



Metformin and vitamin D combination therapy ameliorates type 2 diabetes mellitus-induced renal injury in male Wistar rats

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Abstract

Diabetic kidney disease is a major microvascular diabetes mellitus (DM) complication clinically associated with a gradual renal function decline. Although metformin is a common drug for managing DM, however, monotherapy treatment with any antidiabetic drug will necessitate dosage increment since type 2 DM (T2DM) deteriorates over time due to the increasing pancreatic β -cell dysfunction and will eventually require a combination therapy approach with another antidiabetic medication. Vitamin D is a food supplement that has been proven to have antidiabetic and reno-protective activities. Hence, we explore the combination of vitamin D and metformin on T2DM-induced renal dysfunction. Thirty male Wistar rats were randomized into five (5) groups: control, diabetes untreated, diabetics treated with metformin, vitamin D, and vitamin D + metformin. Vitamin D and metformin significantly reversed DM-induced hyperglycemia, electrolyte imbalance, and dyslipidemia. Also, vitamin D and metformin reversed T2DM-induced increase in serum creatinine and urea and renal lactate, LDH, and oxido-inflammatory response. These observed alterations were accompanied by an increase in proton pump activities and modulation of Nrf2/Nf- κ B and XO/UA signaling. This study revealed that vitamin D and/or metformin ameliorated T2DM-induced renal injury.

Keywords Diabetes mellitus · Diabetic kidney disease · Electrolyte imbalance · Proton pump dysfunction · Vitamin D

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Abbreviations

DKD	Diabetic kidney disease
GPx	Glutathione Peroxidase
HFD	High-fat diet
MET	Metformin
Nf- κ B	Nuclear factor kappa B
Nrf2	Nuclear factor erythroid-2
ROS	Reactive oxygen species
T2DM	Type 2 diabetes mellitus

Introduction

Diabetic kidney disease (DKD) is a major microvascular diabetes mellitus (DM) complication clinically associated with a gradual renal function decline (with or without proteinuria) resulting from prolonged hyperglycemia. DKD has been diagnosed in 25–40% of DM and is a leading contributing factor to end-stage renal disease (Akhtar et al. 2020). Also, DKD has been identified as a major contributing factor to cardiovascular dysfunction (Groop et al. 2009; Orchard et al. 2010). As a major chronic disorder, about 537 million

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people have diabetes, and this number is projected to reach 643 million by 2030, and 783 million by 2045 (International Diabetic Forum, 2021), indicating that more people are expected to suffer from DKD. Although several synthetic drugs have been clinically proven to effectively slow down DKD progression (Blazek and Bakris 2023), this diabetic complication remains incurable (Luo et al. 2023).

Maintaining optimal plasma glucose levels is a major factor in reducing the development of DM complications (such as DKD) (Ahmad, F and Joshi, 2023). Prospective DM study data recommends lifestyle modification (such as diet and exercise) as the first line of treatment probably because it is the safest therapy, followed by hypoglycemic and/or antihyperglycemic medications (Chiasson et al. 2001). The biguanide metformin is the preferred drug commonly used for managing DM (Jones et al. 2022). However, monotherapy treatment with any antidiabetic drug will eventually necessitate dosage increment since type 2 DM (T2DM) deteriorates over time due to the increasing pancreatic β -cell dysfunction (Chiasson et al. 2001) and will eventually require a combination therapy approach with another antidiabetic medication (Rhee et al. 2017). Unfortunately, these synthetic antidiabetic drugs have been reported to have their side effects (Olayaki et al. 2023). In fact, in a survey performed by Ji et al. (2015), about 32% of DM patients changed their drugs within a year, due to intolerable side effects.

Hence, this study was designed to explore a combination approach of metformin with a lifestyle modification therapy (such as a diet supplement). Vitamin D is a fat-soluble vitamin acquired from sunlight, diet, and food supplements. Vitamin D in its active form (1,25-dihydroxyvitamin D) has been shown to impact different biological processes, including cell differentiation and immune-inflammatory responses (Liu et al. 2023). In addition to its anti-inflammatory activities, vitamin D also acts as an antioxidant by suppressing reactive oxygen species (ROS) generation and endogenous antioxidant activities (Odetayo et al. 2024a). Also, vitamin D regulates circulatory insulin, restores the activities of pancreatic β -cell, and prevents oxidative stress and apoptosis in type 2 diabetic animals (Fathi et al. 2022). Clinically, vitamin D has been shown to potentially suppress or prevent T2DM (Gu et al. 2022; Hu et al. 2023; Hsia et al. 2023). In fact, Krisnamurti et al. (2023) reported that multiple studies have consistently associated a decrease in serum vitamin D with diabetes. Interestingly, analysis of the collective findings from different studies established that vitamin D supplementation can reduce the risk of developing T2DM in prediabetic patients (Bouillon et al. 2022; Fathi et al. 2022; Krisnamurti et al. 2023). Nevertheless, the combination therapy of vitamin D and metformin for managing T2DM-induced nephrotoxicity has not been explored. Furthermore, the associated mechanisms of antidiabetic activities of vitamin D remain to be further investigated. Therefore, this

study aimed at determining the protective roles of vitamin D and/or metformin in T2DM-induced nephrotoxicity.

Materials and methods

Chemicals

Vitamin D3 was purchased from Principle Healthcare International Limited in the UK, while metformin was obtained from Oswoth Nigeria Limited in Lagos, Nigeria. Also, STZ was acquired from Sigma-Aldrich in St. Louis, MO, USA.

Ethical consideration.

The animals used in this study were humanely catered for according to the National Institute of Health (NIH), and experimental findings were reported based on ARRIVE guidelines. Also, the guidelines stated by the National Research Council for the Care and Use of Laboratory Animals were strictly followed and the study was approved by the institutional ethical review committee (University of Ilorin Ethical Review Committee: UERC/ASN/2023/2582).

Animal and treatment

Thirty male Wistar rats (age, 10 weeks, and weight, 120–150 g) were purchased from the University of Ilorin. The animals were housed and allowed to acclimatize in a conducive environment for 1 week. After acclimatization, they were randomized into five (5) groups as follows: the control (treated with distilled water), diabetes untreated (DM-U), diabetes treated with 180 mg/kg of metformin (DM + metformin), diabetes treated with 1000 IU/kg of vitamin D (DM + Vitamin D), diabetes treated with 180 mg/kg of metformin and 1000 IU/kg of vitamin (DM + vitamin D + metformin). The treatments were via oral gavage and lasted for 28 days except otherwise stated. The choice of vitamin D dosage was chosen based on the Expert Group on Vitamins and Minerals (Great Britain) (2003) recommendations and it is similar to the dose of Krisnamurti et al. (2023) and Gázquez et al. (2022). Furthermore, the dosage of metformin is within the no observable adverse effect level (NOAEL) of metformin in rats (Sarmiento-Ortega et al. 2018; Quaile et al. 2010), and it is similar to previous studies (Satyam et al. 2023; Olayaki et al. 2023).

