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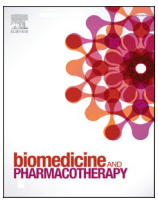

Supplementation of conventional anti-diabetic therapy with alpha-lipoic acid prevents early development and progression of diabetic nephropathy

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ABSTRACT

Background: Diabetic nephropathy (DN) is the leading cause of end-stage renal disease. Current pharmacological interventions only retard DN progression. Alpha-lipoic acid (ALA) is a potent antioxidant with beneficial effect in other diabetic complications. This study investigates whether ALA supplementation prevents early development and progression of DN.

Method: Fifty-eight male Sprague-Dawley rats were randomly assigned to healthy control and diabetic groups and subjected to overnight fasting. Type 2 diabetes mellitus (T2DM) was induced in diabetic group by intraperitoneal administration of nicotinamide (110 mg/kg) and streptozotocin (55 mg/kg). On day 3 after T2DM induction, diabetic rats received oral daily administration of ALA (60 mg/kg), gliclazide (15 mg/kg), ramipril (10 mg/kg) or drug combinations for 6 weeks. Untreated diabetic rats served as diabetic control. Blood, kidneys and pancreas were harvested for biochemical and histological analyses.

Result: Induction of T2DM resulted in hypoinsulinemia, hyperglycemia and renal pathology. ALA supplementation maintained β-cell function, normoinsulinemia and normoglycemia in diabetic rats, and prevented renal pathology (PAS, KIM-1, plasma creatinine, total protein, blood urea nitrogen, uric acid and urine albumin/creatinine ratio) and triglycerides level compared to diabetic control ($p < 0.001$). Additionally, ALA supplementation significantly prevented elevated serum and tissue malondialdehyde, collagen deposition, α-SMA expression, apoptosis and serum IL-1β and IL-6 levels while it markedly increased renal glutathione content and plasma HDL-C compared to diabetic control group ($p < 0.001$).

Conclusion: ALA supplementation prevents early development and progression of DN by exerting anti-hyperglycemic, antioxidant, anti-inflammatory, anti-fibrotic and anti-apoptotic effects. Our findings provide additional option for clinical treatment of DN in T2DM patients.

1. Introduction

Diabetic nephropathy (DN), also called diabetic kidney disease, is a significant and long-term complication of diabetes mellitus. It is currently the leading cause of end-stage renal disease (ESRD) and a major contributor to mortality of diabetic patients globally [1,2]. It affects about 20–30% of type 1 diabetes mellitus (T1DM) patients and 35–40% of type 2 diabetes mellitus (T2DM) patients globally [3]. DN is assessed clinically through a five-stage criteria system, with each stage featuring a distinct set of changes of structural, functional and renal function markers [4]. It is characterised by clinical features such as persistent albuminuria, decreased glomerular filtration rate, hypercreatininemia, uremia and elevated blood pressure [5]. Recent studies have shown that a major molecular mechanism underlying the pathogenesis and progression of DN is excess production of reactive oxygen species (ROS) of which little therapeutic avenues have been explored [6–12]. ROS are free radicals which are principally produced by mitochondria during metabolic processes, and their over-production leads to oxidative stress, which mediates tissue injury as observed in pathological conditions such as diabetes mellitus. Interestingly, oxidative stress has been established in the pathogenesis and progression of DN in T2DM in human patients [13,14].

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Current management of DN includes non-pharmacological and pharmacological approaches. The former approach is based on lifestyle modifications such as diet (reduction in sugar and sodium intake), physical exercise, psychological and social interventions and diabetes self-management education [15]. The pharmacological interventions are based on reduction of hyperglycemia using anti-diabetic drugs and blockade of the renin-angiotensin-aldosterone system using angiotensin-converting enzyme inhibitors and angiotensin II receptor blockers, and non-dihydropyridine calcium channel blockers, thus ensuring strict glycemic and blood pressure control to attenuate proteinuria and slow down progression of DN to ESRD and cardiovascular complications [9,16]. Whereas these pharmacological agents have been approved as cornerstone for the management of DN, they only retard DN progression but do not reverse or prevent it. Therefore, there is urgent need to identify novel pharmacological agents and strategies to target signalling pathways including ROS-induced oxidative stress. Alpha-lipoic acid (ALA), also known as thioctic acid, is a sulphur-containing natural antioxidant and a cofactor for several important mitochondrial multienzyme complexes involved in mitochondrial bioenergetics and amino acid metabolism [17]. It is endogenously produced by lipoic acid synthase in the mitochondria of tissues including the kidney and is also derived from plant and animal sources or given as a dietary supplement [18,19]. There are studies showing that circulating ALA levels are decreased in diabetic patients, and that ALA supplementation is beneficial in the management of both T1DM and T2DM and diabetic complications such as diabetic peripheral neuropathy in preclinical and clinical settings [20,21]. ALA has also been shown to improve blood glucose, lipid profiles and gamma-glutamyl transferase in pregnant women with gestational diabetes mellitus [22,23]. Rodent models also show acceleration of DN following genetic inhibition of lipoic acid synthase [24] and that ALA supplementation attenuated development and progression of DN [25–28] while it further showed beneficial effect in ESRD patients on hemodialysis [29]. However, its effect with conventional anti-diabetic therapy against DN in T2DM has not been described. Therefore, we hypothesize that ALA supplementation would prevent hyperglycemia and protect the kidney beyond that offered by conventional anti-diabetic therapy.

