Co-administration of metformin and vitamin C or E in improves diabetes prognosis in streptozotocin-induced diabetes rats

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Abstract

The prevalence of diabetes mellitus has increased astronomically in recent years. This has resulted in increased disease adjusted life years (DALYs), mortality and decrease in life expectancy. Some of the fall outs of diabetic disease in patients include hyperlipidaemia, oxidative stress, renal and hepatic dysfunction. This study investigated the combined effects of metformin with either vitamin C or E on hyperglycemia-induced hyperlipidemia, oxidative stress, renal and hepatic dysfunction.

Diabetes mellitus was induced in the animals fasted overnight with a single dose of streptozotocin (40 mg/kg, intraperitoneal). Animals with fasting blood glucose (FBG) levels >200 mg/dl after 48 hours of streptozotocin administration were marked diabetic and were randomly grouped into 6 groups of 5 diabetic rats each. Treatments were administered orally once daily for two weeks. FBG levels, and other parameters were determined.

The results showed that the various treatments significantly and comparably reduced the FBG levels on both day 7 ($p<0.05$, $P<0.01$) and day 14 ($p<0.001$). All the treatments significantly reduced hyperglycemia-induced increase in the levels of triglyceride (TG), total cholesterol (TC), alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatinine and malondialdehyde (MDA). The antioxidant enzyme activity of superoxide dismutase (SOD), glutathione reductase (GSH), and catalase (CAT) was enhanced by the combined effects of metformin and vitamin C or E. Vitamin E and combination of metformin and vitamin E did not prevent diabetes induced-weight loss throughout the study compared to diabetic control.

The findings have demonstrated that metformin and vitamin C co-administration

exhibits synergistic effects on blood glucose lowering effect, antioxidant activity and potential advantage of preventing multi-organ damaging effects of type 2 diabetes.

Key words: diabetes mellitus, oxidative stress, semen analysis, metformin, vitamin C, vitamin E.

Introduction

Worldwide, diabetes mellitus (DM) remains a serious challenge and the prevalence is on the increase, particularly in the low- and middle-income countries (Cho *et al.,* 2018). The rise is attributed to aging population, rapid urbanization, unhealthy diet, increasing sedentary lifestyles and obesity (Basu *et al.,* 2013). It was shown that over 425 million people are diabetics, with 1.6 million deaths resulting from this disease, representing the 7th leading cause of death in 2016 (WHO, 2017).

Diabetes mellitus (adult onset) often lead to multiorgan deleterious effects. The deleterious effects include the formation of advanced glycated end-products, activation of protein kinase C, hexosamine pathway, increased glucose flux through polyol pathway and increased oxidative stress, culminating in micro-vascular (diabetic nephropathy, retinopathy and neuropathy) and macrovascular (cardiovascular dysfunction) complications (Maitra, 2015). The management of Diabetes and its complications involves the use of antidiabetic agents and some vitamin supplements.

Metformin, an oral hypoglycemic biguanide anti-diabetic drug, suppresses glucose production from the liver, gastrointestinal glucose absorption, and improves sensitivity to insulin. Vitamins are commonly recommended as food supplements in management of diabetics. Vitamins C and E are well-known for their antioxidant effect and are co-factors and co-enzymes in many metabolic pathways. The appraisal of the effect of co-administration of both vitamins with metformin on hyperglycemia-induced hyperlipidemia, oxidative stress, renal and hepatic dysfunction in diabetic rats was investigated in this work. The outcome of this study may give new insights in the management of DM patients and contribute to the body of scientific knowledge.

Materials and Methods

Materials

Metformin tablets (S.L Poligome Merck, Barcelona, Spain), vitamin C (Cartivalue Chemical Ltd, India), vitamin E (Puritan Pride Inc., USA), streptozotocin (Santa Cruz

Biotechnology, Germany), sodium citrate and citric acid (BDH Chemicals, Uk), glucometer and glucometer strips (Accu-Chek® Active, Roche Diagnostic, USA), pH meter, digital weighing balance, chloroform (analytical grade) were used for this study. All the assay kits for biochemical assessments were gotten from Randox Laboratories Ltd (UK).

