



Evaluation of Antioxidants Supplementation on Renal, Hepatic and Cardiac Function Markers in Alloxan Induced Diabetes Mellitus Wister Rats

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Authors' contributions

This work was carried out in collaboration among all authors. Author AEBC designed the study. Author IE performed the statistical analysis and wrote the protocol. Authors AAS, NLD and II wrote the first draft of the manuscript. Authors EON and AMA managed the analyses of the study. Both authors read and approved the final manuscript.

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ABSTRACT

Aim: To evaluate antioxidants supplementation (selenium and vitamin E) on renal, hepatic and cardiac function markers in alloxan induced diabetes mellitus in Wister rats.

Study Design: Rats were randomly assigned into 5 groups with each group consisting of 5 rats. The treatment pattern involved the induction of hyperglycaemia in the rats followed by oral administration of selenium and vitamin E supplements singularly and in combination. The groups are as follow:

Group A: Alloxan induced diabetic Rats treated with selenium (0.02 mg/kg) for 35 days.

Group B: Alloxan induced diabetic Rats treated with Vitamin E (70.0 mg/kg) for 35 days.

Group C: Alloxan induced diabetic Ratstreated with both Selenium and Vitamin E (0.02 mg/kg + 70.0 mg/kg) for a period of 35 days.

Group D: Alloxan induced diabetic Rats without any treatment (Positive control) for 35 days.

Group E: Rats in this group were fed normally for 35 days without induction and treatment (Negative control).

Place and Duration of the Study Area: The study was carried out in the Department of Medical Laboratory Science, Rivers State University, Port Harcourt, Nigeria over a period of 9 months (January, 2019 – September, 2019).

Methodology: After the inducement of hyperglycaemia in the rats (Group A – D) with a single dose intraperitoneal (IP) injection of 140mg/kg body weight of alloxan hydrate, treatment with the antioxidants (selenium and Vitamin E) was performed for 35 days. At the end of 35 days, the animals were allowed to fast for 18 hours and sacrificed. Plasma specimen collected was used for the assay of Na⁺, K⁺, HCO₃, urea, creatinine, cardiac troponin I, LDH, AST, ALT, ALP, bilirubin, protein, albumin globulin and MDA while renal, hepatic and cardiac tissues collected were used for histological investigations.

Results: Significantly lower values were seen in Na⁺, K⁺, HCO₃ and conjugated bilirubin in the diabetic rats without antioxidants supplementation (group D) when compared to diabetic rats with antioxidants supplementation of selenium and vitamin E (group A, B & C) and non-diabetic control group (group E). There were no significant differences seen when Group A, B, C and E were compared among one another. Also, significantly higher values were seen in AST, ALT, ALP, Unconjugated bilirubin, urea, creatinine, cardiac troponin I and MDA in the diabetic rats without antioxidants supplementation (group D) when compared with diabetic rats treated with antioxidants supplements of selenium and vitamin E (group A, B & C) and non-diabetic control group (group E). However, no significant differences were seen in LDH, total protein, albumin, globulin and total bilirubin at *P*=.05. Histological findings in the kidneys, liver and cardiac tissues of the rats treated with antioxidants supplement showed recovery tendencies compared to diabetic rats without antioxidant supplementation.

Conclusion: Results obtained suggest that the use of selenium or vitamin E singularly or in combination has ameliorative effect on cardiac, renal and hepatic function markers in alloxan-induced diabetic rats. However, the combination of selenium and vitamin E had no synergistic advantage over the use of selenium or vitamin E alone.

Keywords: Vitamin E; selenium; renal; hepatic; cardiac; diabetic; alloxan hydrate.

ABBREVIATIONS

AGE : Advanced Glycated End-products
ALP : Alkaline phosphatase
ALT : Alanine aminotransferase
AST : Aspartate aminotransferase
Crt : Creatinine
cTn-I : Cardiac Troponin I
CVD : Cardiovascular disease
DM : Diabetes Mellitus
LDH : Lactate Dehydrogenase
MDA : Malondialdehyde
ROS : Reactive Oxygen Species

1. INTRODUCTION

Diabetes Mellitus (DM) is a chronic pathological condition that is characterized by an abnormal increase in glucose concentration primarily due to a defect in glucose metabolism [1]. A defect in the normal functions of the pancreatic beta cells of Langerhans, which is majorly involved in the

production of insulin, has been found to be responsible for this chronic pathological condition. DM is classified into two forms: type I (insulin dependent) and type II (non-insulin dependent). DM Type 1 is a very severe pathological condition that has a much lower prevalence globally than DM Type 2 which has an increasing prevalence globally leading to hyperglycemia, ketoacidosis, microvascular and macrovascular complications, and oxidative stress [2].

Oxidative stress is a condition which is characterized by an abnormally elevated concentration of free radicals that leads to the generation of reactive oxygen species (ROS) formed through various oxidative processes [2]. Oxidative stress is associated with high presence of free radicals in the body which are capable of damaging membranes of vital organs such as those of the kidneys, liver, cardiac, blood vessels and so on [3]. Cellular membrane damages in

oxidative state occurs as these reactive oxygen species or free radicals attack macromolecules or structural components of organs in the body such as proteins, lipids, carbohydrates and DNA thereby altering their normal structure and function [3,4]. Studies have also shown that hyperglycemic induced oxidative stress has been one of the major link between diabetes and diabetic complications; ranging from endothelial dysfunctions, alterations and distortions of pancreatic beta cells; insulin resistance and as well as microvascular and macrovascular complications [4]. In diabetic patients the most commonly known oxidative processes are glycoxidation and peroxidation which results to the formation of advanced glycated end-products (AGE) and Lipid peroxyradicals respectively [3, 4,5]. More so, free radicals can lead to pathological conditions like dyslipidaemia, nephropathy, retinopathy, neuropathy, hepatopathy, and cardiomyopathy, which are major microvascular complications. These complications are the primary cause of morbidity and mortality worldwide [3]. However, oxidative stress associated with the generation of ROS can be attributed to reduction in antioxidants activities. Invariably this means that when the concentration of free radicals exceeds the concentration of antioxidants in the human body, it can potentially lead to disease conditions [6].

Antioxidants are chemical compounds which are produced by the body or sourced from foods [7, 8]. Antioxidants have played important roles in the elimination and inhibition of the formation of ROS and also act as chain breaker. Vitamin E for example acts as an important antioxidant in the human body. Vitamin E is a fat soluble vitamin with anti-oxidative properties. It exists in eight different isomers: alpha-, beta-, gamma-, and delta- tocopherol; and alpha-, beta-, gamma-, delta- tocotrienol. In humans, the alpha tocopherol is the most active form with highest bioavailability. Vitamin E halts the formation of lipid peroxy radicals during peroxidation thereby acting as a chain breaking inhibitor [7,8]. It has been reported that the α -tocopherol form is the most important lipid-soluble antioxidant, and that it protects membranes from oxidation by reacting with lipid radicals produced in the lipid peroxidation chain reaction [9]. Another example of antioxidant is selenium. Selenium is a trace element which acts as an antioxidant derived from dietary sources [6]. It plays a vital role in glutathione antioxidant functioning in cells. The absence or low level of selenium has been

implicated in oxidative damages to cells and in cancers affecting the prostate, cervix, colon, thyroid and so on [10]. Selenium has been reported in several clinical trials in the management of dyslipidaemia, hyperglycaemia, and other chronic metabolic disorders [6,10].