2. Materials and methods

2.1. Ethical statement

The experimental protocol was approved by the University of Ghana Institutional Animal Care and Use Committee (Protocol ID: UG-IACUC 002/19–20). The experiment was done in the Laboratory Animal Facility (with Office of Laboratory Animal Welfare assurance number A7604–01) of the Noguchi Memorial Institute for medical research, adhering to protocol and maintaining quality assurance in accordance with good laboratory practice. All procedures and techniques used in this study were in accordance with the National Institute of Health Guidelines for the care and use of laboratory animals.

2.2. Animal handling

Fifty-eight male Sprague-Dawley rats weighing 150 – 200 g and between 6 and 8 weeks old were obtained from the Department of Animal Experimentation, Noguchi Memorial Institute for Medical Research, University of Ghana, Legon, Accra. Rats were housed in standard cages in the same Department at 21 – 25 °C ambient temperature and relative humidity of 45 – 55% at a 12:12 h light: dark cycle. They were fed with standard rat chow (Agricare, Kumasi) and tap water ad libitum and allowed to acclimatise for seven days before the commencement of the experiment.

3. Experimental protocol

3.1. Induction of T2DM

The rats were subjected to 12 h of overnight fast, and fasting blood glucose was measured the next day. As described in previous studies [30,31], T2DM was induced in 51 out of 58 rats by intraperitoneal administration of 110 mg/kg nicotinamide (purchased from Nanjing Yasnt Bio-Tech Co. Ltd - Nanjing, China). Fifteen minutes later, a single dose of 55 mg/kg streptozotocin (STZ; purchased from Sigma Aldrich, Missouri, USA and dissolved in freshly prepared 0.1 M citrate buffer of pH 4.5) was administered intraperitoneally. Fasting blood glucose was measured on day 3 with a portable hand-held glucometer (One Touch Select Plus®; LifeScan Inc., Zug, Switzerland) after T2DM induction. Rats with fasting blood glucose levels exceeding 13.9 mmol/L (250 mg/dL) were considered diabetic and included in this study as previously described [31]. The remaining seven rats served as healthy control.

3.2. Treatment groups

Following confirmation of T2DM on day 3, six groups of diabetic rats were either treated with daily oral administration of 15 mg/kg gliclazide (GLC; Servier Laboratories Limited, France; n = 7), 10 mg/kg ramipril (RAM; Teva Pharmaceuticals, United Kingdom; n = 7), 60 mg/kg alpha-lipoic acid (ALA; Nanjing Yasnt Bio-Tech Co. Ltd - Nanjing, China; n = 7) or combinations of these drugs for 6 weeks while another group of diabetic rats (Diabetic control; n = 9) received distilled water. Body weights and blood glucose were measured on days 7, 14, 21, 28, 35 and 42.

3.3. Euthanasia and organ harvest

After 6 weeks of treatment, rats were euthanized with ketamine: xylazine intraperitoneally. About 8 mL of blood sample was obtained from each rat via cardiac puncture and transferred into EDTA and Eppendorf tubes. The kidneys and pancreas were harvested, weighed and stored for analysis. The right kidney was snap-frozen in liquid nitrogen and transferred into a − 80 °C freezer while the left kidney and pancreas were stored in 10% neutral buffered formalin for histological analysis. Relative kidney weight was calculated by expressing the weight of the kidneys as a percentage of the rat's body weight. Urine samples were also collected on the day of sacrifice via bladder puncture for analysis.