T2DM induction

As described by Okesina et al. (2024b), “the rats were fed a HFD (maize = 5.5 kg, wheat = 0.5 kg, ground nut cake = 5.5 kg, soya meal/ cake/full fat = 12.5 kg, palm kernel cake = 5.0 kg, bone meal = 0.5 kg, methionine = 0.25,

lysine = 0.25) for 5 weeks,” and after overnight fasting, they received a single intraperitoneal dosage of STZ (35 mg/kg b.w.) dissolved in a cold citrate buffer (Olayaki et al. 2023). After 72 h of STZ injection, a glucometer (Fine test) was used to measure the rats’ blood glucose, and rats with blood glucose level above 250 mg/dl were classified as diabetic (Srinivasan et al. 2005; Okesina et al. 2024a). Additionally, the control group received 0.5 mL of citrate buffer via the same route.

Collection of samples

After 12 h from the last dosage, the animals were euthanized via an intraperitoneal injection of 40 and 4 mg/kg of ketamine and xylazine respectively (Fatai and Luqman 2022). Blood samples were obtained through cardiac puncture into heparinized bottles, and plasma was obtained via centrifugation at a revolution of 5000 rpm for 15 min. Furthermore, the left kidneys were collected into a sufficient amount of phosphate buffer and homogenized for biochemical assays. The right kidney was also obtained for histological processing.

Biochemical analysis

Fasting blood glucose

Fasting blood glucose was estimated by glucose oxidase method using a digital glucometer (On Call®Plus ACON Laboratories, Inc. San Diego, CA).

Electrolytes and proton pump activities

Potassium, magnesium, and calcium (Fortress Diagnostics, Antrim, UK) and phosphorus (Abbott Laboratories, IL, USA) were assayed as directed by their respective manufacturers. Renal transmembrane protein (Na⁺/K⁺-ATPase and Ca²⁺/Mg²⁺-ATPase) activities were determined spectrophotometrically as previously established (Torlińska & Grochowalska 2004; Odetayo and Olayaki 2024).

Renal histology

The renal histology was processed as previously described with some modifications (Azemi et al. 2021; Odetayo et al. 2024b). The right kidneys were fixed in 10% formal saline, dehydrated with ethanol series, and cleared with toluene. The kidney was embedded at room temperature and blocked in paraffin wax incubated overnight in a 60 °C incubator. Afterward, hematoxylin and eosin (H&E) stain was applied to the kidney’s 3-µm-thick paraffin sections.

Renal injury markers

Urea and creatinine were determined in the plasma using a standard colorimetry method as prescribed by the manufacturer (Randox Laboratory Ltd., UK) while renal lactate and lactate dehydrogenase (LDH) were determined using the ELISA method (Aggape Diagnostic, Switzerland).

Lipid profile

Serum and renal total cholesterol (TC), triglycerides (TG), and high-density lipoproteins (HDL) were determined using the colorimetry method (Randox Laboratory Ltd., UK).

Oxidative stress and inflammatory markers

Renal glutathione peroxidase (GPx) was determined using a colorimetry method (Fortress Diagnostic Kit, Switzerland). Renal malondialdehyde (MDA) was estimated according to the method of Odetayo et al. (2023a), Hamed et al. (2022), and Akhigbe et al. (2021). Renal catalase (CAT), superoxide dismutase (SOD), glutathione (GSH), tumor necrotic factor- α (TNF- α), interleukin 6 (IL-6), interleukin 1 beta (IL-1 β), interleukin 10 (IL-10), Nrf2, and NF- κ B were determined using an ELISA method (Elabscience, USA).

Xanthine oxidase (XO) and uric acid (UA)

Renal XO activities were estimated according to a previously reported method (Odetayo et al. 2023b) and UA concentration was also estimated using colorimetric methods (Precision, UK).

Statistical analysis

Statistical analysis was performed using the one-way analysis of variance (ANOVA) and Tukey’s post hoc test using GraphPad PRISM 5 software (GraphPad Software, La Jolla, CA, USA). Data were reported as mean $\pm$ standard deviation (SD). Values of $P < 0.05$ were considered statistically significant.

Results

There was a significant increase in FBS of DM-U animals compared with their counterparts in the control group (Fig. 1). However, this observed increase was reversed in animals treated with vitamin D, metformin, and vitamin D + metformin, although an enhanced ameliorative effect was observed in animals treated with vitamin D and metformin compared with those treated with vitamin D or metformin alone.

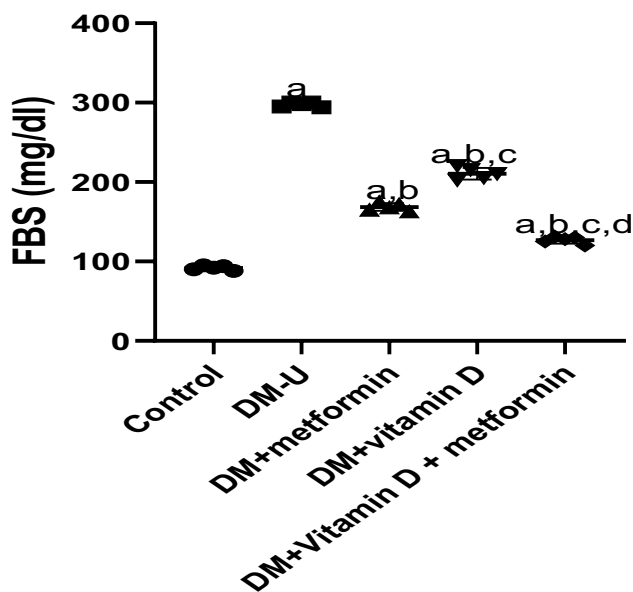


Fig. 1 Effect of vitamin D and/or metformin on FBS following T2DM induction. ^a $P < 0.05$ vs control, ^b $P < 0.05$ vs DM-U, ^c $P < 0.05$ vs DM+met, ^d $P < 0.05$ vs DM+vit D. Data ($n=6$) were analyzed by one-way ANOVA and Tukey's post hoc test and reported as mean $\pm$ SD. DM-U diabetic untreated

Also, a rise in serum potassium and phosphorus and a decrease in calcium and magnesium were observed in the DM-U group compared with animals of the control group (Table 1). This observed electrolyte imbalance was accompanied by a decrease in Na⁺/K⁺ ATPase and Ca²⁺/Mg²⁺ ATPase activities. These distortions were ameliorated by vitamin D and/or metformin, although a better ameliorative effect was observed in animals in the DM+MET+VIT D.