The integrity of the kidney is important in regulating homeostasis, removal of metabolic by-products, maintenance of cellular fluid, production of hormones such as renin and erythropoietin, electrolyte balance, as well as acid-base balance [11,12]. Oxidative stress and ROS most times impairs the functional integrity of the kidney [13]. In assessing the renal functional integrity, biochemical parameters such as electrolytes, urea and creatinine are used [Bakker]. In this study, electrolyte such as sodium, potassium, bicarbonate and chloride were considered as well urea and creatinine.

Cardiac function is essential for the circulation of blood viz-a-viz oxygen to tissues and cells of the body. Cardiovascular disease (CVD) is a class of diseases that involve the heart and blood vessels [14,15]. Diabetic complications such as CVD are one of the leading cause of morbidity and mortality for some population in developed and developing countries of the world with hypertension and heart failure having the highest admissions [16]. Therefore, having a better understanding of the antioxidant supplementations and cardiovascular disease could pave way for better management of CVD and other health conditions. Cardiac troponin I and Lactate Dehydrogenase are markers considered in this study.

Liver is a key organ and site where the metabolism of carbohydrates, lipids and proteins take place [17]. Activities of ROS also affect the liver and therefore the need to assess liver function. In this study, bilirubin, aminotransferases and proteins were considered to assess the hepatic function of the rats. Bilirubin is an endogenous anion formed by the catabolism of heme. It is measured as conjugated bilirubin and unconjugated bilirubin. Conjugated bilirubin is water soluble and unconjugated bilirubin is not soluble in water and it requires solubilizer such as alcohol. Hence when the reaction is carried out in alcohol then total bilirubin is estimated. Unconjugated bilirubin is estimated by subtracting conjugated bilirubin from total bilirubin [18]. The aminotransferases are enzymes that catalyze reversible transfer of the amino group from an amino acid to a

ketoacid in which Pyridoxal-5'-phosphate (PLP) serves as a cofactor [17,18]. Estimation of ALT and AST are sensitive and relatively specific test for hepatocyte damage. Their activities in serum rises even in a small damage of the liver cell, caused by increased permeability of the cell membrane [17,18]. Alkaline phosphatase (ALP) is a family of zinc metalloenzymes, with a serine at the active center; they release inorganic phosphate from various organic orthophosphates. ALP activity is mainly from the liver with contribution from bone [17]. Highest levels of ALP occur in cholestatic disorders, osteomalacia, osteoblastoma, metastatic carcinoma of bone and hyperparathyroidism [17]. Proteins are synthetic products of the liver. They are associated with progressive distortion of the hepatic tissue which in turn induced immunological or inflammatory responses [19]. Albumin as a major transporter protein in the plasma is usually low when hepatic parenchymal tissues are lost or when there are progressive hepatic distortions. In addition, increase in globulin is usually increased in immunological reactions as means of the body defense mechanism which is targeted towards foreign bodies or hepatic distortion [19].

Since antioxidants have reported to play important roles in the elimination and inhibition of the formation of ROS/free radicals and as such their absence could lead to pathological conditions like dyslipidaemia, nephropathy, retinopathy, neuropathy, hepatopathy, cardiomyopathy and other major microvascular complications [7,8]. It is therefore important to evaluate the effect of antioxidants like vitamin E and selenium on renal, hepatic and cardiac function markers in alloxan induced diabetes mellitus in Wistar rats.

2. MATERIALS AND METHODS

2.1 Materials

Materials used in this study include Albino rats, Selenium, Vitamin E, plain bottles, commercially sold protein, albumin, glucose, LDH, troponin I, MDA, Urea and creatinine reagents. Protein, albumin, LDH, urea and creatinine reagents were purchased from Randox diagnostics, UK. MDA and cardiac troponin I reagents were purchased from Elascience, Houston, Texas, USA. Rats specific reagents were used where appropriate. Other materials used include SFRI Ion Selective Electrode (ISE) 4000 Electrolyte analyser (SFRI medical Diagnostics, France), Biotechnica

Spectrophotometer BT 224 (MedWrench), Accu-Chek Active glucometer (Roche, Germany), Ohaus Scout-Pro Electronic weigh balance (Ohaus Corporation, New Jersey, USA), bucket centrifuge (MPW, Poland), incubator at 37°C (Merment, Germany), Microplate reader Stat-Fax 4500 (Awareness Incorporated, California, USA), polypropylene gavage tubes (Intech Laboratory Incorporated, Plymouth Meeting, USA), Haier thermocool refrigerator (China), Shandon AS325 rotary microtome (Fisher Scientific, United Kingdom), digital Olympus microscopic with Camera (Olympus, Tokyo, Japan), automatic pipettes, hypodermic syringe and chloroform.

2.2 Place and Location of the Study

The study was carried out in the Department of Medical Laboratory Science, Rivers State University, Port Harcourt, Nigeria from January, 2019 – September, 2019.

2.3 Experimental Rats

A total of 25 Wistar rats weighing approximately 0.15 kg were used for this study. The rats were obtained from the Department of Medical Laboratory Science, Rivers State University, Port Harcourt, Nigeria and were housed in well ventilated cages with access to water and food (chow) *ad libitum*. They were left to acclimatize under normal laboratory conditions (temperature 24-30°C, relative humidity 60% and 12 hours light-dark cycle) to their new environment for 2 weeks before treatment.

2.4 Experimental Design

After acclimatization, the rats were randomly separated into 5 groups with each group consisting of 5 rats. The treatment pattern involved the induction of hyperglycaemia in the rats followed by administration of selenium and vitamin E supplements singularly and in combination. Glucose concentrations were taken before and after inducement with Alloxan, to ascertain if the Wistar rats were actually hyperglycaemic or diabetic. Our positive control comprises of the rats induced with without any treatment while our negative control were not treated with alloxan or the supplements. The summary of our study design is shown below:

Group A: Alloxan induced diabetic Rat treated with Selenium (0.02 mg/kg) for 35 days.

Group B: Alloxan induced diabetic Rat treated with Vitamin E (70.0 mg/kg) for 35 days.

Group C: Alloxan induced diabetic Rats treated with both Selenium and Vitamin E (0.02 mg/kg + 70.0 mg/kg) for a period of 35 days.

Group D: Alloxan induced diabetic Rats without any treatment (Positive control) and allowed for a period of 35 days.

Group E: The rats in this group were fed normally for a period of 35 days without induction and treatment (negative control).

2.5 Induction of Diabetes Mellitus

Hyperglycaemia was induced in the experimental animals of Group A - D after 2 weeks of acclimatization with a single dose intraperitoneal (IP) injection of 140mg/kg body weight of Alloxan hydrate. Hyperglycaemia was confirmed by determining glucose concentration in the fasting whole blood collected by pricking from the tail region using lancet. Fasting blood glucose before induction was at 4.90 ± 0.58 mmol/L while after the induction, fasting blood glucose was at 24.62 ± 7.34 mmol/L. The determination of the fasting blood glucose was done using Accu-Chek Active glucometer.

