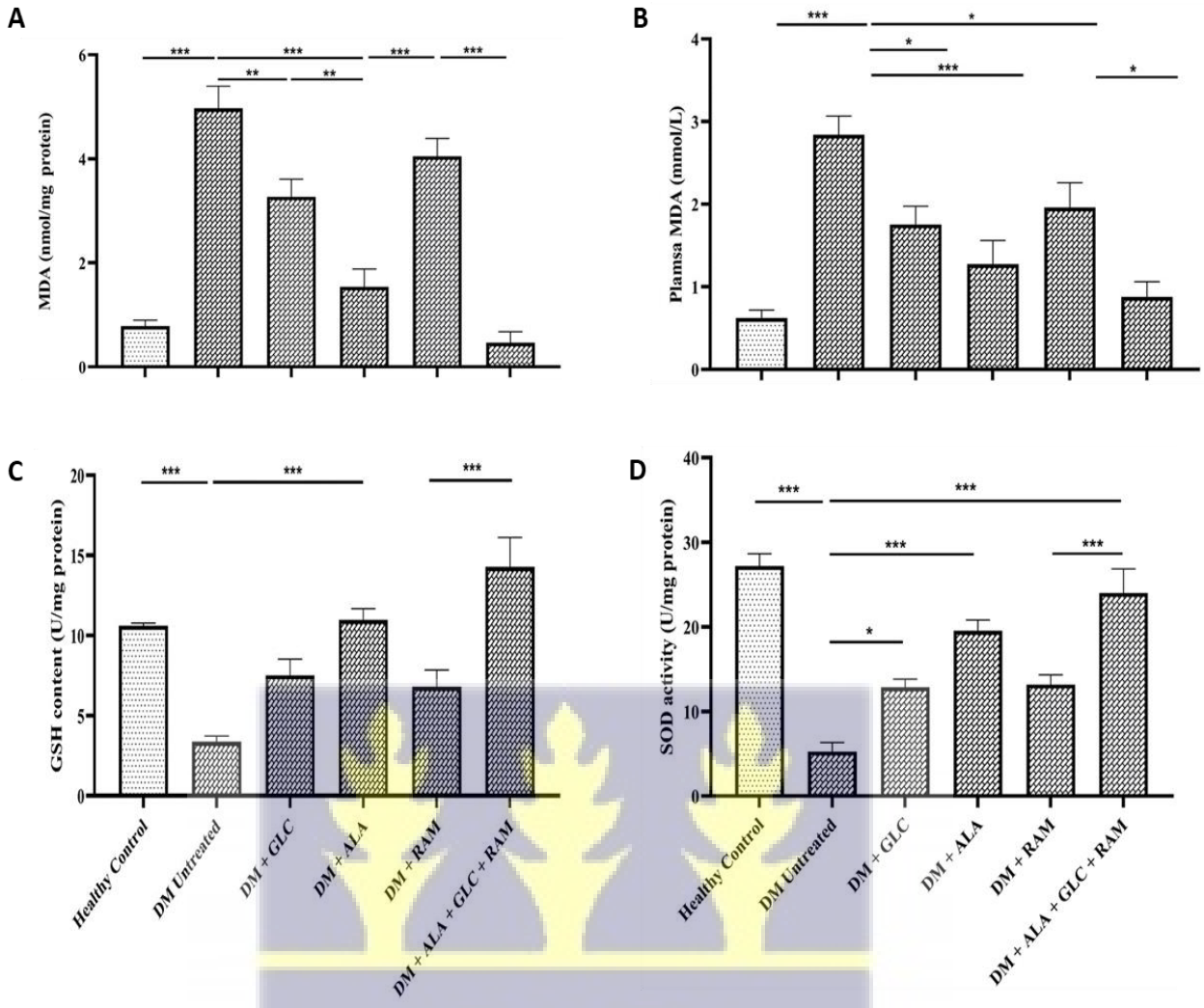

Figure 2. Antioxidants status in the heart and plasma. (A) Malondialdehyde (MDA) level in cardiac tissue, (B) MDA levels in plasma, (C) Glutathione content and (D) Superoxide dismutase activity.