As shown in Fig. 2, DM disrupted renal histology evidenced by severe degeneration of tubular epithelium, shrinkage of the glomeruli, and thinning of the parietal layer of diabetic rats. These observed distortions were abrogated in animals in the treated groups. While ameliorative effects were observed in all the treatment groups, animals in the

DM+MET+VIT D renal histology were comparable with their counterparts in the control group.

Additionally, a rise in serum creatinine, urea, renal lactate, and LDH was observed in animals in the DM-U group compared with the control (Figs. 3 and 4). These observed increases were blunted in animals treated with vitamin D or metformin alone and in combination, although a more pronounced effect was observed in animals treated with vitamin D and metformin.

Also, a significant increase in triglyceride and cholesterol and a decrease in HDL were observed in animals in the DM-U group compared with the control (Table 2). This observed dyslipidemia was reversed in animals treated with metformin and/or vitamin D.

Furthermore, T2DM induction significantly elevated renal MDA, IL-1 β , IL-6, TnF- α , and Nf- κ b and a decrease in GPx, CAT, SOD, IL-10, and Nrf2 compared with the control (Figs. 5, 6, and 7). These observed alterations were ameliorated in vitamin D and/or metformin-treated animals, although more ameliorative effects were observed in animals that received vitamin D and metformin treatment. In the same vein, the significantly elevated XO and UA activities in T2DM animals compared with the control were ameliorated in all the treatment groups (Fig. 8).

Discussions

T2DM is a metabolic disorder with increasing incidence globally (Okesina et al. 2024b). According to previous basic and clinical studies, vitamin D may possess some beneficial effects on the management and prevention of T2DM (Tian et al. 2021; Fathi et al. 2022; Hu et al. 2023; Hsia et al. 2023). Also, a meta-analysis predicted the ameliorative effects of vitamin D on T2DM-induced hyperglycemia and the associated complications (Farahmand et al. 2023). However, the combination of vitamin D and metformin for managing T2DM-induced nephrotoxicity and its associated mechanisms has not been explored. In this study, vitamin D and/or metformin were indicated to ameliorate glucose

Table 1 Effect of vitamin D and/or metformin on serum electrolytes and renal proton pump activities following T2DM induction

	CTRL	DM-U	DM+MET	DM+VIT D	DM+MET+VIT D
Potassium (mmol/l)	4.29 $\pm$ 0.20	12.47 $\pm$ 1.25 ^a	6.64 $\pm$ 0.24 ^{a,b}	7.75 $\pm$ 0.13 ^{a,b}	5.44 $\pm$ 0.16 ^{a,b,c,d}
Phosphorus (mmol/l)	4.70 $\pm$ 0.24	13.06 $\pm$ 0.19 ^a	7.98 $\pm$ 0.15 ^{a,b}	8.38 $\pm$ 0.15 ^{a,b}	5.55 $\pm$ 0.29 ^{a,b,c,d}
Calcium (mmol/l)	16.14 $\pm$ 1.28	6.12 $\pm$ 0.34 ^a	9.64 $\pm$ 0.64 ^{a,b}	7.92 $\pm$ 0.44 ^{a,b,c}	16.47 $\pm$ 0.72 ^{b,c,d}
Magnesium (mmol/l)	8.70 $\pm$ 0.21	2.08 $\pm$ 0.17 ^a	6.02 $\pm$ 0.14 ^{a,b}	4.35 $\pm$ 0.15 ^{a,b,c}	7.80 $\pm$ 0.21 ^{a,b,c,d}
Na/K-ATPase (umol/g)	0.35 $\pm$ 0.02	0.14 $\pm$ 0.02 ^a	0.28 $\pm$ 0.02 ^{a,b}	0.22 $\pm$ 0.01 ^{a,b,c}	0.32 $\pm$ 0.02 ^{b,c,d}
Ca/Mg-ATPase (umol/g)	0.29 $\pm$ 0.01	0.12 $\pm$ 0.01 ^a	0.19 $\pm$ 0.01 ^{a,b}	0.16 $\pm$ 0.01 ^{a,b,c}	0.23 $\pm$ 0.02 ^{a,b,c,d}

^a $P < 0.05$ vs control, ^b $P < 0.05$ vs DM-U, ^c $P < 0.05$ vs DM+met, ^d $P < 0.05$ vs DM+vit D. Data were analyzed by one-way ANOVA and Tukey's post hoc test. DM diabetic mellitus, DM-U diabetic untreated, met metformin, vit D vitamin D

Fig. 2 Histology of the kidney. *Control*: photomicrograph of the kidney section of the control group showed close to normal architecture: glomerulus (G), Bowman's capsule (BC), proximal (P) and distal (D) convoluted tubules (CT). *DM-U*: severe degeneration of tubular epithelium, shrinkage of the glomeruli, and thinning of the parietal layer. *DM + metformin*: mild degeneration of the tubules in the form of cytoplasmic vacuolation and distortion of the renal corpuscles. *DM + vitamin D*: shows tubular dilatation and mild atrophy. *DM + metformin and vitamin D*: showed pre-served renal histoarchitecture. Mg X 800