2.6 Vitamin E and Selenium Preparations

Vitamin E in the form of DL- α -tocopherol acetate and selenium were purchased from Sigma Aldrich (Germany) and Rotamedics pharmacy (Nigeria) respectively. In terms of Vitamin E, 1.0 ml of the vitamin E contains 500mg of DL- α -tocopherol acetate (water soluble form of vitamin E). In the preparation, the dose for the rats was extrapolated from the human dose of 11.2 mg/kg/day for 60 kg weight (i.e 670 mg/day) to give 70 mg/kg for 0.15 kg rat weight. The 70 mg/kg was obtained by administering 0.02 ml (10.5 mg) in 0.15 kg rats. In terms of selenium, the dose for the rat was also extrapolated from the human dose of 0.003 mg/kg/day for 60 kg weight (i.e 0.2 mg/day) to give 0.02 mg/kg for 0.15 kg rat weight. In the preparation, 0.2 mg of selenium was dissolved in 10.0 ml of water which gives an equivalent of 0.003 mg in 0.15 kg rats by administering 0.15 ml of the selenium solution. The conversion of human equivalent dose (HED) to animal equivalent dose (AED) as seen was obtained using the equation: $HED (mg/kg) = AED (mg/kg) \times 0.16$ as described by Nair and Jacob [20].

2.7 Treatment, Specimen Collection and Preparation

After the inducement of hyperglycaemia in the rats (Group A - D), treatment with the antioxidants (selenium and Vitamin E) was performed orally using gavage tube for 35 days. At the end of 35 days, the animals were allowed to fast for 18 hours and later anaesthetized using chloroform after which cardiac puncture was performed and 5ml of fasting blood samples was collected into lithium heparin bottles. The whole blood was centrifuged at 4000 rpm for 5 minutes to obtain plasma. The plasma was collected into plain bottles for analyses for electrolytes, urea, creatinine, cardiac troponin I, LDH, AST, ALT, ALP, protein, albumin globulin and MDA. Renal, hepatic and cardiac tissues were also collected and fixed in 10% formol-saline, section stained using Haematoxylin & Eosin stain and examined with digital Olympus microscopic with Camera.

2.8 Laboratory Analysis of Biochemical Parameters and Histological Examination

Blood analytes like AST, ALT, ALP, albumin, total protein, LDH, conjugated, and total bilirubin were measured using spectrophotometer while cardiac troponin I and MDA concentrations were measured using Microplate reader. Urea concentration was determined using Berthollet's method as described by Patton and Crouch [21] while creatinine concentration was determined using Jaffe's slot alkaline picrate reaction as documented by Vaishya et al. [22]. Electrolytes (Na^+ , K^+ , HCO_3^-) were analyzed using Ion Selective Electrode (ISE) Method as described by Buck and Linder [23]. Glucose concentration was based on glucose oxidase method as documented by Trinder [24]. Determination of alkaline phosphatase was based on method described by Kind and King [25]. ALT and AST were determined as described by Reitman and Frankel [26]. Determination of total, conjugated and unconjugated bilirubin were based on Malloy and Eveyln's method as described by Dangerfield and Finlayson [27]. Determination of MDA and troponin I was based on ELISA technique described by Engvall and Perlmann [28]. Determination of LDH was based on enzymatic methods described by Ghosh and Mitra [29]. Total Protein was determined by biuret method as described by Henry [30] while albumin determined by bromocresol green dye-binding method described by Speicher et al. [31]. Globulin was determined by subtracting albumin from total protein as described by Busher [32].

Renal, hepatic and cardiac tissues were examined using Haematoxylin & Eosin stain. Blood analytes like AST, ALT, ALP, Albumin, Total Protein, LDH, Conjugated and Total bilirubin were measured using spectrophotometer.

2.9 Statistical Analysis

Values of Raw data obtained were evaluated statistically using GraphPad Prism Version 8.02 (California, USA). Descriptive statistical tools used are mean and Standard Deviation (SD). Inferential statistics was done using one Way ANOVA with Post-Hoc carried out using Turkey Multiple Comparison Test. Statistical significance was set at $P=.05$. Results obtained were expressed as Mean \pm SD.

3. RESULTS

3.1 Results of Renal Function Parameters in Rats

When parameters of renal function was evaluated, Na^+ and K^+ indicated significantly lower values in the diabetic rats without supplementation (group D) when compared to diabetic rats with antioxidants supplementation of selenium and vitamin E (group A, B, & C) and non-diabetic control group (group E). There were no significant differences seen when Group A, B, C and E were compared among one another. More so, when HCO_3^- was considered, there were no significant difference in the comparison between groups A, B, C, and E. However, there was a significantly lower value seen in Group D when Group E was compared. The comparison of Group A, B, C and D did not indicate significant difference at $P=.05$. In addition, when creatinine and urea were considered, significantly higher values were seen in the diabetic rats without supplementation (group D) when compared to diabetic rats treated with antioxidants supplements of selenium and vitamin E (group A, B and C) and non-diabetic control group (group E). There were no significant differences seen when Group A, B, C and E were compared among one another (Table 1).

3.2 Results of Hepatic Function Parameters in Rats

When liver enzymes were considered, AST, ALT, and ALP indicated significantly higher values in

the diabetic rats without supplementation (group D) when compared to diabetic rats treated with antioxidants supplements (groups A, B & C) and non-diabetic control group (group E). There were no significant differences seen when Group A, B, C and E were compared among one another at $P=.05$. In addition, when total protein, albumin and globulin proteins as well as total bilirubin were considered, there were no significant differences. More so, when conjugated bilirubin was considered, there was a significantly lower value seen in diabetic rats treated with selenium and vitamin E combined (group C) as well as non-diabetic control (group E) when compared with diabetic rats not treated with antioxidant supplements (group D) at $P=.05$. The comparison between group A, B and D as well as the comparison between groups A, B, C and D did not indicate any significant difference. at $P=.05$. In addition, when unconjugated bilirubin was considered, significantly higher value was seen in group D when compared with diabetic rats with antioxidants supplementation of selenium (group A) and non-diabetic control (group E). The comparison between group B, C and D did not indicate any significant difference as well as the comparison between group A, B, C and E at $P=.05$ (Table 2).