Experimental animals

Thirty-five healthy Wistar rats, all males with body weight of 200 - 250 g, were purchased from the animal breeding house in the Pharmacology & Toxicology Department, Faculty of Pharmacy, University of Benin, Benin City, Edo State, Nigeria. Animals were housed in the plastic rat cages, in a well-ventilated animal room at temperature of 24 ± 2ºC with a fixed 12/12 hour light/dark cycle. The animals had free access to water and rodent pellet diet. All experiments were performed in accordance with the National Institute of Health Guidelines for the care and use of laboratory animals and approval was obtained from the Faculty of Pharmacy Animal Ethics Committee, University of Benin, Nigeria (EC/FP/018/35).

Experimental induction of diabetes mellitus

Type 2 DM was induced in the animals fasted overnight by an intraperitoneal injection of a single dose of streptozotocin (STZ) 40 mg/kg, dissolved in 0.1M citrate buffer at pH of 4.5 that was freshly prepared. After STZ administration, the animals had water and feed *ad libitum*. After 48 hours, blood glucose levels of the animals were taken and those with fasting blood glucose (FBG) levels >200mg/dl were considered diabetic and selected for the study (Mostafavinia *et al.,* 2016).

Experimental treatment design

Thirty five diabetic animals were randomly assigned to 6 groups of five animals each. Group 1 and 2 served as the normal (non-diabetic) and diabetic control rats that received distilled water (0.5 ml). Group 3 to 7 were the diabetic rats that received vitamin C (1000 mg/kg), vitamin E (600 mg/kg), metformin (500 mg/kg), metformin (500 mg/kg) and vitamin C (1000 mg/kg), and metformin (500 mg/kg) and vitamin E (600 mg/kg) respectively. Drug administration commenced 48 hours after the confirmation of diabetes and the treatment was carried out once daily for 14 days via oral route.

Body weight determination

Body weight of each rat was determined on day 1, 7 and 14 of the experiment using a digital weighing balance. Change and percentage change in their body weights were calculated and recorded following Uchendu's method (Uchendu *et al.,* 2016).

Biochemical analysis

The FBG levels were measured according to Marques method (Marques *et al.,* 2016) using Accu-check glucometer (mg/dl) following manufacturer's instructions. This was carried out on day 0 (before commencement of treatments), day 7 and day 14 after overnight fasting of the animals.

On the 15th day, the overnight fasted animals were euthanized under chloroform anesthesia and blood was collected from the abdominal aorta. The blood was centrifuged at 3500 rpm for 15 minutes. The serum obtained for each group was used to assay for lipid profiles (total cholesterol (TC), , low-density lipoprotein (LDL) high-density lipoprotein (HDL) and triglyceride (TG)), liver function (alanine aminotransferase (ALT), alkaline phosphatase (ALP aspartate aminotransferase (AST)), renal function (creatinine, urea) and oxidative stress parameters (malondialdehyde (MDA), superoxide dismutase (SOD) glutathione reductase (GSH), and , glutathione peroxidase (GPx), catalase (CAT)) as stated by the details provided in the Randox kit's instructions.

Statistical Analysis

Data were expressed as mean ± standard error of mean (S.E.M). One-way analysis of variance (ANOVA) was used to determine the significant effect among the treatment groups. Tukey's multiple post–hoc test was used for comparisons of subsequent results. Graph pad prism version 6.0 software was used for statistical analysis. The $p < 0.05$ was considered significant.

Results

Effects on Body weight

A persistent reduction in body weight was witnessed in diabetic control group over the period of the investigation, compared to non-diabetic control group (Table 1). Treatments with vitamin C ($p<0.05$), metformin ($p<0.001$) and metformin + vitamin C ($p<0.001$) combination significantly reduced the body weight loss compared to diabetic control. Treatments with vitamin E, and metformin + vitamin E combination did not significantly improve body weight in diabetic rats.

Effects on Blood glucose levels

The various treatments significantly lowered the mean FBG levels of the diabetic animals at day 7 ($p<0.05$, $p<0.01$) and day 14 ($p<0.001$) when compared to the diabetic control animals (Figure 1). The combination of metformin and vitamin C displayed a comparatively greater reduction of FBG compared to metformin alone.