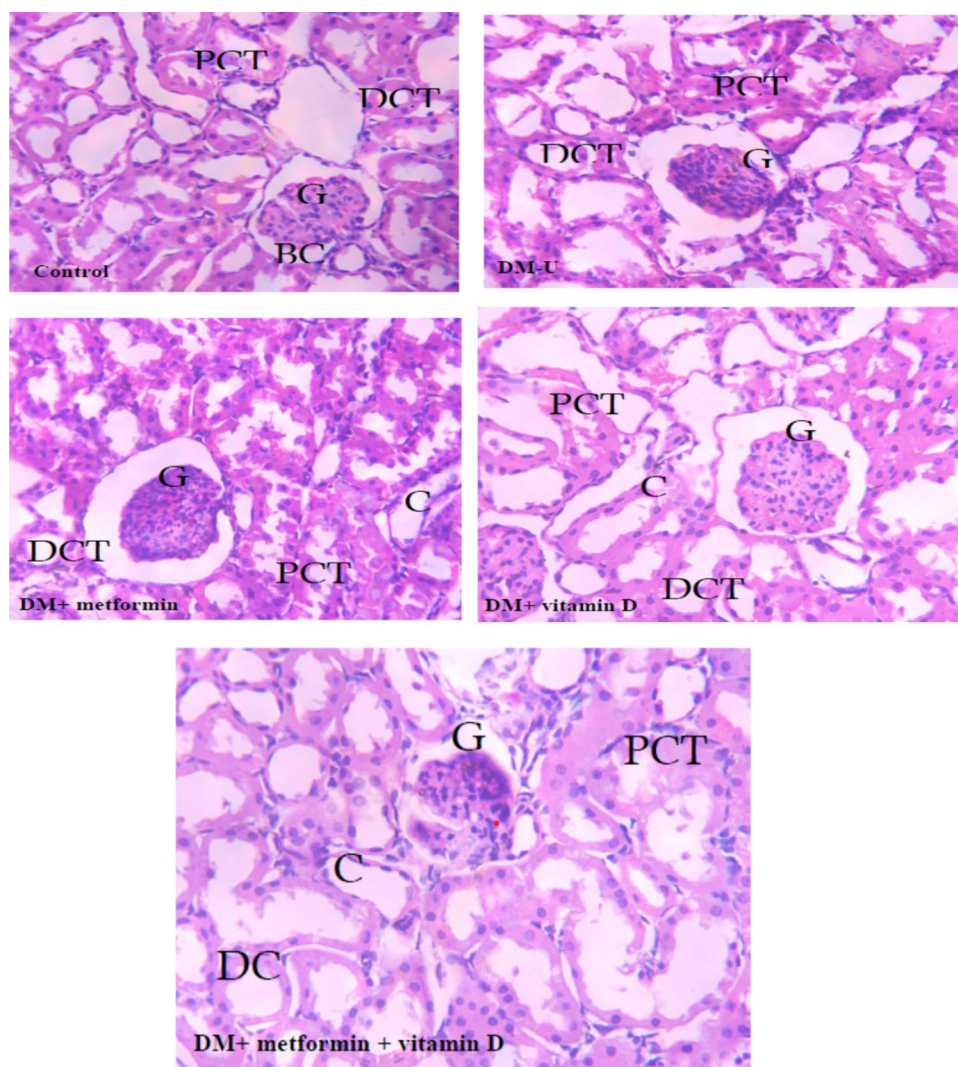


Fig. 3 Effect of vitamin D and/or metformin on serum urea and creatinine following T2DM induction. ^a $P < 0.05$ vs control, ^b $P < 0.05$ vs DM-U, ^c $P < 0.05$ vs DM + met, ^d $P < 0.05$ vs DM + vit D. Data ($n = 6$) were analyzed by one-way ANOVA and Tukey's post hoc test and reported as mean $\pm$ SD. *DM-U* diabetic untreated

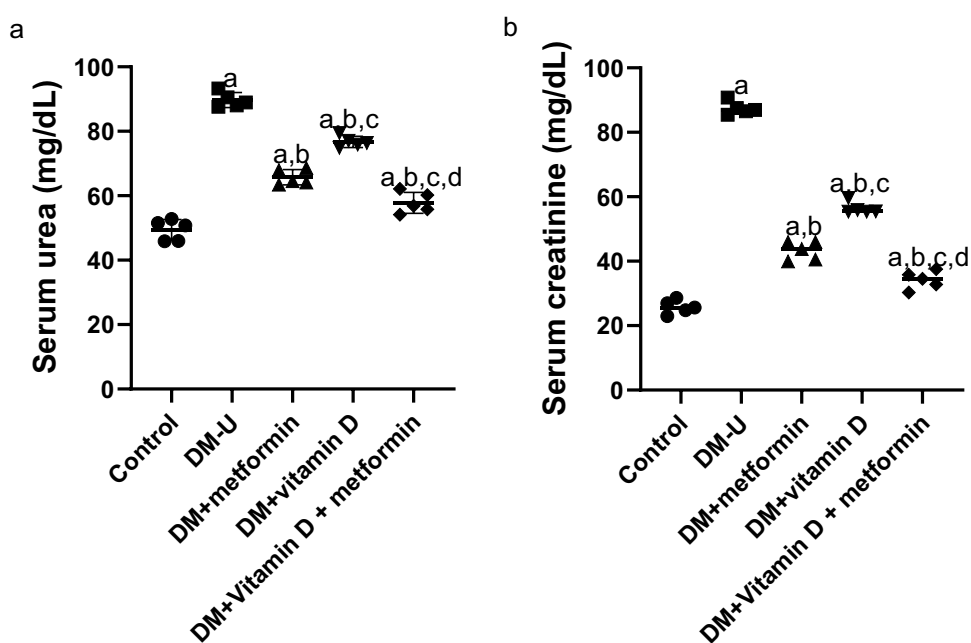


Fig. 4 Effect of vitamin D and/or metformin on renal lactate and lactate dehydrogenase (LDH) following T2DM induction. ^a $P < 0.05$ vs control, ^b $P < 0.05$ vs DM-U, ^c $P < 0.05$ vs DM + met, ^d $P < 0.05$ vs DM + vit D. Data ($n = 6$) were analyzed by one-way ANOVA and Tukey's post hoc test and reported as mean $\pm$ SD. DM-U diabetic untreated

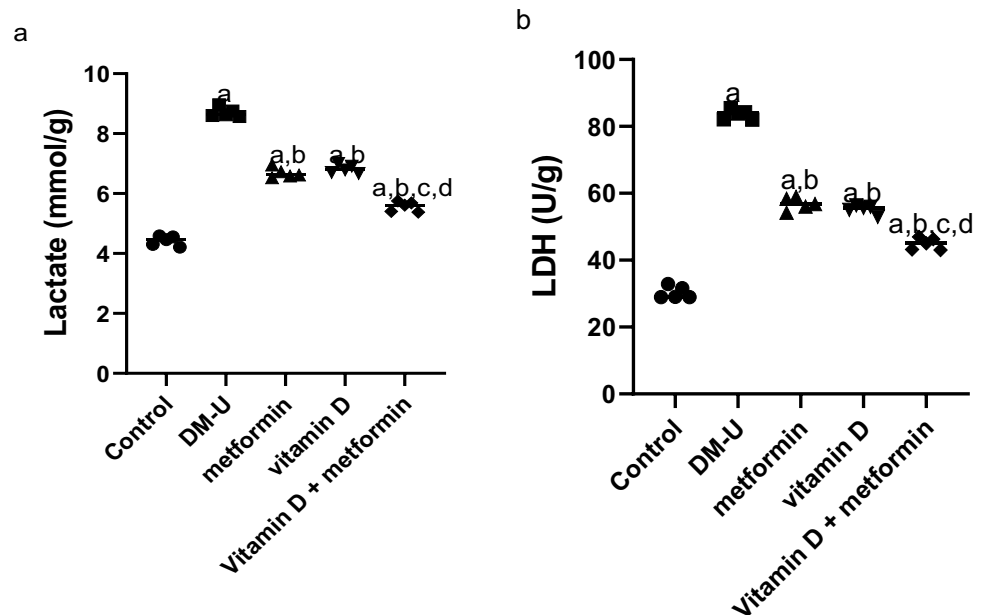


Table 2 Effect of vitamin D and/or metformin on lipid metabolism following T2DM induction