3.4. Plasma/serum preparation and biochemical analysis

The blood samples in EDTA and Eppendorf tubes were centrifuged at 3000 rpm for 15 min at 4 °C. Plasma and serum samples obtained from centrifugation were stored in EDTA and Eppendorf tubes, respectively at − 20 °C. Plasma insulin levels were determined using a radioimmunoassay (RIA) kit in accordance with the manufacturer's instructions (Atom High-Tech Co., Ltd., Beijing, China). Plasma creatinine, total protein, blood urea nitrogen, uric acid, triglycerides (TG) and high-density lipoproteins-cholesterol (HDL) were also measured as we previously described [32] and according to the manufacturer's instructions (Mindray BS-200 Biochemistry Auto-analyzer, Shenzhen, China). Serum levels of interleukin-1β and interleukin-6 (IL-1β and IL-6; inflammatory markers) were also measured by ELISA as previously described [33] using a DuoSet Kit following the manufacturer's instructions (Quantikine, R&D Systems, Minneapolis, MN, USA). Urine samples from the various groups were collected via bladder puncture upon euthanasia. Urinary albumin levels were measured using an automatic chemical analyser (Dirui, Changchun, China) and urinary albumin/creatinine ratio of each group was calculated. β-cell function was assessed by computing HOMA-β using the formula: 20 × Insulin (μ IU/mL) ÷ (Glucose (mmol/L) − 3.5) as reported by Deora et al [72].

3.5. Tissue processing and histological analysis

Histological analysis was performed as we previously described [35,36]. Following fixation with 10% neutral buffered formalin, the left kidney and pancreas were transferred onto labelled tissue cassettes and put into a tissue processor. The tissues were then dehydrated in increasing order of alcohol concentration (70%, 80%, 90% and 100%) followed by dehydration in xylene and finally embedded in molten paraffin wax. The paraffin-embedded tissues were cut from similar site (renal cortex) of the tissue and in similar plane into 4 μm-thick sections. The sections were floated on water at a temperature of 46 °C and were picked with glass slides. The tissue slides were labelled and dried in an oven at a temperature 60 °C for 3 h. Next, the tissue slides were deparaffinised in xylene and hydrated in decreasing series of alcohol (100%, 95%, 80%) and then in distilled water. Following the hydration procedure, the tissue slides were subjected to periodic acid Schiff (PAS) stain as we previously described [35]. Briefly, the tissue slides were placed in periodic acid for 5 min and then in Schiff's reagent for 20 min, and finally under running water for 60 s. The tissues were then counter-stained with hematoxylin for 60 s, and washed under running water for 5 min, dehydrated in increasing series of alcohol (80%, 95% and 100%) and cleared in xylene. Finally, the slides were mounted using a mounting medium, dried and viewed under a light microscope. Kidney sections were examined independently and in a double-blinded fashion under light microscope by two experienced renal pathologists. Semi-quantitative scoring of glomerular damage from 0 to 4 was done in 100 glomeruli based on glomerular basement membrane thickening (doubling), glomerular hypertrophy, mesangiolysis and polar vasculosis, which are characteristic lesions of DN. Tubulointerstitial damage was quantified based on tubular dilatation, atrophy of tubular epithelial cells and widening of tubular lumen as we previously described [35,36].

3.6. Immunohistochemistry

Following the standard protocol before PAS staining protocol described above, kidney sections were subjected to immunohistochemical staining as we previously described [35,36]. Briefly, antigen retrieval was done by placing the kidney sections in 0.1 M Tris/HCl buffer (pH 9.0) for overnight incubation at 80 °C and then blocked in 500 μL of 30% H_2O_2 for 30 min. Next, the sections were incubated in kidney injury molecule-1 (KIM-1; goat polyclonal, diluted 1:50 v/v, Santa Cruz, Netherlands) antibody, a renal tubular damage marker, alpha-smooth muscle actin (α-SMA; Dako; Carpinteria, CA, United States) antibody, a marker for fibrosis, and Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL; a marker of apoptosis purchased from Beyotime Biotechnology, Shanghai, China) for 60 min at room temperature. The samples were then washed with phosphate buffered saline (PBS) and then incubated in appropriate secondary and tertiary antibodies for 30 min at room temperature. After another washing with PBS, the sections were incubated in DAB for 15 min, and covered in Depex mounting medium and cover slips applied. The sections were viewed and quantified under a light microscope in a double-blinded fashion by two experienced renal pathologists. The quantification was done based on the staining intensity as follows: 0 = none or weak staining; 1 = stained area < 25% or weak-moderate staining; 2 = stained area within 25–50% or moderate staining; 3 = stained area within 50–75% or moderate-strong staining; 4 = stained area > 75% or strong staining. To assess the degree of tubulointerstitial collagen deposition, sections were stained with Masson Trichrome and the area of fibrosis was expressed as the percentage of the blue-stained area.