Effects on lipid profile

The result revealed a significant ($p<0.001$) reduction on serum TC and TG levels in all the animal groups that received some form of treatment, compared to diabetic control group. Also, the serum LDL level was significantly reduced in groups treated with vitamin C, metformin, and metformin plus vitamin E combination when compared to diabetic animal group as shown in Figure 2.

Effects on renal function

The creatinine levels were significantly ($p<0.05$) reduced in groups treated with Vitamin E metformin, and combination of metformin + vitamin C. Treatment with Vitamin C, and metformin n + vitamin E showed no significant difference in creatinine levels compared to diabetic control. Furthermore, a significant increase in serum potassium concentration was found in diabetic rats treated with metformin alone compared to the diabetic control. No significant difference was observed in serum urea. This is depicted in Table 2.

Effects on liver function.

The serum levels of ALP, ALT and AST were significantly increased in the diabetic control group compared to normal control. All the treatments significantly decreased ALP, ALT and AST levels as compared to the diabetic rats, with the lowest figures recorded with Metformin/Vitamin C combination. This is shown in table 3.

Effects on Oxidant level and antioxidant activity

There was significant increase in MDA level in the diabetic animal control group, compared to normal control animals. However, all the treatments significantly ($P<0.0001$) decreased the serum MDA level compared to the diabetic control animals. Serum SOD, CAT, and GSH enzyme activities in the diabetic control rats were significantly reduced compared to the normal rats. All the treatments except metformin significantly elevated the serum SOD activity in the diabetic animals compared to the diabetic control animals. A significant increase in serum CAT and GSH activities was observed in the diabetic animals treated

with metformin, vitamin C, vitamin E, and combination of metformin and vitamin C or E. Interestingly, the increased activities of serum SOD, CAT and GSH observed in combination of metformin and vitamin C or E group were greater than metformin alone. Meanwhile, all the various treatments showed no significant change in the serum GPx activity compared the normal control.

Table 1. Effect of treatments on body weight in streptozotocin (STZ)-induced diabetic rats.

Treatments	**Day 1**	**Day 7**	**Day 14**
	Body weight (g)	**Change in weight (g)**	**Change in weight (g)**
Normal control	209.00±1.84	39.50±3.84	49.34±9.72
Diabetic untreated	238.15±7.09	-21.55±9.70	-33.20±10.86
Vitamin C	235.70±11.30	2.72±2.31*	2.05±7.13*
Vitamin E	248.18±3.79	-6.30±6.32	-9.34±14.19
Metformin	275.13±6.36	15.25±7.79***	-1.43±8.60
Metformin + Vitamin C	248.72±4.57	12.00±2.73***	4.92±7.26
Metformin + Vitamin E	231.86±6.69	-3.22±10.11	-4.58±10.19

Values are expressed as mean ± SEM.*$p<0.5$, **$p<0.01$, ***$p<0.001$ when compared with diabetic control, n=5.