3.3 Results of Cardiac Function and Oxidative Parameters in Rats

When cardiac and oxidative markers were considered, LDH indicated no significant difference when the groups when compared with one another at $P=.05$. However, there were non-significantly higher values of LDH in the diabetic rats not treated with antioxidant supplements (group D) compared to the diabetic rats with antioxidants supplements (Group A, B and C) as well as the non-diabetic control group (group E). In addition, when cardiac troponin I was evaluated, significantly higher values were seen in diabetic rats without supplements (group D) compared to the diabetic rats treated with antioxidants supplements (Group A, B and C) as well as the non-diabetic control group (group E). More so, significantly higher values were also seen in diabetic rats treated with antioxidants supplements (Group A, B and C) when compared with the non-diabetic control group (group E). However, there were no significant difference when group A, B and C when compared against one another at $P=.05$. Furthermore, when MDA was evaluated, significantly higher values of MDA were seen in the diabetic rats without antioxidant

Table 1. Results of renal function parameters in rats

Parameters	Group A	Group B	Group C	Group D	Group E	Fvalue	Pvalue	Remark
Na ⁺ (mmol/L)	128.50±3.42 ^a	126.25±3.50 ^{ac}	124.75±6.29 ^{ace}	90.50±13.70 ^{bdfg}	134.00±4.62 ^{aceh}	21.962	<0.001	S
K ⁺ (mmol/L)	4.13±0.10 ^a	4.40±0.50 ^{ac}	4.43±0.39 ^{ace}	2.78±0.41 ^{bdfg}	4.28±0.69 ^{aceh}	9.087	0.001	S
HCO ₃ (mmol/L)	18.50±2.08 ^a	20.50±3.70 ^{ab}	19.50±1.29 ^{abc}	16.50±2.64 ^{abcd}	22.50±1.29 ^{abce}	3.529	0.032	S
Crt (μmol/L)	59.50±7.19 ^a	63.25±10.75 ^{ac}	69.75±9.18 ^{ace}	131.25±6.99 ^{bdfg}	55.25±7.50 ^{aceh}	55.469	<0.001	S
Urea(mmol/L)	3.28±0.67 ^a	3.18±0.62 ^{ac}	2.50±0.61 ^{ace}	7.15±0.26 ^{bdfg}	2.48±0.22 ^{aceh}	58.058	<0.001	S

Na, K, Cr & Ur: Values in the same row with different superscript (a, b) differ significantly when group A was compared with other groups. Values in the same row with different superscript (c, d) differ significantly when group B was compared with other groups. Also, values in the same row with different superscript (e, f) differ significantly when group C was compared with other groups. More so, values in the same row with different superscript (g, h) differ significantly when group D was compared with Group E. HCO₃: Values in the same row with same superscript (a) do not differ significantly when group A was compared with other groups. Again, values in the same row with same superscript (b) do not differ significantly when group B was compared with other groups. Also, values in the same row with same superscript (c) do not differ significantly when group C was compared with other groups. Finally, values in the same row with different superscript (c, d) differ significantly when group B was compared with other groups. S=significant at P=.05. Crt = Creatinine

Table 2. Results of hepatic function parameters

Parameters	Group A	Group B	Group C	Group D	Group E	Fvalue	P value	Remark
ALT (U/L)	26.00±2.94 ^a	25.75±6.70 ^{ac}	26.50±1.73 ^{ace}	59.25±5.90 ^{bdfg}	29.50±3.42 ^{aceh}	4.358	0.015	S
AST (U/L)	86.25±12.74 ^a	98.25±29.72 ^{ac}	80.75±22.38 ^{ace}	161.25±7.25 ^{bdfg}	85.50±19.28 ^{aceh}	3.846	0.007	S
ALP (U/L)	217.75±4.99 ^a	212.25±6.80 ^{ac}	206.00±13.14 ^{ace}	263.25±15.13 ^{bdfg}	202.75±17.37 ^{aceh}	11.99	<0.001	S
Albumin (g/dl)	46.75±1.89 ^a	47.75±0.95 ^{ab}	48.25±0.50 ^{abc}	46.75±0.96 ^{abcd}	46.25±2.99 ^{abcd}	0.686	0.613	NS
T. Protein (g/dl)	71.00±6.16 ^a	77.00±2.23 ^{ab}	73.00±1.41 ^{abc}	79.0±5.01 ^{abcd}	74.25±2.80 ^{abcd}	1.535	0.242	NS
Globulin (g/dl)	24.25±4.26 ^a	30.23±1.28 ^{ab}	24.75±0.91 ^{abc}	33.25±4.05 ^{abcd}	27.12±9.18 ^{abcd}	0.857	0.672	NS
T. Bilirubin (μmol/L)	3.00±0.82 ^a	3.65±2.06 ^{ab}	4.34±2.06 ^{abc}	5.06±0.15 ^{abcd}	3.90±0.82 ^{abcd}	1.238	0.156	NS
C. Bilirubin (μmol/L)	1.25±0.96 ^a	1.00±0.82 ^{ac}	2.00±0.27 ^{acd}	0.55±0.50 ^{bcef}	1.75±0.50 ^{aceg}	2.995	0.038	S
Unconj. Bilirubin (μmol/L)	2.64±0.86 ^a	2.45±1.24 ^{ac}	2.34±1.68 ^{acd}	4.63±0.51 ^{bdef}	2.26±0.32 ^{acd}	3.098	0.031	S

ALT, AST, & ALP: Values in the same row with different superscript (a, b) differ significantly when group A was compared with other groups. Also, values in the same row with different superscript (c, d) differ significantly when group B was compared with other groups. Again, values in the same row with different superscript (e, f) differ significantly when group C was compared with other groups. More so, values in the same row with different superscript (g, h) differ significantly when group D was compared with Group E.

Albumin, T. protein, globulin, & T. bilirubin: Values in the same row with different superscript (a) do not differ significantly when group A was compared with other groups. Again, values in the same row with same superscript (b) do not differ significantly when group B was compared with other groups. Also, values in the same row with same superscript (c) do not differ significantly when group C was compared with other groups. Finally, values in the same row with different superscript (d) differ significantly when group D was compared with group E

C. bilirubin&Unconj. Bilirubin: Values in the same row with different superscript (a, b) differ significantly when group A was compared with other groups. Also, values in the same row with different superscript (c, d) differ significantly when group B was compared with other groups. Again, values in the same row with different superscript (d, e) differ significantly when group C was compared with other groups. More so, values in the same row with different superscript (f, g) differ significantly when group D was compared with Group E. S=significant at P=.05.

C. bilirubin=conjugated bilirubin, T. bilirubin= Total Bilirubin, Unconj. Bilirubin=Unconjugated bilirubin

Table 3. Results of cardiac function and oxidative markers

Parameters	Groups A	Group B	Group C	Group D	Group E	Fvalue	Pvalue	Remark
LDH (U/L)	34.40±11.59 ^a	33.91±10.75 ^{ab}	39.48±8.68 ^{abc}	43.00±21.99 ^{abcd}	33.15± 3.04 ^{abcd}	0.45	0.77	NS
cTn-I (ng/ml)	0.12±0.01 ^a	0.18±0.15 ^{ac}	0.19± 0.15 ^{ace}	0.29±0.17 ^{bceg}	0.05±0.01 ^{bdfh}	4.52	0.025	S
MDA(ng/ml)	63.36±21.13 ^a	67.25±17.19 ^{ac}	66.12±13.22 ^{acd}	104.1±40.31 ^{bdef}	57.24±24.01 ^{aceh}	4.84	0.004	S

LDH: Values in the same row with different superscript (a) do not differ significantly when group A was compared with other groups. Again, values in the same row with same superscript (b) do not differ significantly when group B was compared with other groups. Also, values in the same row with same superscript (c) do not differ significantly when group C was compared with other groups. Finally, values in the same row with different superscript (c, d) differ significantly when group B was compared with other groups.

cTn-I: Values in the same row with different superscript (a, b) differ significantly when group A was compared with other groups. Also, values in the same row with different superscript (c, d) differ significantly when group B was compared with other groups. Again, values in the same row with different superscript (e, f) differ significantly when group C was compared with other groups. More so, values in the same row with different superscript (g, h) differ significantly when group D was compared with Group E. MDA: Values in the same row with different superscript (a, b) differ significantly when group A was compared with other groups. Also, values in the same row with different superscript (c, d) differ significantly when group B was compared with other groups. Again, values in the same row with different superscript (e, f) differ significantly when group C was compared with other groups. More so, values in the same row with different superscript (g, h) differ significantly when group D was compared with Group E. S=significant at P=.05. MDA=Melondialdehyde, cTn-I= Cardiac troponin I

treatment (group D) compared to the diabetic rats treated with antioxidants supplements (Group A, B and C) as well as non-diabetic control group (group E). There were no significant differences in MDA when Group A, B, C and E were compared among one another at $P=.05$ (Table 3).