3.7. Determination of MDA levels and renal glutathione content

Measurement of serum and renal tissue malondialdehyde (MDA) was done as previously described [37,38] with some modifications. Briefly, a volume of 0.5 mL of serum was mixed with 0.5 mL of ice-cold 10% trichloroacetic acid. An amount of 50 mg of kidney tissue was homogenized and centrifuged at 2000 rpm for 3 min at 4 ºC. A volume of 0.5 mL of the supernatant was drawn into labelled tubes and mixed with 0.5 mL of ice-cold 10% trichloroacetic acid. A volume of 1 mL of 0.67% thiobarbituric acid was subsequently added to the mix (serum and renal tissue) in separate tubes each containing either serum or supernatant from kidney homogenate. The mixture was warmed in a waterbath at 100 ºC for 60 min and then allowed to cool. A volume of 4 mL of n-butanol was then added to the mix, vortexed for 30 s and centrifuged at 2000 rpm for 10 min at 4 ºC. The supernatant obtained was pipetted into a 96 well-plate in duplicates. The absorbance was then measured at 532 nm. The method was repeated using serum samples and lipid peroxidation was measured at the same optical density of 532 nm.

Renal glutathione (GSH) content was determined in kidney homogenates of each group using GSH-Glo™ kit from Promega (Madison, WI, USA) and as was previously described [34]. In brief, about 100 mg of frozen kidney tissue from each group was homogenized in 1 mL of ice-cold 0.5% potassium chloride and sonicated for 1 min. The homogenates were centrifuged at 3000 rpm at 4 °C for 10 min, and the supernatant used together with GSH standard in the test kit. Renal GSH content was quantified by chemiluminescence in a SpectraMax 2 plate reader.

3.8. Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM). Statistical analysis was evaluated using one-way analysis of variance (ANOVA) followed by Bonferroni's post-hoc test using Prism software (Prism 6, GraphPad Software, Inc., San Diego, CA, USA). A p-value of less than 0.05 between groups is considered statistically significant.

4. Results

4.1. Addition of ALA to anti-diabetic therapy maintained body weight, normoinsulinemia and normoglycemia

Induction of T2DM resulted in a significant weight loss, hypoinsulinemia and hyperglycemia (Fig. 1A-1 C). Except for the RAM only and RAM+ALA groups, the percentage change in body weight of all other treatment groups significantly increased compared to diabetic control group (Fig. 1A; $p < 0.001$). Remarkably, triple combination therapy (ALA+GLC+RAM) restored the body weights of rats to values comparable to those of healthy control group (Fig. 1A). Also, mean blood glucose level was markedly elevated in untreated diabetic rats due to reduced β-cell function and insulin level compared to that in healthy control rats (Fig. 1B-1D; $p < 0.001$). However, mono- and dual therapies significantly increased β-cell function and insulin levels and reduced mean blood glucose levels compared to diabetic control group (Fig. 1B-1D; $p < 0.05$) although did not reach the levels in the healthy control rats (Fig. 1B-1D; $p < 0.05$). Interestingly, triple combination therapy prevented these significant changes, and maintained β-cell function and insulin and glucose to levels comparable to healthy control value (Fig. 1B-1D; $p > 0.05$). The significantly reduced insulin and elevated mean blood glucose levels in the untreated diabetic control group reflects destruction of pancreatic islets of Langerhans, which affected insulin-producing β-cells compared to the intact islets in the healthy control group (Fig. 1E). Mono- and dual therapies resulted in some protection of the islets of Langerhans. However, superior protection was observed in rats which received triple combination therapy and was comparable to that in the healthy control group (Fig. 1E). Thus, triple combination therapy with ALA, GLC and RAM maintained body weight and preserved pancreatic islets of Langerhans and β-cell function in T2DM, leading to maintenance of normoinsulinemia and normoglycemia.