	CTRL	DM-U	DM + MET	DM + VIT D	DM + MET + VIT D
Triglyceride (mg/dl)	45.60 $\pm$ 3.362	79.00 $\pm$ 2.55 ^a	58.00 $\pm$ 3.67 ^{a,b}	67.40 $\pm$ 2.70 ^{a,b,c}	49.40 $\pm$ 1.67 ^{b,c,d}
Cholesterol (mg/dl)	24.00 $\pm$ 1.41	55.40 $\pm$ 3.13 ^a	35.60 $\pm$ 1.51 ^{a,b}	44.60 $\pm$ 2.70 ^{a,b,c}	30.60 $\pm$ 1.67 ^{a,b,c,d}
HDL (mg/dl)	30.33 $\pm$ 1.96	12.44 $\pm$ 2.05	25.45 $\pm$ 2.56	19.26 $\pm$ 1.72	29.87 $\pm$ 0.58

^a $P < 0.05$ vs control, ^b $P < 0.05$ vs DM-U, ^c $P < 0.05$ vs DM + met, ^d $P < 0.05$ vs DM + vit D. Data were analyzed by one-way ANOVA and Tukey's post hoc test. DM diabetic mellitus, DM-U diabetic untreated, met metformin, vit D vitamin D

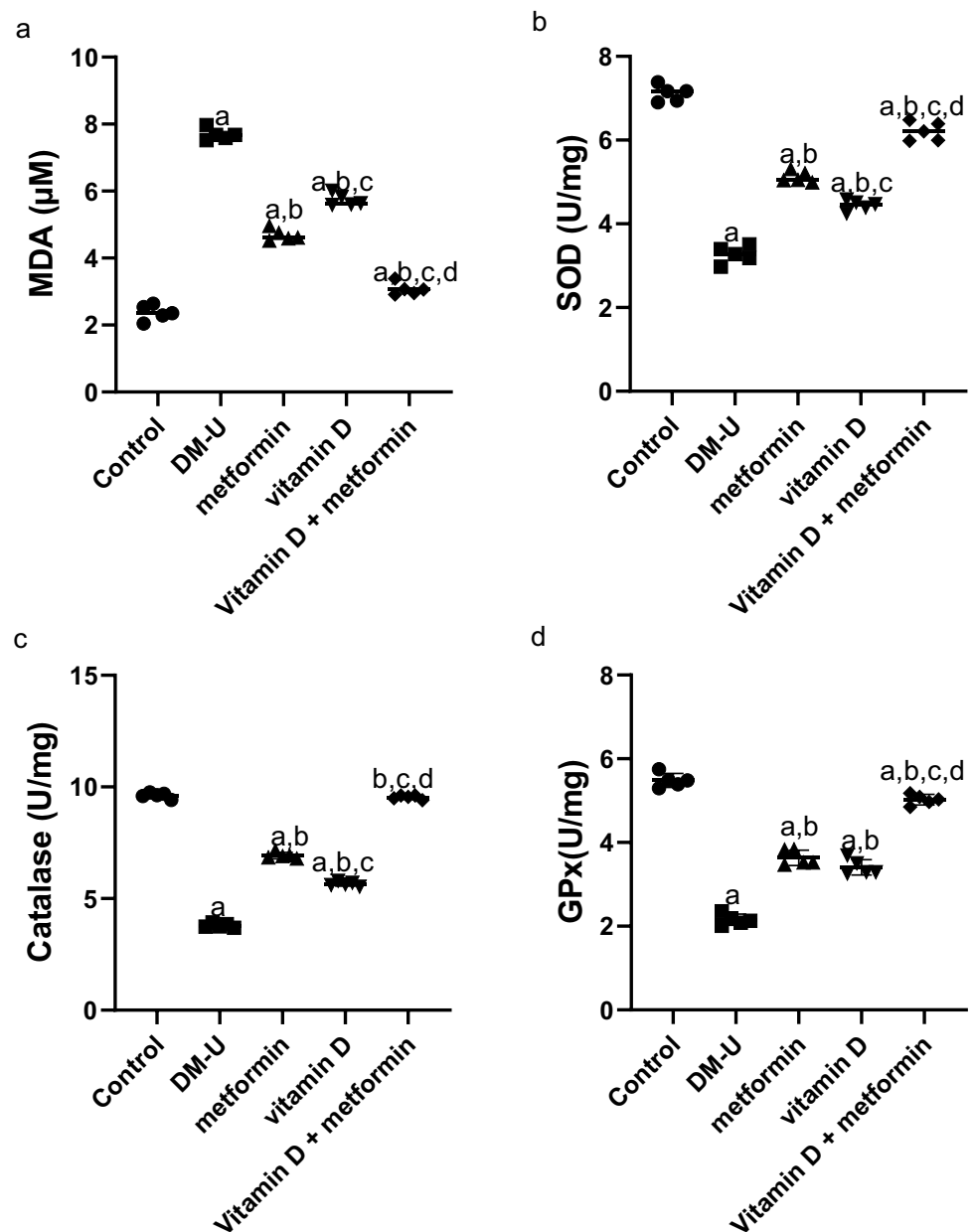
dysmetabolism and electrolyte imbalance by inhibiting oxido-inflammatory response.

In this study, HFD was combined with STZ to induce T2DM in Wistar rats. After STZ administration, the rats in the diabetic groups were fed with HFD for another 5 weeks since it takes about 3 weeks for the kidney to recover from the acute mild nephrotoxic effect of STZ (Zhiqing et al. 2014). The observed glucose dysmetabolism in the diabetes animals confirmed the induction of T2DM since hyperglycemia is the hallmark of DM. This observed hyperglycemia was ameliorated in diabetic animals treated with vitamin D and/or metformin. These findings are in agreement with previous studies (Pereira et al. 2023; Odetayo et al. 2024a) that revealed that vitamin D and metformin therapy significantly reduced T2DM-induced hyperglycemia.