3.4 Histological Examination of Renal, Hepatic and Cardiac Tissues

Histological examinations of renal, hepatic and cardiac tissues in this study are shown in Figs. 1-3.

4. DISCUSSION

The research was carried out in order to assess the ameliorative effects of vitamin E and selenium in diabetic hepatopathy, nephropathy and cardiomyopathy in which diabetic Wistar rats served as model. DM in the rats was induced with Alloxan hydrate. Alloxan hydrate is a chemical compound that destroys the pancreatic β cells of Langerhans, due to this destruction; insulin production becomes low resulting to a dysfunction in glucose metabolism leading to an increase in the concentration of glucose in blood (hyperglycemia). Fasting blood glucose before

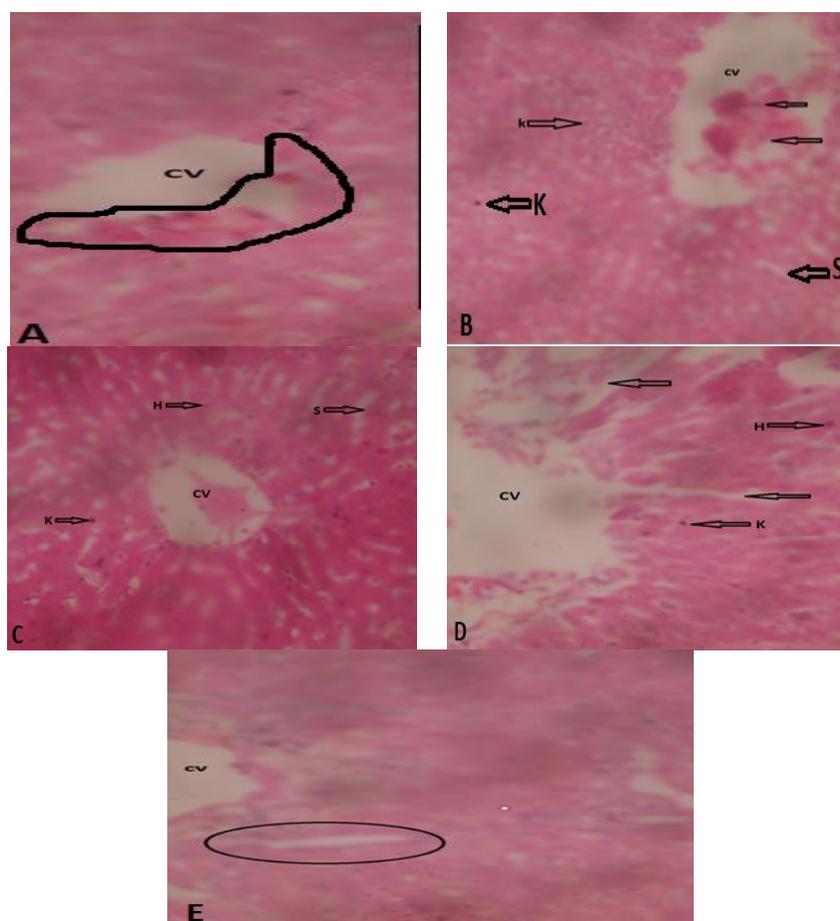


Fig. 1. Histology of hepatic tissues

A. Alloxan+Selenium. Section showed central vein (CV) with mild infiltration (irregular circled area) and Kupffer cells. Hepatocytes and hepatic plates appear normal with intact sinusoids (S). B. Alloxan+vitamin E. The central vein (CV) indicated infiltration of parenchymal materials alongside Kupffer cells (arrows). The sinusoids (S) appear normal with hepatocytes properly arranged alongside the hepatic plates. C. Alloxan+Selenium+vitamin E. The central vein (CV) showed mild infiltration of parenchymal materials. The Sinusoids (S), Hepatocytes (H) and hepatic arrangements are intact. D. Alloxan only. Section showed distorted central vein (CV). Sinusoids (arrows) were also distorted due to mild vacuolation with presence of Kupffer cells (K). Hepatocytes (H) appear hypertrophic within distinct hepatic plates. E. No alloxan+No selenium+No vitamin E. Section showed normal central vein (CV), sinusoids, hepatocytes and hepatic plates. Hepatic portal tract (circled area) appears normal. H&E stain, X400