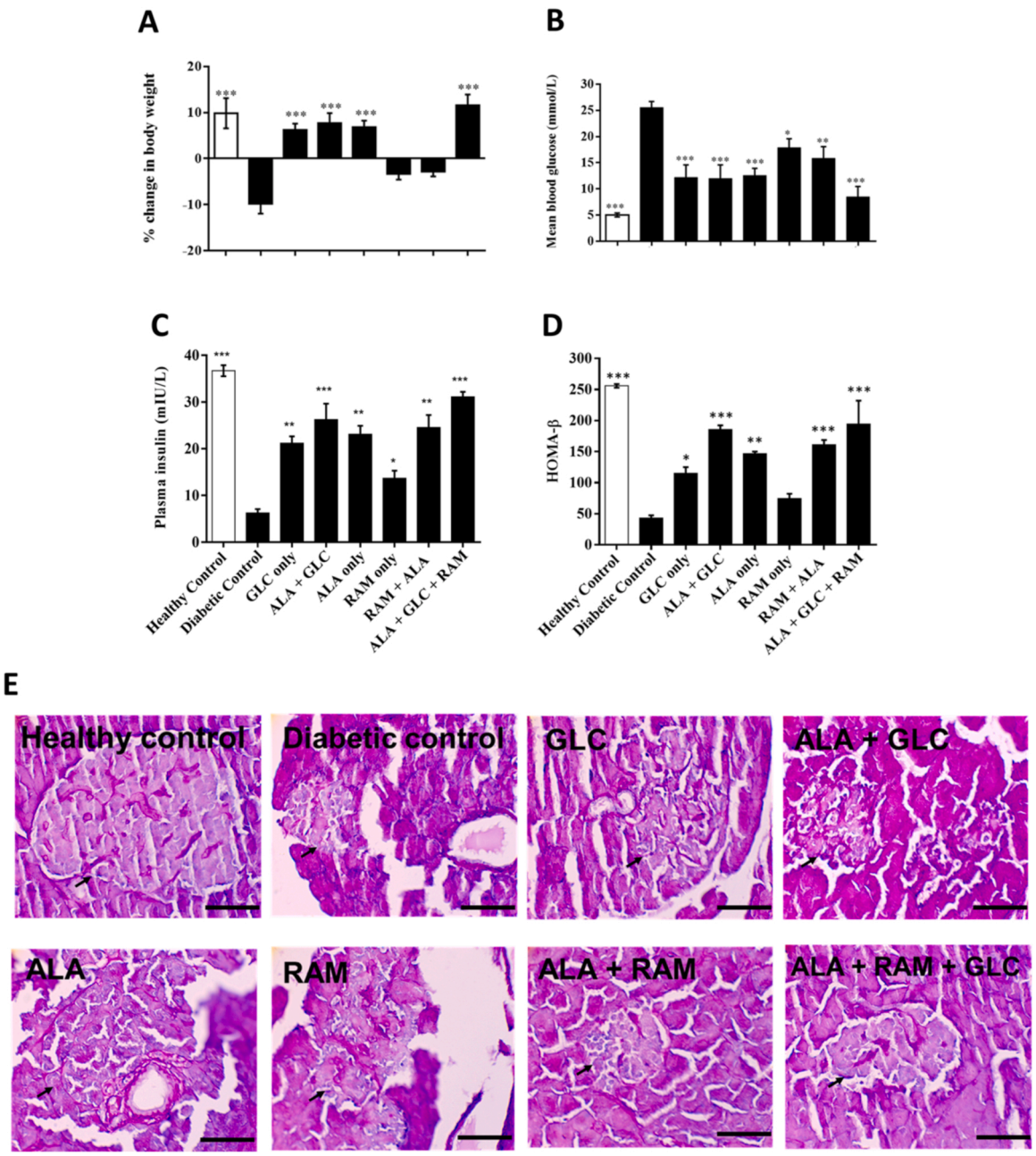

Fig. 1. Changes in body weight and mean glucose level. (A) Percentage change in body weight, (B) mean blood glucose levels, (C) plasma insulin (D) β-cell function and (E) representative photomicrographs of PAS-stained images of pancreatic tissues from all groups. Scale bars in right lower corner represent 50 μm. Arrows point to pancreatic islets of Langerhans. Healthy control; Diabetic control; GLC = Diabetic rats treated with gliclazide; ALA+GLC = Diabetic rats treated with alpha-lipoic acid and gliclazide; ALA = Diabetic rats treated with alpha-lipoic acid; RAM = Diabetic rats treated with ramipril; ALA+RAM = Diabetic rats treated with alpha-lipoic acid and ramipril; ALA+GLC+RAM = Diabetic rats treated with alpha-lipoic acid, gliclazide and ramipril. *$p < 0.05$ vs. diabetic control, **$p < 0.01$ vs. diabetic control, ***$p < 0.001$ vs. diabetic control.