The decline in renal functions was observed in diabetic untreated rats evidenced by the observed electrolyte imbalance. Although renal function can be determined by the glomerular filtration rate and urine output, nephrotoxicants can impair kidney functions without altering these traditional renal function markers (Kwon et al. 2023; Adelakun et al. 2024). Electrolyte balance which is majorly regulated by

the kidney is extremely important for the proper functioning of various biological systems (Moldakozhayev and Gladyshev 2023). Consequently, renal dysfunction can distort electrolyte homeostasis and may become life-threatening if not managed properly. Accumulation of potassium (hyperkalemia) is an indication of declined renal functions and can distort cardiac electrophysiology which may lead to death (Larivée et al. 2023). Also, previous findings obtained from CKD patients reported a 20% increase in death probability for every 1 mg/dl rise in serum phosphorus (Centeno et al. 2023). Additionally, an increase in serum phosphorus could account for the decrease in serum calcium since they are inversely related and are majorly regulated by the kidney (Wu et al. 2016). Also, the decrease in calcium can be related to hypomagnesemia, since decreased magnesium can impair magnesium-dependent adenylyl cyclase generation of cyclic adenosine monophosphate (cAMP) responsible for maintaining parathyroid hormone secretion that maintains calcium level (Gazzaz and Alghamdi 2021). Hence, the observed electrolyte imbalance following T2DM-induction is an indication of renal dysfunction, and the ability of vitamin D with or without metformin to restore renal function/

Fig. 5 Effect of vitamin D and/or metformin on renal oxidative stress markers following T2DM induction. ^a $P < 0.05$ vs control, ^b $P < 0.05$ vs DM-U, ^c $P < 0.05$ vs DM + met, ^d $P < 0.05$ vs DM + vit D. Data ($n = 6$) were analyzed by one-way ANOVA and Tukey's post hoc test and reported as mean $\pm$ SD. DM-U diabetic untreated

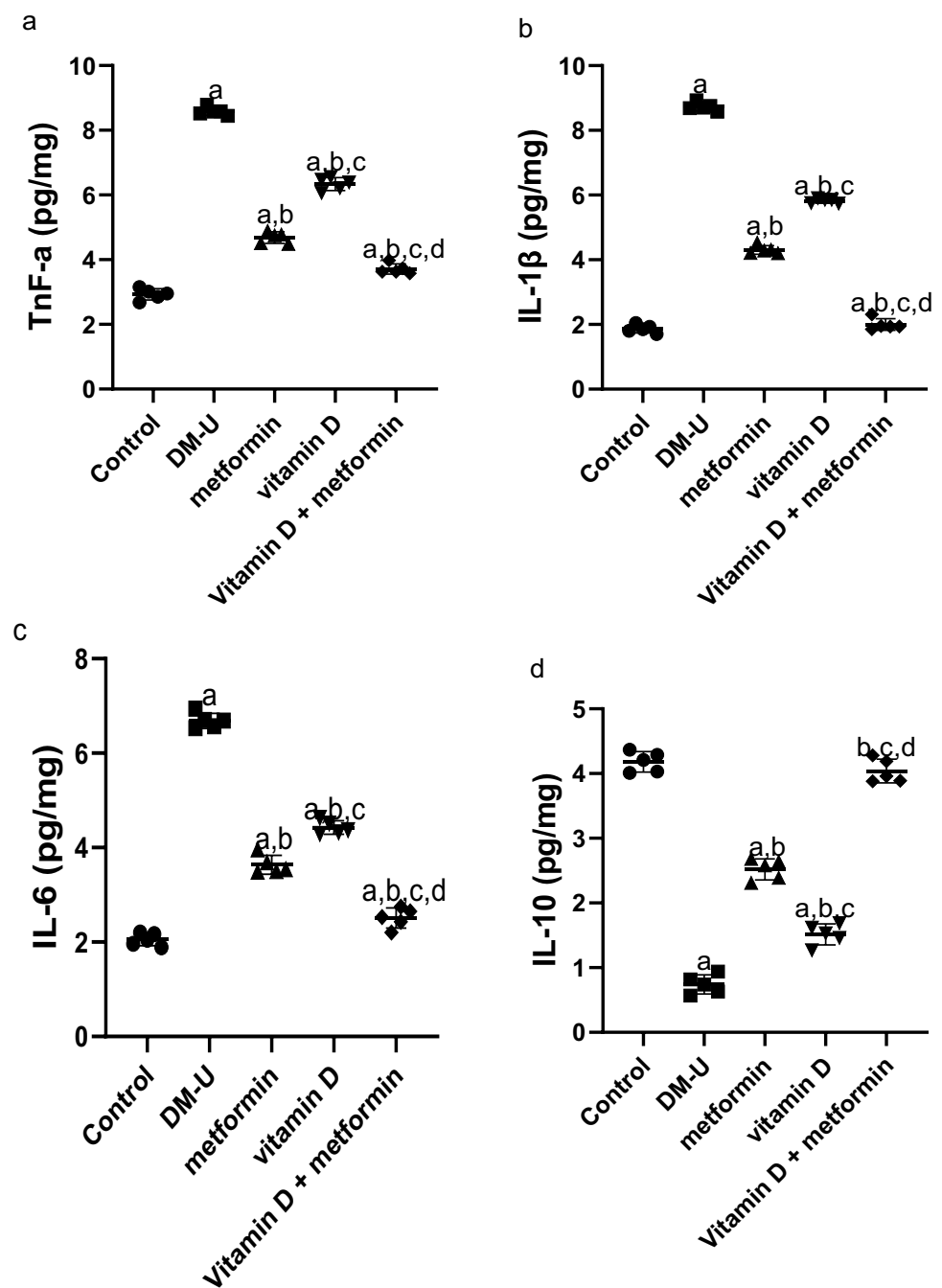


electrolyte balance after T2DM-induced nephrotoxicity suggests its renoprotective effects in T2DM animals. This could be attributed to its ability to restore proton pump activities. Na^+/K^+ ATPase and $\text{Ca}^{2+}/\text{Mg}^{2+}$ ATPase are enzymes present in the distal convoluted tubules and the basolateral membranes of the kidney, respectively (Adelakun et al. 2024). The kidney maintains serum electrolyte balance among other functions via the ion gradient produced by the Na^+/K^+ ATPase (Obafemi 2022), while $\text{Ca}^{2+}/\text{Mg}^{2+}$ ATPase maintains intracellular calcium concentration required for maintaining Ca^{2+} and Mg^{2+} homeostasis among other renal functions (Rodenbeck et al. 2017). Our findings that vitamin D restored proton pump activities agreed with those of Cross and Peterlik (1983) that reported

that vitamin D stimulated Na^+/K^+ ATPase activities in chicks. Hence, it is plausible that vitamin D enhanced metformin activities in T2DM-induced nephrotoxicity by upregulating proton pump activities.

The observed declined renal functions were consistent with our observed distortion in renal histology and injury markers. Our findings reported a disrupted renal histology which was ameliorated in animals treated with vitamin D with or without metformin. These findings corroborated those of Song et al. (2021) that reported improved renal histology following vitamin D treatment. Additionally, serum creatinine and urea are specific kidney function markers and the observed increase in these markers following T2DM is an indication of declined renal functions (Gowda

Fig. 6 Effect of vitamin D and/or metformin on inflammatory markers following T2DM induction. ^a $P < 0.05$ vs control, ^b $P < 0.05$ vs DM-U, ^c $P < 0.05$ vs DM + met, ^d $P < 0.05$ vs DM + vit D. Data ($n = 6$) were analyzed by one-way ANOVA and Tukey's post hoc test and reported as mean $\pm$ SD. DM-U diabetic untreated



et al. 2010). Also, lactate and LDH are other injury markers (Allen et al. 2021) that can suggest renal dysfunctions. Hence, vitamin D-induced reduction in these markers suggests its renoprotective effects in diabetic rats.