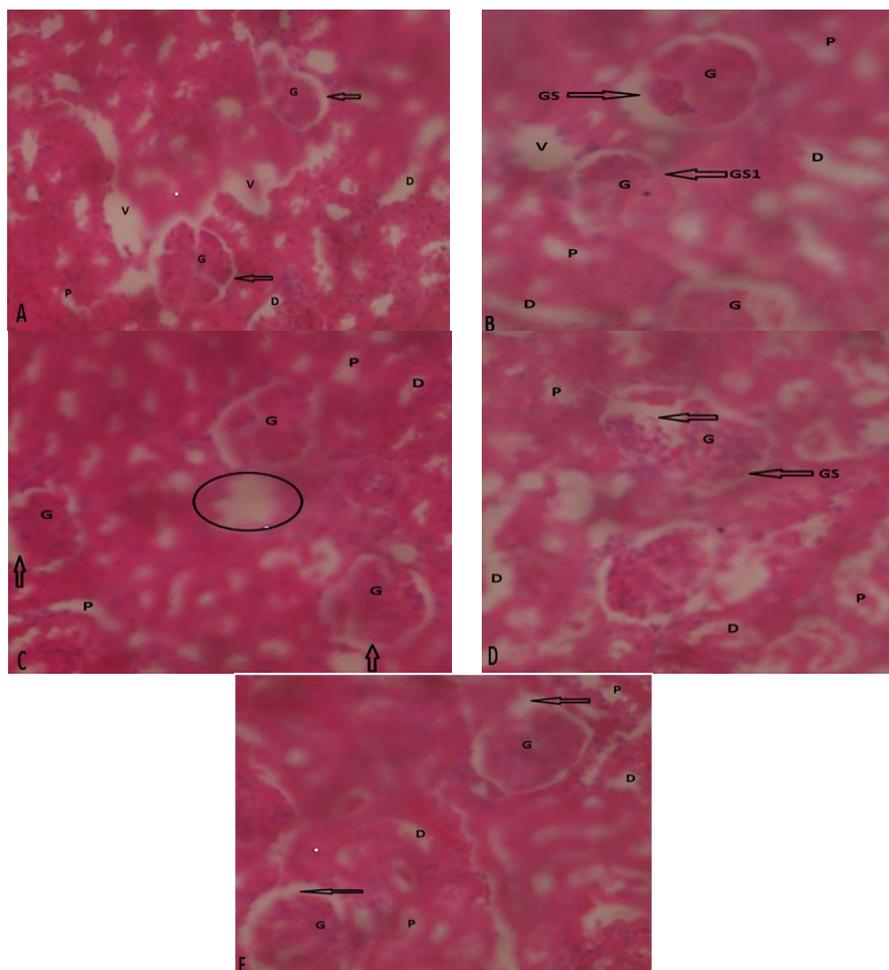


Fig. 2. Histology of renal tissue

A. Alloxan+Selenium. Glomerulus (G) appears normal with hypercellularized mesangial cells. The glomerular space appears normal but distorted at some point (arrow). The proximal tubules (P) and distal tubule (D) appears normal. Loss of parenchymal materials due to vacuolation (V). B. Alloxan+Vitamin E. Glomerulus (G) appears mildly distorted. The glomerular space appears dilated (GS) and obstructed (GS1) at some points. Presence of mild vacuolation (V). The proximal tubules (P) and distal tubule (D) appears normal. C. Alloxan+Selenium+Vitamin E. Glomerulus (G) appears normal alongside distorted glomerular space (arrows). The proximal tubules (P) and distal tubule (D) appears normal. The circled area indicates mild loss of parenchymal materials. D. Alloxan only. Glomerulus (G) appears distorted with vacuolation (arrow) and presence of hypercellularized mesangial cells. The glomerular space (GS) appears normal. The proximal tubules (P) indicate the presence of tubular cast (obstructions). Distal tubule (D) appears dilated at some point. E. Glomerulus (G) appears normal with slightly distorted glomerular space (arrows) at some points. The proximal tubules (P) and distal tubule (D) appears normal. H&E stain, X400

induction was at 4.90 ± 0.58 mmol/L while after the induction, fasting blood glucose was at 24.62 ± 7.34 mmol/L. Therefore, by using a fasting blood reference range of (2.7-7.5 mmol/L) the rats were confirmed to be diabetic.

From our results, the significantly lower values of Na^+ and K^+ and HCO_3^- (Table 1) seen in the diabetic rats without supplementation (group D)

compared to the diabetic rats with antioxidants supplementation of selenium and vitamin E (Group A, B and C) as well as the non-diabetic control group (group E) is in agreement with the findings of [5,9]. They all reported in their respective studies concerning the alleviation of diabetic nephropathy in rats supplemented with a dose of vitamin E. However, rats not supplemented with vitamin E had severely

deranged nephritic biochemical parameters as seen in the Na^+ and K^+ and HCO_3^- . Furthermore, significantly higher values of creatinine and urea were seen in the diabetic rats without supplementation (group D) compared to the diabetic rats with antioxidants supplementation of selenium (Group A, B and C) as well as the non-diabetic control group (group E) which is in agreement with the findings of [5,9]. They [5, 9] reported alleviation of diabetic nephropathy in rats supplemented with a dose of vitamin E. However, rats not supplemented with vitamin E had severely deranged nephritic biochemical parameters as seen in the creatinine and urea. In another related work, [6] also reported significant increase in the level of creatinine and urea not treated with selenium and vitamin E supplementation compared to rats in which selenium and vitamin E supplemented in rats induced nephrotoxicity using cisplatin.

As reported by Prohp and Onoagbe [33], one of the functions of a healthy kidney is to maintain constant blood electrolyte concentrations despite changes in physiological conditions. The results obtained showed that antioxidants supplementation help with the prevention of excessive loss of electrolytes at the proximal tubules. The antioxidants supplementation may have helped increase the electrolyte concentration in the diabetic animals that were treated with antioxidant supplements. Our finding is also in line with the findings of Eteng et al. [34]. They reported that antioxidants such as vitamin E and selenium are vital in ameliorating the depletion of electrolytes under diabetic conditions. More so, the significantly lower value seen in bicarbonate in the diabetic rats without supplementation (group D) compared to the diabetic rats with antioxidants supplementation (Group A, B and C) as well as the non-diabetic control group (group E) could be due metabolic acidosis as a result of ketoacids commonly encountered in diabetic situations in which there is depletion of bicarbonate acting as buffer in trying to cushion the acidosis tendencies. The significant fall also seen in Na^+ and K^+ in the diabetic rats without supplementation (group D) further predicts the presence of acidosis in this group of rats which further suggest nephritic derangements. The significant increase in creatinine and urea in the diabetic rats without supplementation (group D) could be attributed to nephritic derangements particularly of the glomerular region. The significant reduction in potassium seen in group D compared to the other groups could be as a result of loss of large

amount of potassium in the urine due to glucosuria. As potassium moves from intracellular to extracellular space, they (potassium) were also excreted and poorly re-absorbed by the distal tubules due to the inability of it (distal tubules) to build up Hydrogen ion concentration gradient between the distal lamina and the laminal cells. So, the low potassium level with a corresponding low bicarbonate level seen in our results is suggestive of deranging distal renal tubules due to distal renal acidosis. In diabetics, increased proteolysis which is commonly associated with the release of amino acids that are glycogenic which are deaminated in the liver, resulting to an increase in the concentration of urea in systemic circulation. The diabetic rats with antioxidants supplementation of selenium (Group A, B and C) had creatinine and urea values not significant compared to the non-diabetic control group (group E). This explains that the antioxidants supplementation helped in preventing or ameliorating of diabetic nephropathy in diabetic animals by limiting oxidative damages of the nephrons. Furthermore, the non-significant differences seen in our results between the diabetic rats with antioxidants supplementation of selenium (group A), supplementation of Vitamin E (group B) and combined supplementation of selenium and vitamin E (Group C) suggests that combination of selenium and vitamin E does not have any synergetic effect with respect to their antioxidant properties. In other words, the use of selenium or vitamin E singly will give same effect when selenium and vitamin E were combined.

More so, significantly higher values of AST, ALT, and ALP as well as unconjugated bilirubin (Table 2) were seen in the diabetic rats without supplementation (group D) compared to diabetic rats with antioxidants supplementation of selenium and vitamin E (Group A, B and C) as well as the non-diabetic control group (group E). Our observation is in agreement with the findings of Abdelhalim et al. [35]. Abdelhalim et al. [35], reported significant increase in AST, ALT and ALP in rats not treated with vitamin E compared to rats in which vitamin E was supplemented in the treatment of myocardial infarction induced by isoprenaline in albino rats. In addition, our finding is accordance with the reports of Helal et al. [36]. Helal et al. [36], recorded ameliorative effect of Vitamin E in rats in which oxidative stress was induced using Bisphenol A compared to rats not treated vitamin E. Again, our observation correlates with the work of [7]. They [7], also reported significant increase in AST, ALT and

ALP in rats not treated singularly or in combination with vitamin E or selenium compared to rats where vitamin E and selenium were supplemented in the treatment of hepatotoxicity induced by malathion. In addition, the non-significantly higher values seen (Table 2) in the proteins (total proteins, albumin and globulin) in the diabetic rats without supplementation (group D) compared to diabetic rats with antioxidants supplementation (Group A, B and C) as well as non-diabetic control group (group E) is contrary to the reports of Mardy et al. [37]. They reported increase in total protein and albumin levels due to increased production of additional inflammatory cytokines in rats with hepatic and renal toxicity induced by abamectin pesticide. However, they also reported significantly lower values of albumin in rats supplemented with Vitamin E and C combined as an ameliorative effect of these antioxidants.