4.2. Supplementation of anti-diabetic therapy with ALA prevented progression of early diabetic nephropathy

We examined histopathological changes in the kidneys following 6 weeks of T2DM induction and treatment. Induction of T2DM resulted in substantial injury in the glomerular and tubular compartments of the kidneys as assessed by PAS, KIM-1, α-SMA, TUNEL and Masson's trichrome stains compared to kidneys of healthy control rats (Fig. 2A-2 F; $p < 0.001$). With the exception of RAM only group, mono- and dual therapies with GLC, ALA and RAM significantly prevented the histopathological changes in comparison with untreated diabetic control rats (Fig. 2A-2 C; $p < 0.01$) but not comparable to healthy control rats. However, triple combination with ALA, GLC and RAM markedly prevented these morphological changes to a level comparable to healthy control group (Fig. 2A-2 C; $p > 0.05$). Also, mono- and dual therapies markedly reduced collagen deposition and α-SMA expression and protected renal tubular cells against apoptosis compared to untreated diabetic rats (Figs. 2A, 2D-2 F; $p < 0.05$), with the most effect observed in rats that received triple combination drug therapy, which is comparable to kidneys of healthy control rats (Figs. 2A, 2D-2 F; $p > 0.05$). Next, we measured plasma levels of creatinine, uric acid, total protein, blood urea nitrogen (BUN), and calculated urine albumin/creatinine ratio as markers of renal function as well as relative kidney weight. Corresponding with the histopathological assessment, T2DM markedly