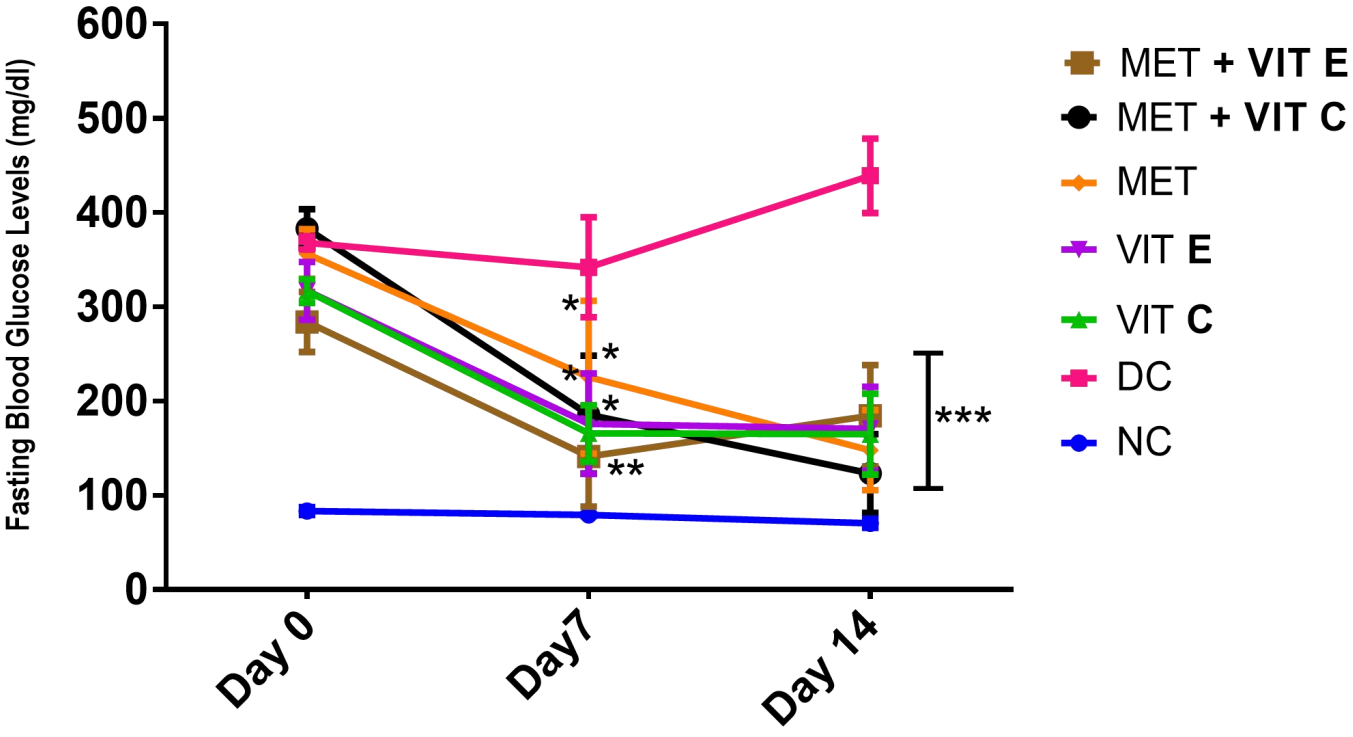


Figure 1. Blood glucose levels in STZ-induced diabetic rats treated with vitamin C (VIT C), vitamin E (VIT E) , metformin (MET) , metformin + vitamin C (MET + VIT C), and metformin + vitamin E (MET + VIT E). NC, normal control group; values are expressed as mean ± SEM. *$p<0.05$, **$p<0.01$, ***$p<0.001$ when compared to diabetic control, n=5.