4.4 Impact of treatment with ALA, gliclazide and ramipril on fibrotic pathway in the heart

To investigate whether fibrosis is implicated in our diabetic cardiomyopathy model, we used western blot technique to measure transforming growth factor-beta 1 (TGF-β1) and its effector proteins Smad2 and Smad3 in the heart tissue as a major fibrotic pathway. DM untreated rats expressed substantial amount of TGF-β1, Smad2 and Smad3 in comparison with the expression in the heart tissue of healthy control rats (Fig. 8; $p < 0.001$). Whereas monotherapies with either

ALA, gliclazide or ramipril afforded some level of protection by reducing the expression levels of these fibrotic proteins, treatment with all three drugs in the last group strongly inhibited this fibrotic pathway (Fig. 8).

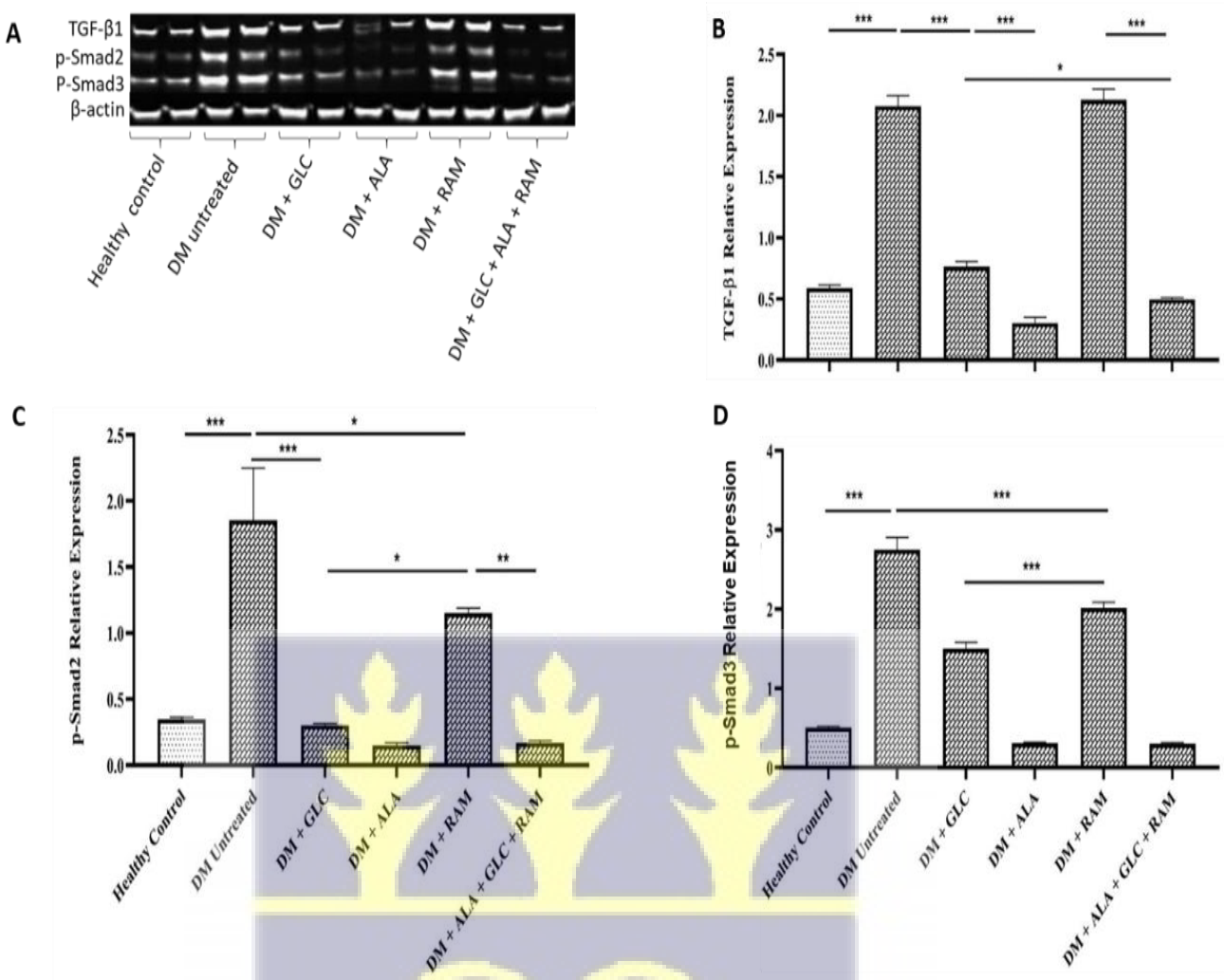

Figure 3. Fibrosis in the heart showing **(A)** expressing level of fibrotic proteins on immunoblot, and measurement of the quantity of **(B)** transforming growth factor-beta 1 (TGF-β1), **(C)** Smad2 and **(D)** Smad3.