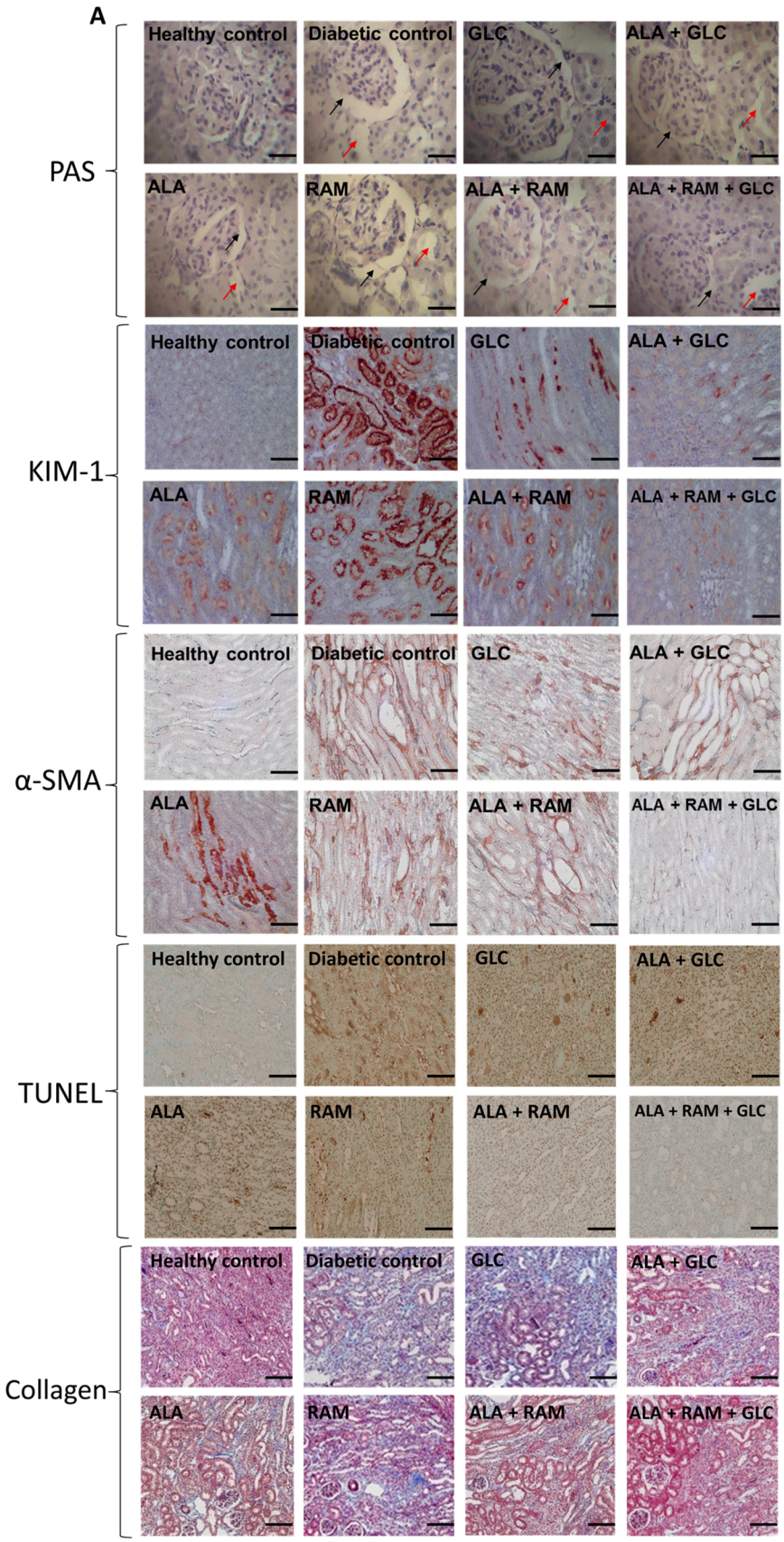

Fig. 2. Renal histopathology and quantification. (A) Representative photomicrograph of renal cortical section of all groups showing PAS, KIM-1, α-SMA, TUNEL and Masson's trichrome stainings. Positive areas are stained brown and collagen in Masson's trichrome stain is stained green-blue. Black arrows in PAS stain show expansion and thickening of glomerular basement while red arrows show tubular dilatation, atrophy of tubular epithelial cells and widening of tubular lumen. Scale bars in right lower corner represent 100 μm. (B-F) Quantification of immuno(histochemical) stainings of (B) PAS, (C) KIM-1, (D) α-SMA, (E) TUNEL and (F) Masson's

trichrome. Healthy control; Diabetic control; GLC = Diabetic rats treated with gliclazide; ALA+GLC = Diabetic rats treated with alpha-lipoic acid and gliclazide; ALA = Diabetic rats treated with alpha-lipoic acid; RAM = Diabetic rats treated with ramipril; ALA+RAM = Diabetic rats treated with alpha-lipoic acid and ramipril; ALA+GLC+RAM = Diabetic rats treated with alpha-lipoic acid, gliclazide and ramipril. *p < 0.05 vs. diabetic control, * *p < 0.01 vs. diabetic control, * **p < 0.001 vs. diabetic control.(For interpretation of the references to colour in this figure, the reader is referred to the web version of this article.)