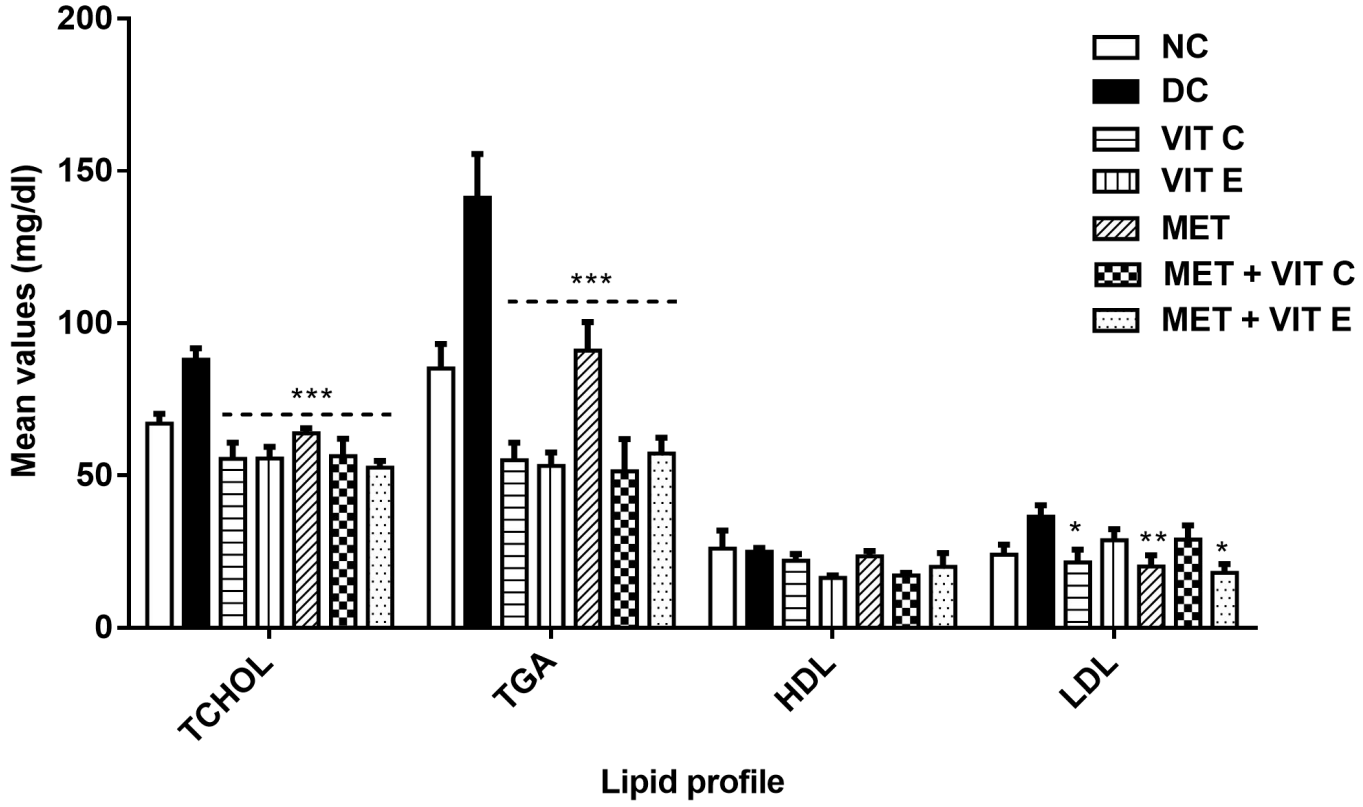


Figure 2. Lipid profile of the treatments in STZ-induced diabetic rats compared to diabetic control. TCHOL, total cholesterol; TGA, total triglycerides; HDL, high density lipoproteins cholesterol; LDL, low density lipoproteins cholesterol; NC, normal control; DC, diabetic control; VIT C, vitamin C; VIT E, vitamin E; MET, metformin; MET + VIT C, metformin and vitamin C; MET + VIT E, metformin and vitamin E . Values are mean ± SEM. *$p<0.05$, **$p<0.01$, ***$p<0.001$, n = 5.

Table 2. Effects of the treatments on renal function in STZ-induced diabetic rats.

GROUPS	Urea (mg/dl)	Creatinine (mg/dl)	Na^+ (mmol/L)	K^+ (mmol/L)	$HCO3^-$ (mmol/L)	Cl^- (mmol/L)
NC	30.20 ± 1.36	0.50 ± 0.03	144.00 ± 0.89	5.50 ± 0.14	23.00 ± 3.32	107.60 ± 0.40
DC	49.25 ± 9.28	0.63 ± 0.03	140.00 ± 1.00	6.03 ± 0.20	25.50 ± 0.50	102.25 ± 2.18
VIT C	56.50 ± 4.78	0.58 ± 0.07	141.00 ± 3.06	6.33 ± 0.50	23.83 ± 1.30	104.50 ± 1.73
VIT E	60.60 ± 5.96	0.42 ± 0.04*	144.80 ± 1.46	6.82 ± 0.57	21.60 ± 1.03	105.80 ± 2.50
MET	53.17 ± 2.60	0.42 ± 0.04*	141.17 ± 2.15	7.35 ± 0.41*	20.50 ± 1.03	104.33 ± 2.45
MET + VIT C	50.33 ± 3.42	0.45 ± 0.06*	141.50 ± 0.96	6.93 ± 0.29	21.11 ± 0.79	105.50 ± 2.08
MET + VIT E	55.00 ± 6.96	0.50 ± 0.07	139.80 ± 3.02	6.18 ± 0.19	21.20 ± 1.86	102.60 ± 2.04