The observed renal injury following T2DM could be attributed to dyslipidemia and redox imbalance. Dyslipidemia is a common comorbidity of T2DM, which is characterized by a significant increase in TG, TC, and LDL and a decrease in HDL levels (Kane et al. 2021). In agreement with our findings, previous reports established a

link between vitamin D and lipid metabolism (Gong et al. 2022; Shafie et al. 2022), although recent findings reported that vitamin D did not significantly influence TC, LDL, and HDL levels. However, the same findings suggested that higher doses of vitamin D could decrease TG and fasting blood sugar levels (Cai et al. 2021). Here, we reported that vitamin D with or without metformin supplementation markedly lowered renal TG, TC, and HDL and increased HDL levels in T2DM-exposed animals. These findings could at least in part account for the reno-protective effect

Fig. 7 Effect of vitamin D and/or metformin on Nrf2/Nfkb signaling following T2DM induction. ^a $P < 0.05$ vs control, ^b $P < 0.05$ vs DM-U, ^c $P < 0.05$ vs DM+met, ^d $P < 0.05$ vs DM+vit D. Data ($n=6$) were analyzed by one-way ANOVA and Tukey's post hoc test and reported as mean $\pm$ SD. DM-U diabetic untreated

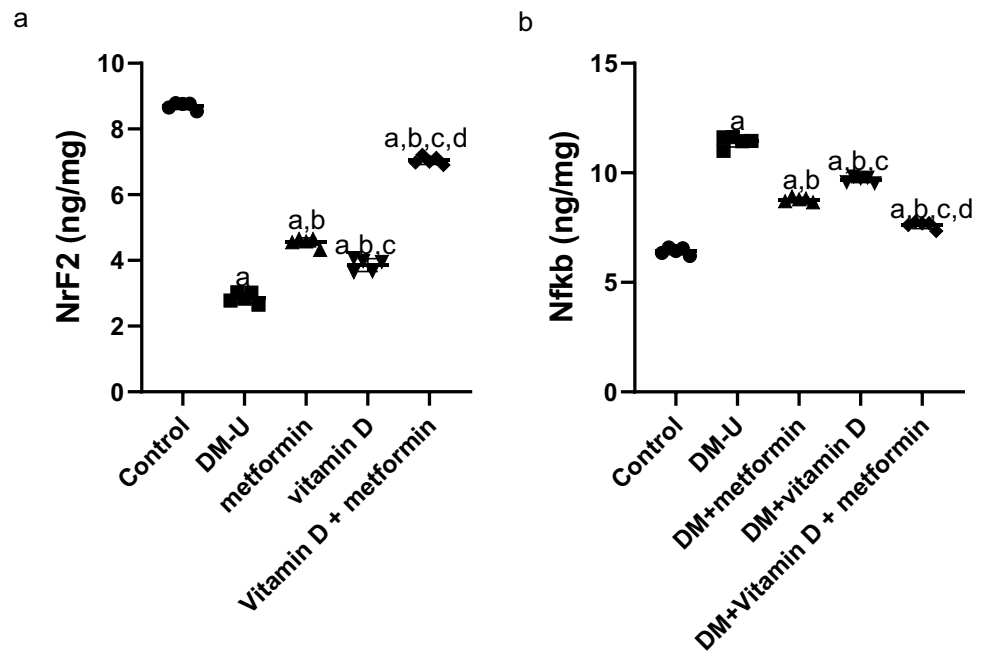
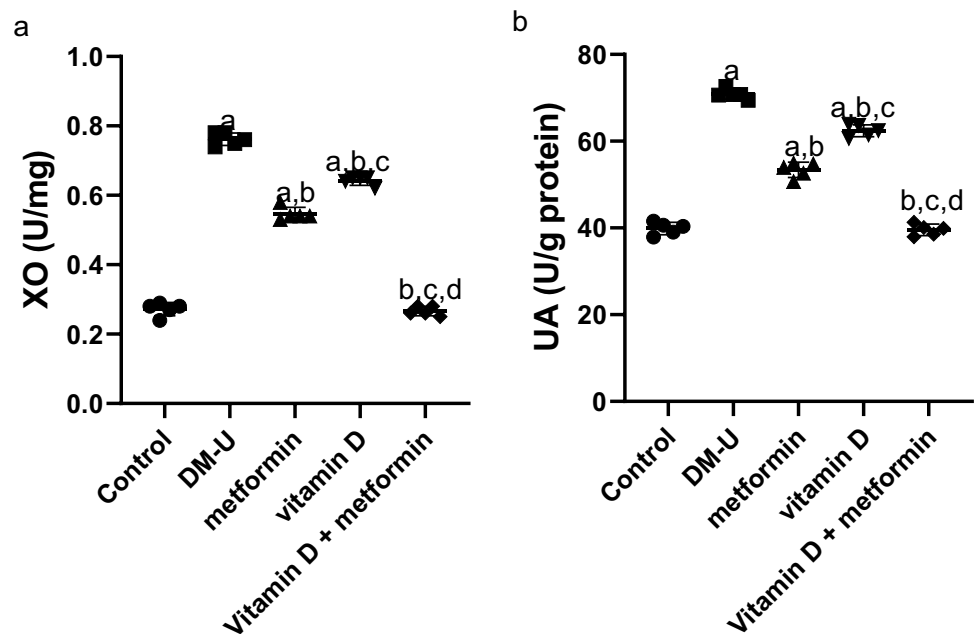


Fig. 8 Effect of vitamin D and/or metformin on xanthine oxidase (XO)/uric acid (UA) signaling following T2DM induction. ^a $P < 0.05$ vs control, ^b $P < 0.05$ vs DM-U, ^c $P < 0.05$ vs DM+met, ^d $P < 0.05$ vs DM+vit D. Data ($n=6$) were analyzed by one-way ANOVA and Tukey's post hoc test and reported as mean $\pm$ SD. DM-U diabetic untreated



of vitamin D in T2DM-induced animals since dyslipidemia is one of the main risk factors in DKD leading to nephrotic syndrome or renal insufficiency and glomerular damage (Mooradian 2009; Palyginet al. 2019). Additionally, elevated TC and TG observed in T2DM have been identified as an independent factor for renal disease progression in humans (Vergès, 2015). Hence, the ability of vitamin D to reduce T2DM-induced dyslipidemia could contribute to its ameliorative effect on T2DM-induced renal dysfunction.