The significantly higher values seen in AST, ALP, and ALP in the diabetic rats without supplementation (group D) compared to the diabetic rats with antioxidants supplementation (Group A, B and C) as well as the non-diabetic control group (group E) is probably due to inflammation of the hepatic cells. The supplementation of vitamin E and selenium helped in ameliorating the oxidative damages induced by DM. However, use of vitamin E or selenium separately was observed to have similar outcome when vitamin E and selenium were combined in the supplementation process. Also, the significantly higher and lower values of unconjugated bilirubin and conjugated bilirubin respectively seen in the diabetic rats without supplementation (group D) compared to the diabetic rats with antioxidants supplementation (Group A, B and C) as well as the non-diabetic control group (group E) is due to very poor or loss of hepatic function of conjugation as a result of the induced oxidative stress leading to parenchymal damages of the hepatic tissue. Conjugation is an essential function of the hepatic cells where toxins, water insoluble substances are converted into non-toxic, water soluble substances to enhance their excretion preventing bio-accumulation of toxicant in the biological systems.

Again, the significantly higher values of cardiac troponin I (Table 3) in the diabetic rats without supplementation (group D) compared to the diabetic rats treated with antioxidants supplements (Group A, B and C) as well as the non-diabetic control group (group E) is in

agreement with the findings of [8]. They also reported significant increase in cardiac troponin I in rats not treated with antioxidants supplements compared to rats in which vitamin E, C and combination of Vitamin E and C were supplemented in the treatment of cardiotoxicity induced by methidathion. They further reported synergistic beneficial effect on reducing oxidative stress when vitamin E and vitamin C were combined unlike our work where the combination of selenium and vitamin E had no synergy on alleviating cardiac troponin I. More so, [35], also reported significant increase in cardiac troponin I in rats not treated with vitamin E compared to rats in which vitamin E was supplemented in the treatment of myocardial infarction induced by isoprenaline. Furthermore, our study showed that cardiac troponin I were still significantly increased in the diabetic rats with antioxidants supplementation of selenium (Group A, B and C) compared to the non-diabetic control group (group E) but not in similar degree when compared to diabetic rats without supplementation (group D). This finding is contrary to the reports of [8,35]. They reported significant reversal of cardiac troponin I in antioxidant supplemented groups compared to non-diabetic control group.

In addition, the non-significant differences seen in LDH (Table 3) in the diabetic rats without supplements (group D) compared to diabetic rats with antioxidants supplementation of selenium (Group A, B and C) as well as non-diabetic control group (group E) is contrary to the findings of Abdelhalim et al. [35]. Abdelhalim et al. [35], reported significant increase in LDH in rats not treated with vitamin E compared to rats in which vitamin E was supplemented in the treatment of myocardial infarction induced by isoprenaline in albino rats. [7], also reported significant increase in LDH in rats not treated singularly or in combination with vitamin E or selenium compared to rats in which vitamin E and selenium were supplemented in the treatment of hepatotoxicity induced by malathion in rats. Finally when MDA was considered (Table 3), significantly higher values of MDA were seen in the diabetic rats without antioxidant supplements (group D) compared to diabetic rats with antioxidants supplements (Group A, B and C) as well as non-diabetic control group (group E). The result observed is in agreement with the findings of [9]. They reported alleviation of diabetic nephropathy in rats supplemented with a dose of vitamin E and as such decreased MDA values were noticed. However, rats not supplemented with vitamin E had severely increased

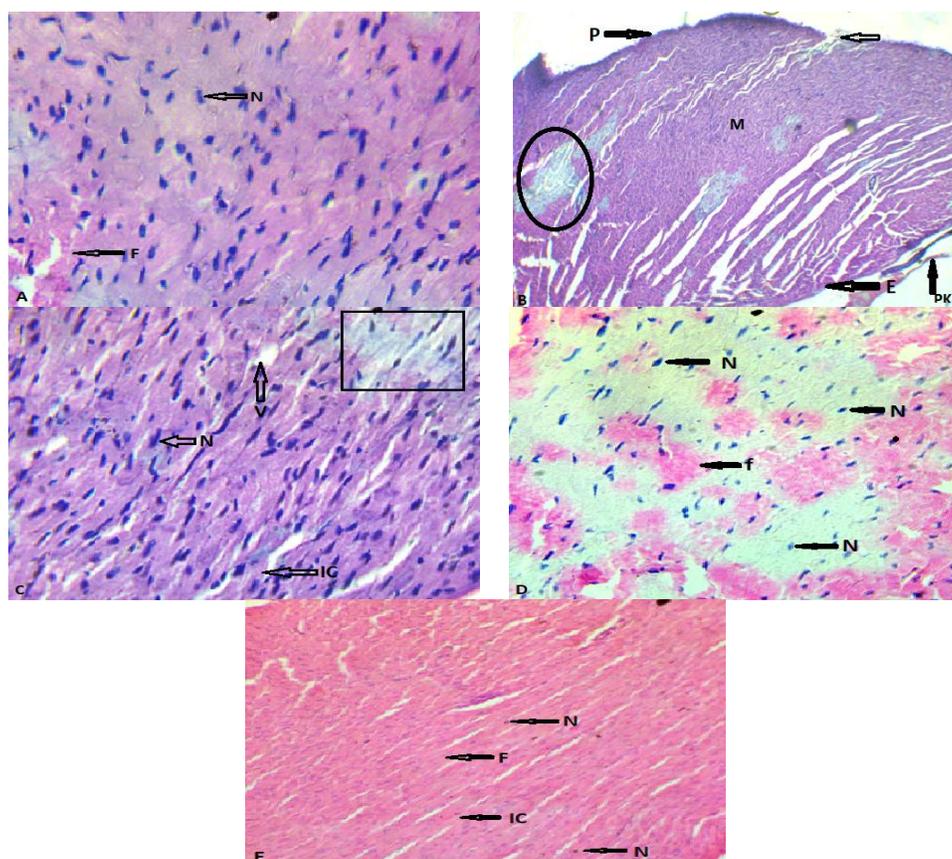


Fig. 3. Histology of cardiac tissue

A. Alloxan+Selenium. Cardiac tissue (endocardium) indicates hypertrophied nucleus (N) of cardiomyocytes. Fibrous connective tissues (F) are distinct. H&E stain, X400. B. Alloxan+Vitamin E. cardiac tissue showing the pericardium (P), myocardium (M) and Endocardium (E) with the purkinje fibre (PK). The epicardial surface of the pericardium indicates some form of distortion (arrow). Within the myocardium, slight loss of cardiomyocyte due to necrosis was seen (circled area). H&E stain, X200 C. Alloxan+Selenium+Vitamin E. cardiac tissue (myocardium) showed hyperplasia of cardiomyocytes and hypertrophied nucleus (N). The myofiber are distinct linked by the intercalated disc (IC). Mild loss of parenchymal materials was also seen (rectangular area) as well as vacuolation (V). H&E stain, X400 D. Alloxan only. Cardiac tissue (endocardium) indicates hyperplasia of cells nuclei (N). Loss of fibrous connective tissues (f) was also seen. H&E stain, X400. E. Cardiac tissue (myocardium) indicates the presence of normal cardiomyocyte with nucleus (N). The myofibrille (F) are distinct and intercalated disc (IC). H&E stain, X200

MDA value. The significant increase in MDA indicates oxidative stress precisely of lipid peroxidation source. Therefore, the non-significant increases of MDA seen in antioxidants supplemented groups compared to non-diabetic control group (group E) indicate ameliorative effect of the antioxidant on the oxidative stress induced due to DM or hyperglycaemia in the rats.