NC, normal control; DC, diabetic control; VIT C, vitamin C; VIT E, vitamin E; MET, metformin; MET + VIT C, metformin and vitamin C; MET + VIT E, metformin and vitamin E; Na^+, sodium; K^+, potassium; HCO_3^-,bicarbonate; Cl^-, chloride. Values are mean ± SEM, *$p<0.05$ as compared to diabetic control, n = 5.

Table 3. Effects of the treatments on liver function in STZ-induced diabetic rats.

GROUPS	ALP (u/L)	ALT (u/L)	AST (u/L)	ALB (g/dl)	TB (mg/dl)	CB (mg/dl)	TP (g/dl)	GLO (g/dl)
NC	237.00±29.13	32.40±5.98	33.60±3.23	4.34±0.16	0.28±0.04	0.09±0.01	7.06±0.38	2.72±0.26
DC	932.00±169.46	62.50±3.23	100.00±44.77	3.85±0.13	0.30±0.04	0.13±0.03	6.80±0.29	2.95±0.35
VIT C	474.83±64.52**	33.83±7.52*	69.00±16.13	3.65±0.16	0.27±0.03	0.12±0.02	7.17±0.25	3.52±0.24
VIT E	436.80±10.23**	27.60±5.80**	42.00±6.80*	4.00±0.19	0.28±0.04	0.14±0.02	7.62±0.30	3.62±0.45
MET	700.33±110.49*	31.17±5.94*	37.50±6.56**	3.70±0.28	0.23±0.02	0.11±0.02	7.52±0.29	3.78±0.35
MET + VIT C	388.67±7.96***	25.67±3.63**	28.17±4.00**	4.07±0.22	0.23±0.02	0.10±0.02	7.37±0.36	3.30±0.23
MET + VIT E	700.80±130.15*	27.60±2.38**	34.40±1.54**	3.46±0.34	0.32±0.04	0.14±0.2	6.94±0.21	3.48±0.29

NC, normal control; DC, diabetic control; VIT C, vitamin C; VIT E, vitamin E; MET, metformin; MET + VIT C, metformin and vitamin C; MET + VIT E, metformin and vitamin E; ALP, serum alkaline phosphatase; ALT, serum alanine aminotransferase; AST, serum aspartate aminotransferase; ALB, albumin; CB, conjugated bilirubin; TP, total protein, GLO, globulin. Values are expressed as means ± S.E.M, n=5, *$p<0.05$, **$p<0.01$, ***$p<0.001$ compared to diabetic control.

Table 4. Effects of the treatments on oxidant and antioxidant parameters in STZ-induced diabetic rats

GROUPS	MDA (nmol/mg)	SOD (U/mg)	CAT (U/mg)	GSH (µg/mg)	GPx (U/mg)
NC	14.57 ± 0.03	3.07 ± 0.06	109.40 ± 0.08	2.41 ± 0.16	2.13 ± 0.01
DC	27.00 ± 0.07$^{\#\#\#\#}$	1.69 ± 0.02$^{\#}$	88.71 ± 0.10$^{\#\#\#}$	1.80 ± 0.05$^{\#\#\#}$	2.16 ± 0.01
MET	15.58 ± 0.07****	2.51 ± 0.01	110.00 ± 0.23***	2.13 ± 0.01*	2.12 ± 0.01
VIT C	18.36 ± 0.02****	4.17 ± 0.34****	122.40 ± 0.85****	2.32 ± 0.05***	2.05 ± 0.06
VIT E	15.09 ± 1.13****	3.95 ± 0.43***	116.00 ± 4.06****	2.17 ± 0.08*	2.27 ± 0.01
MET + VIT C	13.87 ± 1.59****	3.11 ± 0.27*	125.40 ± 0.77****	2.25 ± 0.05**	2.21 ± 0.03
MET + VIT E	18.35 ± 1.85****	3.32 ± 0.35**	118.30 ± 5.41****	2.25 ± 0.08**	2.18 ± 0.04