CHAPTER FIVE

DISCUSSION AND CONCLUSSION

5.0 DISCUSSION

This study provides a new pharmacotherapeutic strategy to effectively treat or prevent DCM. Our T2DM model in the untreated diabetic rats revealed damaged pancreas, which indirectly culminated in a threefold increase in glycated hemoglobin and a remarkable weight loss, all of which are hallmarks of uncontrolled diabetes mellitus in a clinical situation. In addition to these changes, examination of the heart tissues of this group of rats revealed severe cardiac damage, along with increased damage markers in plasma and heart tissue and activation of fibrotic pathway. Furthermore, biochemical assessment showed impaired lipid profile, decreased antioxidant status and increased inflammation. While single therapy with either alpha-lipoic acid (ALA), gliclazide or ramipril afforded some protection, combined treatment with all three drugs strongly protected and prevented these pathological changes.

Such an anti-hyperglycemic effect and effective protection of the pancreas and heart tissue by the combined treatment suggests that either each of these drugs activated individual protective pathways within these tissues or produced a synergistic action that turned on protective pathways including antioxidant and other pathways that are outside the scope of this thesis and inhibited damaging such as inflammatory, fibrotic, and other unidentified signaling pathways. For example, as ALA is a potent multifunctional antioxidant in the mitochondria, an organelle which also serves as a source of ROS generation, a study by Onozato *et al* (2004) also showed

that gliclazide administration exhibited antioxidant effect by increasing mitochondrial manganese superoxide dismutase, leading to suppression of pro-inflammatory intercellular adhesion molecule-1 (ICAM-1) and improved lipid profile, and consequently protected rats against diabetic nephropathy, another major complication of diabetes mellitus. Importantly, hyperglycemia-induced increase in production of ROS as observed in this study, has been well-established as the central mediator of many pathological pathways including fibrotic, inflammatory, and apoptotic pathways in the developing and progression of DCM and other diabetic complications (Jia *et al*, 2016., Midaoui et al, 2003; Murdoch et al, 2014). Therefore, the addition of ALA to already existing anti-diabetic and anti-hypertensive drugs presents a new pharmacological strategy for effective DCM treatment. Thus, this approach will strongly boost the body's antioxidant defence system at the cellular and subcellular levels and raise a strong resistance against cell death and tissue injury due to hyperglycemia-induced ROS production. Our results also suggest that early detection and early treatment of diabetes mellitus with this combined therapy is an effective way of preventing DCM and possibly other diabetic complications.

Our novel pharmacotherapeutic technique, on the other hand, has certain caveats. Insulin levels in the blood and pancreatic β-cell function could not be measured as a result of technological problems. Measuring these parameters would have provided more insights into understanding the impact of untreated T2DM and the effect of single therapy versus combined therapy. Nevertheless, from the pancreatic histological pictures and glycated hemoglobin levels in the current investigation, it can be deduced that insulin levels in the blood would be much lower and pancreatic β-cell function would be impaired in the untreated diabetic rats, which could have been possibly improved by the combined therapy.

CONCLUSION

Finally, our findings showed that a combination of ALA, ramipril, and gliclazide reduces the with anti-hypertensive drugs such as ramipril in addition to anti-diabetic medications for DCM prevention and treatment. The results of this project add to the current body of information concerning DCM and its pharmacotherapy and management. As a result, the findings from this project may be applied to the human heart to have a better understanding of the pathophysiology of clinical DCM and therapeutic alternatives.

RECOMMENDATION

This research work provides an important addition to the body of existing knowledge about DCM, and its pharmacological treatment. Future research can extrapolate these findings to the human heart to gain insight into the pathophysiology of DCM and its pharmacological treatment.

Also, future studies should include measuring blood insulin and pancreatic B-cell which will give insights into understanding the impact of untreated T2DM and the effect of single therapy versus combined therapy.

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