Another important factor that can contribute to renal dysfunction is oxidative stress since redox imbalance and dyslipidemia have been identified as indispensable features of nephrotoxicity (Jabarpour et al. 2019). Mounting evidence has established that redox imbalance is a common denominator associating renal metabolic dysfunction with impaired renal hemodynamics in DKD (Jha et al. 2017). Renal redox imbalance in DKD is majorly caused by pro-oxidant accumulation and a decrease in antioxidant activities (Piko

et al. 2023). According to Jha et al. (2016), pro-oxidants do not only accumulate in the podocytes and the glomerulus, but also in the tubulointerstitium where they also mediate inflammatory responses. Vitamin D on the other hand could prevent T2DM-induced renal injury via its antioxidative stress and anti-inflammatory properties. Corroborating this guess, an observational study involving 66 obese children established a relationship between insufficient vitamin D and redox imbalance and inflammatory response (Codoñer-Franch et al. 2012). Different in vitro and in vivo studies also found that vitamin D supplementation decreases oxidative stress and inflammatory response and increases the level of antioxidant and anti-inflammatory markers (Zhang, et al., 2014; Akbari et al. 2018; Odetayo et al. 2024a). These observed reno-protective effects were associated with Nrf2/Nf- κ b activities since they have been reported to play a dominant role in T2DM-induced oxidative renal injury (Cui et al. 2017; Lin et al. 2023). Nrf2 maintains redox balance by upregulating the activities of various antioxidant systems (Ajibare et al. 2024a) and preventing inflammatory response by inhibiting Nf- κ b activities (Ajibare et al. 2024b). Nf- κ b is an inflammatory and redox imbalance marker which has been shown to be upregulated in DM (Morcos et al. 2002) and plays a crucial role in diabetic nephropathy pathogenesis (Nakai et al. 2014). The observed Nf- κ b upregulation in DM is closely associated with increased mesangial cell proliferation, which may be a major step in diabetes nephropathy pathogenesis (Zhang et al. 2012). This may participate in renal extracellular matrix accumulation, resulting in renal injury (Kanwar et al. 2011). It may therefore be intriguing that similar to previous studies (Nakai et al. 2014; Liu et al. 2023), our results showed that vitamin D upregulates Nrf2 and downregulates Nf- κ b activities in DM rats. Hence, the upregulation of Nrf2 activities and consequent decrease in Nf- κ b activities in animals treated with vitamin D with or without metformin indicates that vitamin D probably prevents oxidative renal injury via the Nrf2-dependent pathway.

XO/UA signaling is another pathway involved in T2DM-induced oxido-inflammatory response. Mounting evidence shows that XO is involved in T2DM-induced redox imbalance and the consequent inflammatory response. Data from a preclinical study established DM-induced increase in MDA and protein oxidation was suppressed in DM animals treated with allopurinol (XO inhibitor) (Romagnoli et al. 2010). In the same vein, studies involving human subjects revealed an increase in serum XO level in T2DM patients (Kuppusamy et al. 2005; Miric et al. 2016). Additionally, plasma XO is positively correlated with IR (Sunagawa et al. 2019). The T2DM-induced XO activities upregulation in this study accounted for the increased UA concentration since XO metabolized purine nucleotides into UA (Hernandez-Hernandez et al. 2022; Odetayo and Olayaki 2022). This observed hyperuricemia is common in chronic kidney

disease (CKD) (Saliba et al. 2024). Although hyperuricemia could be a result of CKD-impaired glomerular filtration rate (GFR), it can also precede CKD. Previous findings revealed that UA can directly impair renal functions by causing ischemia, tubulointerstitial fibrosis, and oxido-inflammatory response (Piani et al. 2021). Hence, the observed decrease in XO/UA-mediated oxido-inflammatory response in animals treated with vitamin D with or without metformin suggests that vitamin D conferred its reno-protective activities against T2DM-induced renal injury by suppressing the activities of XO/UA signaling. This claim is substantiated by the study of Zhang et al. (2020) that revealed that vitamin D supplementation is inversely related to hyperuricemia. Our claim is also supported by previous studies that reported that vitamin D deficiency can stimulate the release of parathyroid hormone (Emilion and Emilion 2011) which is directly associated with an increase in UA (Neer et al. 2001; Dalbeth et al. 2009).

Our findings that vitamin D exerted its reno-protective effect via oxido-inflammatory-dependent mechanisms could be associated with the agonistic effects of vitamin D on vitamin D receptors (VDRs). VDR agonists have been established to have inhibitory effects on the renin–angiotensin–aldosterone system (RAAS) that has been implicated in IR and oxido-inflammatory response (Ganimusa et al. 2024). Also, VDR activation by their agonists directly inhibits NF- κ B expression via ROS-mediated signalings (Ramalingam et al. 2017). Hence, it is plausible that vitamin D prevented renal injury in T2DM by activating VDRs which in turn maintained redox balance via the inhibition of RAAS and NF- κ B activities.

Conclusion

This study indicated that vitamin D and/or metformin maintained renal function in T2DM animals by maintaining glucose homeostasis, thereby regulating electrolyte balance and urea and creatinine levels by modulating proton pump activities and ROS-inflammatory-dependent mechanisms. These findings provide a possible insight into the protective molecular mechanisms of vitamin D and metformin against T2DM-induced renal dysfunction.

Limitations

Measuring the gene expression of transcription factors such as Nrf2 and Nf- κ b will provide more mechanistic information on the role of vitamin D and metformin on T2DM. However, the protein level of renal Nrf2 and Nf- κ b was assayed. These proteins' expression indicates that the genes

would be expressed too since it is known that proteins are transcriptional and translational products of genes.

Author contributions AHA, OAF, and OLA: Conceptualization, AHA, OAF, OEA, BJD, JFB, GEO, OIF, OLA: Methodology, OAF: Data curation, OAF: Writing- Original draft preparation. AHA, OAF, DAT, AYF, SRO, OPK, OLA: Visualization, Investigation. AHA, OAF, and OLA: Supervision AHA, OAF, OLA Software, Validation.: AHA, OAF, DAT, AYF, SRO, OPK, OLA: Writing- Reviewing and Editing. The authors declare that all data were generated in-house and that no paper mill was used.

Data Availability No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate The animals were carefully handled as stated by the National Institute of Health (NIH), and ARRIVE guidelines for reporting experimental findings were strictly followed. The experimental research protocol was designed according to the National Research Council's guidelines for the Care and Use of Laboratory Animals, and ethical approval was obtained from the University of Ilorin's ethical review committee before the commencement of the research work.

Consent for publication Not applicable.

Competing of interest The authors declare no competing interests.

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