NC, normal control; DC, diabetic control; VIT C, vitamin C (1000 mg/kg); VIT E, vitamin E (600 mg/kg); MET, metformin (500 mg/kg); MET + VIT C, metformin (500 mg/kg) and vitamin C (1000 mg/kg); MET + VIT E, metformin (500 mg/kg) and vitamin E (600 mg/kg); MDA, malondialdehyde; SOD, superoxide dismutase; CAT, catalase; GSH, glutathione reductase; GPx, glutathione peroxidase. Values are expressed as means ± S.E.M, n=5, $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, $^{****}p<0.0001$ compared to diabetic control and $^{\#}p<0.05$, $^{\#\#\#}p<0.001$, $^{\#\#\#\#}p<0.0001$ vs. normal control.

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Discussion

In this study, the various treatments significantly reduced the fasting blood glucose levels compared to diabetic control. It was observed that the treatment with the metformin-vitamin C co-administration produced a greater decline in blood glucose levels compared to metformin-treated group. This result is consistent with the work of Dakhale *et al* (2011) and Adeneye *et al* (2006). The beneficial effect demonstrated by the combination of metformin and vitamin C on glycemic control probably, might be due to the scavenging of free radicals, protection of β-cells from glucotoxicity, preservation of β-cell mass and insulin content by vitamin C (Kaneto *et al.,* 1999; Kaplan et al., 2007). Earlier studies have reported that high doses of vitamin C (≥1000 mg/kg) significantly improved the glycemic control (Eriksson and Kohvakka, 1995; Afkhami-Ardekani *et al.,* 2006). Similarly, our findings demonstrated that the diabetic animals treated with vitamin E alone, and metformin + vitamin E significantly lowered the fasting blood glucose compared to the diabetic untreated. This is in agreement with the work by Manning *et al*.(2004) who proposed that vitamin E improves glycaemia by enhancing insulin sensitivity and it is attributed to its ability to decrease oxidative stress, modify membrane properties, and decrease in inflammatory (Manning *et al.,* 2004). Previous studies by Panda *et al.,* (2016) and Al-Shamsi *et al.,* (2006) are in agreement with our findings.

As shown in Table 2, diabetic untreated animals demonstrated a progressive decrease in body weight throughout the experiment compared to normal rats. Diabetes is often associated with weight loss due to muscle wasting and loss of tissue protein caused by increased gluconeogenesis, glycogenolysis and lipolysis (Nair *et al.,* 1983). It was observed in this study that the body weight loss was significantly minimized in diabetic rats treated with vitamin C alone, metformin alone, and metformin-vitamin C combination when compared to diabetic control. This may be related to their glucose-lowering effects and antioxidant activity. However, vitamin E failed to restore the body weight of the diabetic treated rats. This is consistent with earlier work by Fahami *et al*., who observed that Vitamin E supplementation at dose of 60 mg/kg did not reverse weight loss among humans (Fahami *et al*. 2005).

Our results on lipid profiles showed that TG, TC, and LDL were higher in untreated diabetic rats compared to the normal rats (Figure 2). Abnormalities in insulin action is commonly known to affect lipid metabolism enzymes and pathways, and cause dyslipidemia in diabetes (Wu and Parhofer, 2014). Diabetic dyslipidemia is characterized by high plasma levels of TG, TC, LDL, and low level of HDL. These conditions increase the risk of developing coronary artery disease and atherosclerosis (Al-Ramadhan *et al.,* 2015). In this study, all the treatments significantly decreased the mean TC and TG while vitamin C, metformin and metformin + vitamin E treatments showed significant reductions in serum levels of LDL when compared to diabetic control. There was no significant change observed in the serum levels of HDL in all the treatment groups. This study also revealed that the combination of metformin and vitamin C or E produced greater reduction in the TG and TC levels when compared to metformin alone. This enhanced effect can be credited to vitamin C or vitamin E co-administration since Metformin alone had lower effect (Afkhami-Ardekani *et al.,* 2006; Soliman and Bahagt, 2012; Bamanikar *et al.,* 2016).