In addition, the histological examination of liver, renal and cardiac tissues after 35 days of selenium and vitamin E supplementation showed varying histological alterations in the various groups. Hepatic tissues of rats treated singularly

with selenium and vitamin E as well in combination showed normal histology of the liver with alteration seen in the central vein with mild to moderate infiltration of parenchymal materials and Kupffer cells (Fig. 1A, 1B and 1C). However, the infiltration seen in vitamin E supplemented rats were more pronounced compared to selenium supplemented and selenium+vitamin E supplemented rats (Fig. 1A, 1B and 1C). The histology of the liver of diabetic rats treated with selenium and vitamin E singularly and in combination indicates inflammation but structural alterations seen were milder and the loss of hepatic parenchymal tissues were not obvious

compared to the diabetic rats not treated with antioxidants (group D). Histology of the diabetic rats without supplementation (group D) showed distorted central vein and sinusoids due to vacuolation with presence of Kupffer cells. The hepatocytes appear hypertrophic within distinct hepatic plates. The histological results seen in group D diabetic rats indicate inflammation and loss of hepatic parenchymal tissues (Fig. 1D). Histology of the non-diabetic rats (group E) showed normal central, sinusoids, hepatocytes and hepatic plates. Hepatic portal tract appears normal (Fig. 1E). These findings are also in agreement with the reports of [7]. They also reported hepatic distortions and infiltration of central vein.

When histology of the renal tissue was considered, renal tissues of rats treated with selenium supplements showed normal proximal and distal tubules as well as glomerulus with hypercellularized mesangial cells within. The glomerular space appears normal but distorted at some point. Losses of parenchymal materials due to vacuolation were also observed (Fig. 2A). In rats supplemented with Vitamin E as well as those supplemented selenium+Vitamin E, the glomerulus appears mildly distorted. The glomerular space appears dilated and obstructed at some points. Mild vacuolations were also seen. However, the proximal tubules and distal tubule appeared normal (Fig. 2B and 2C). These alterations seen indicate inflammation but the structural alterations seen were milder. More so, the distortion of the glomerulus itself was not obvious compared to the diabetic rats not treated with antioxidants (group D). Histology of the diabetic rats without supplementation (group D) showed distorted glomerulus with vacuolation and presence of hypercellularized mesangial cells. The proximal tubules indicate the presence of tubular cast (obstructions) while distal tubular dilation was also seen at some points. However, the glomerular space appears normal (Fig. 2D). The histological changes indicate glomerular nephritis. Histology of the non-diabetic rats (group E) showed normal glomerulus, proximal tubules and distal tubule. These histological findings are also in line with the reports of [6]. They [6] also reported distorted glomeruli and tubular dysfunction as well as severe vacuolation. They further reported decreases in degenerative features in the kidneys of rats treated with antioxidants compared to cisplatin-induced nephrotoxicity rats.

When histology of the cardiac tissue was considered, tissues of rats treated with selenium

supplements for a period of 35 days after induction of hyperglycaemia showed hypertrophied nucleus of cardiomyocytes with distinct fibrous connective tissues (Fig. 3A). In rats supplemented with Vitamin E, the epicardial surface of the pericardium indicates some form of distortion while within the myocardium, slight loss of cardiomyocytes due to necrosis were seen (Fig. 3B). In those supplemented selenium+Vitamin E, cardiac tissue (myocardium) showed hyperplasia (increase in number) and hypertrophied (increase in size) nucleus of the cells. The myofibres are distinctly linked by the intercalated disc. In addition, mild loss of parenchymal materials as well as vacuolation was also seen (Fig. 3C). In summary, the histology of diabetic rats treated with antioxidant supplements indicates hyperplasia and hypertrophy of the cardiomyocytes, vacuolation and distorted epicardial surface. These alterations indicate inflammation but these structural alterations seen were milder compared to the diabetic rats not treated with antioxidant supplements (group D). Histology of the diabetic rats without antioxidant supplements (group D) showed the presence of hyperplasia of the cell nuclei alongside the loss of fibrous connective tissues within the endocardium (Fig. 3D). The histological changes seen indicate cardiac inflammatory disturbances. Histology of the non-diabetic rats (group E) showed normal cardiomyocytes with nucleus. The muscle fibres are distinct connected by intercalated disc. These histological findings are also in line with the reports of Abdel-Samia et al. [38]. They also reported decreased histological alterations such as vacuolated cardiac muscle fibres, mitochondria degeneration as well as congested and dilated blood vessels in rats treated with antioxidants supplements compared to doxorubicin-induced cardiotoxicity rats.

The histological results seen in the rats treated with selenium and vitamin E singular or combined compared to the diabetic rats generally indicates recovery tendencies of rats which could be attributed to the reduced or ameliorating effect of the antioxidant supplements. This also explains why the liver enzymes, renal and cardiac parameters as well as MDA indicated a significant fall after a period of 35 days supplementation (Tables 1, 2 and 3) compared to diabetic rats without supplementation. More so, the results obtained indicate that supplementation of selenium and vitamin E separately and in combination ameliorate the

derangements induced by diabetes. In a similar work done by Ben-Chioma and Elekima [39], it was reported that selenium and vitamin E improved the immune function in women with breast cancer as they “mop up” free radicals generated through oxidative processes and lipid peroxidation while degenerative outcomes were observed in women with deficient selenium and vitamin E levels in their serum.

5. CONCLUSION

Results obtained showed that the use of selenium or vitamin E singularly or in combination has ameliorative effect on cardiopathy, nephropathy and hepatopathy in alloxan-induced diabetic rats. However, the effect seen in cardiac markers such as troponin I was not decreased significantly as seen in other organ. Again, the combination of selenium and vitamin E has no synergistic advantage over the use of selenium or vitamin E alone.

6. RECOMMENDATION

The use of selenium or vitamin E in the therapeutic management of diabetics should be encouraged before of their strong antioxidative roles played by these vitamins and trace elements in chronic disease conditions such as dyslipidaemia and hyperglycaemia associated strongly with DM.

7. LIMITATION OF THE STUDY

The study was not designed to cover longer duration rather it was on a short-term period of 35 days.

CONSENT

It is not applicable.

ETHICAL APPROVAL

We hereby declare that the Principles of laboratory animal care (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments concerning the use of animals was approved by the Rivers State University research/ethics committee with file No: RSU/CV/APU/74/VOL.VIII/104.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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