Diabetic nephropathy is one of the complications of diabetes, thus, making assessment of renal function a necessity in management of diabetic patients. The data from this study revealed that the uncontrolled glycaemia in diabetic untreated animals produced high levels of serum creatinine and urea compared to the normal control. Similar findings have been reported by some researchers, demonstrating that elevation of serum urea and creatinine in diabetics denotes progressive renal damage (Aldler *et al.,* 2003; Anjaneyulu *et al.,* 2004; Sirivole and Eturi, 2017). In the present study, we observed a significantly decrease in the creatinine levels in the groups treated with vitamin E, metformin and metformin-vitamin C combination.

ALT, ALP and AST were found elevated in diabetic untreated group compared to the normal control (non-diabetic group). These liver enzymes serve as useful biomarkers of hepatocellular damage, thus, high levels noted in diabetic untreated may suggest inflammation or hepatic injury. Higher activities of ALT, AST and ALP are comparatively higher among type 2 diabetic patients, which is consistent with our findings (Harris, 2005; Mathur *et al.,* 2016). According to Mathur *et al.,* oxidative stress was indicted as one of the contributing factors to the elevation in serum ALT, AST and ALP

(Mathur *et al.,* 2016). In this present study, all the treatments significantly decreased the serum activities of these liver enzymes compared to the diabetic control. Interestingly, it was noted that metformin-vitamin C co-administration produced greater reductions of these liver enzymes compared to metformin alone. Several studies have reported the hepatoprotective effect of vitamin C which was attributed to its antioxidant property (Adikwu and Deo, 2013; Metwally *et al,* 2015) and this may be responsible for the enhanced result in this current study.

There is substantial evidence that oxidative stress largely plays crucial roles in the pathogenesis and development of complications of diabetes mellitus (Ogar *et al.,*2019). Oxidative stress occurs as a result of an abnormally increased formation of free radicals, decreased antioxidant enzyme and defense system or both, leading to increased lipid peroxidation, destruction of cellular organelles and DNA, and induction of insulin resistance (Giacco and Brownlee, 2010; Ogar *et al.,*2019). The degree of lipid peroxidation can be estimated by measuring the concentration of malondialdehyde (MDA), a primary end-product of cellular lipids. Increase in lipid peroxidation (increased MDA level) has been associated to the progression of DM complications (Miaffo *et al.,* 2019). The present study showed that the serum MDA level was significantly ($p<0.0001$) elevated in diabetic untreated rats compared to normal rats, indicating increased peroxidation of lipids, increased formation of free radicals and consequently increased oxidative stress. Oral administration of metformin, vitamin C or E, and the combination of metformin and vitamin C or E significantly ($p<0.0001$) reduced the increased MDA level in the serum of diabetic rats. The low level of antioxidant enzyme (such as SOD, CAT, GSH) activities in DM is suggestion of oxidative stress. In the present study, there was significant decrease in SOD, CAT, and GSH activities in the serum of diabetic untreated rats compared to the normal control rats. Oral administration of all the treatments administered to the diabetic rats were found to significantly increase the serum SOD, CAT and GSH levels compared to the diabetic control. Oral administration of metformin alone failed to elevate the serum SOD activity in the diabetic rats. It was interestingly noted that the inclusion of vitamin C or E to metformin markedly increased antioxidant enzymes potentials of in the serum of diabetic rats compared to metformin alone. It therefore appears that the combination of metformin and vitamin C or E

would offer a better scavenging of hyperglycemia-induced free radicals, and attenuation of oxidative stress injury in DM than metformin alone.

In conclusion, our findings have elucidated the effect of Vitamin C and metformin co-administration in achieving better glycemic control, reducing oxidative stress, improved lipid profile, and potential advantage of preventing multi-organ damaging effect of Type 2 diabetes.

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Authors' contributions

A.P.U. conceptualised, designed and participated in the experiments, analysed the data and wrote the manuscript. E.K.O.

Competing interest

The authors declared that they have no competing interest.

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