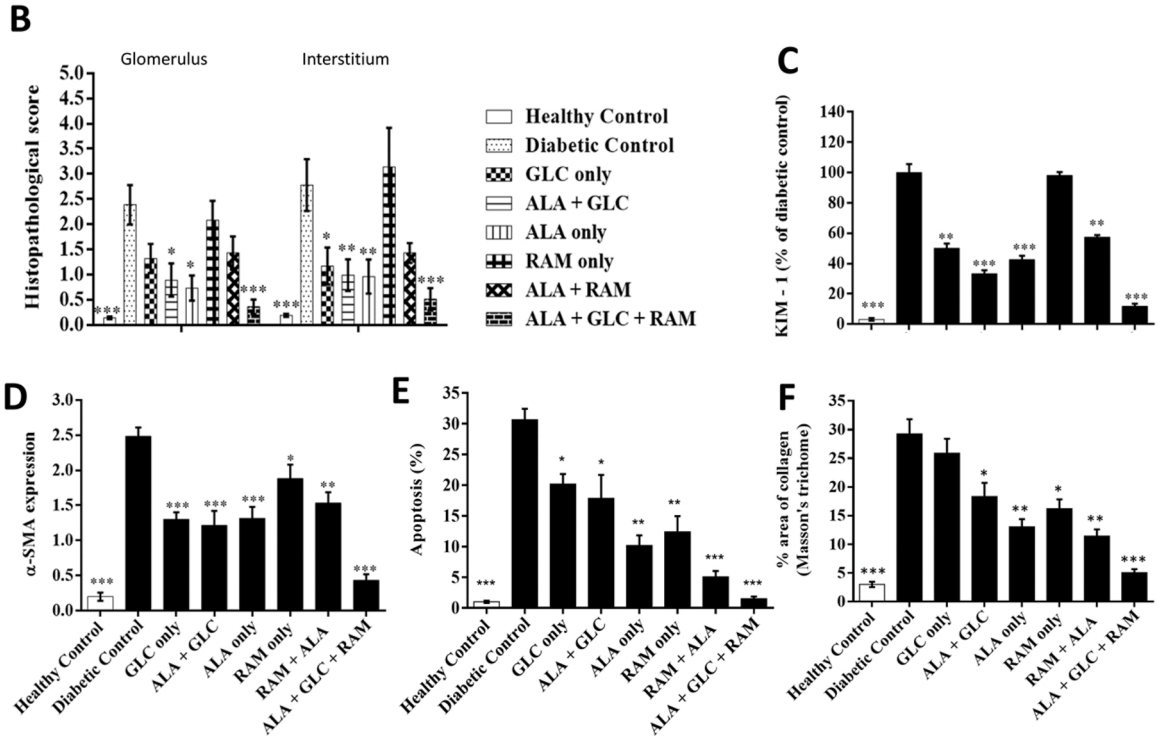

Fig. 2. (*continued*).

increased plasma levels of creatinine, BUN, uric acid and urine albumin/creatinine ratio and significantly decreased plasma total protein compared to the levels in healthy control rats (Fig. 3A-3E; $p < 0.05$). Interestingly, while mono- and dual therapies reduced the levels of these renal function markers compared to untreated diabetic control rats, monotherapy with RAM did not significantly reduce plasma creatinine level compared to the untreated diabetic control group (Fig. 3A; $p > 0.05$). Remarkably, triple combination therapy (ALA+GLC+RAM) reduced these markers of renal function to the levels in healthy control rats with the exception of BUN, which was significantly higher than in the healthy control group (Fig. 3B; $p < 0.05$). We also evaluated relative kidney weight by computing kidney-to-body weight ratio expressed as a percentage. The relative kidney weight was significantly higher in the untreated diabetic control rats compared to the healthy control rats and the rats that received various treatments (Fig. 3F; $p < 0.05$). Collectively, these results demonstrate that treatment of T2DM with ALA+GLC+RAM protects against progression of early diabetic nephropathy beyond that offered by mono- and dual therapies.

4.3. Combination therapy with ALA improved lipid profile and suppressed ROS production and inflammation

To determine whether alteration in lipid metabolism was associated with diabetic nephropathy, we measured the plasma levels triglycerides and high-density lipoprotein-cholesterol (HDL-C). Induction of T2DM resulted in about three-fold increase in plasma levels of triglycerides compared to healthy control rats (Fig. 4A; $p < 0.001$). Mono- and dual therapies reduced plasma triglycerides level significantly to near healthy control value while a triple combination drug therapy (ALA+GLC+RAM) reduced plasma triglycerides to a level slightly lower than healthy control level (Fig. 4A; $p > 0.05$). Interestingly, our measurement of plasma HDL-C level revealed no statistical difference between untreated diabetic control and healthy control rats. However, mono- and combination therapies significantly increased plasma HDL-C levels relative to healthy and untreated diabetic controls with the exception of RAM only group (Fig. 4B; $p < 0.05$). To determine the mechanisms by which the drugs protect against diabetic nephropathy, we explored antioxidant, anti-inflammatory and anti-fibrotic pathways through measurement of serum and tissue malondialdehyde (MDA; an indicator of ROS production), tissue glutathione (GSH) content, serum interleukin-1β (IL-1β) and interleukin-6 (IL-6), and α-SMA staining of the kidney. As with plasma triglycerides and HDL-C, levels of serum and renal MDA as well as serum IL-1β and IL-6 were markedly elevated in untreated diabetic control rats in comparison with healthy control group (Figs. 4C, 4E and 4F; $p < 0.01$). Mono- and combination drug therapies significantly reduced the increased MDA, IL-1β and IL-6 to near healthy control levels, with the exception of RAM only, which did not significantly change IL-6 compared to untreated diabetic control group (Figs. 4C, 4E and 4F; $p < 0.05$). In addition, renal content of GSH substantially decreased in the untreated diabetic control group relative to healthy control rats (Fig. 4D; $p < 0.001$). However, mono- and dual therapies significantly raised GSH content in the renal tissue to near healthy control value (Fig. 4D). Interestingly, renal GSH content of rats that received triple drug therapy (ALA, GLC and RAM) was significantly higher than that in healthy control kidneys (Fig. 4D; $p < 0.05$). Taken together, triple combination therapy with ALA, GLC and RAM improved lipid profile and suppressed the levels of ROS production, pro-inflammatory mediators and fibrosis while increasing renal antioxidant status.

5. Discussion

The present study shows that supplementation of conventional anti-diabetic therapy with alpha-lipoic acid (ALA) normalizes body weight,

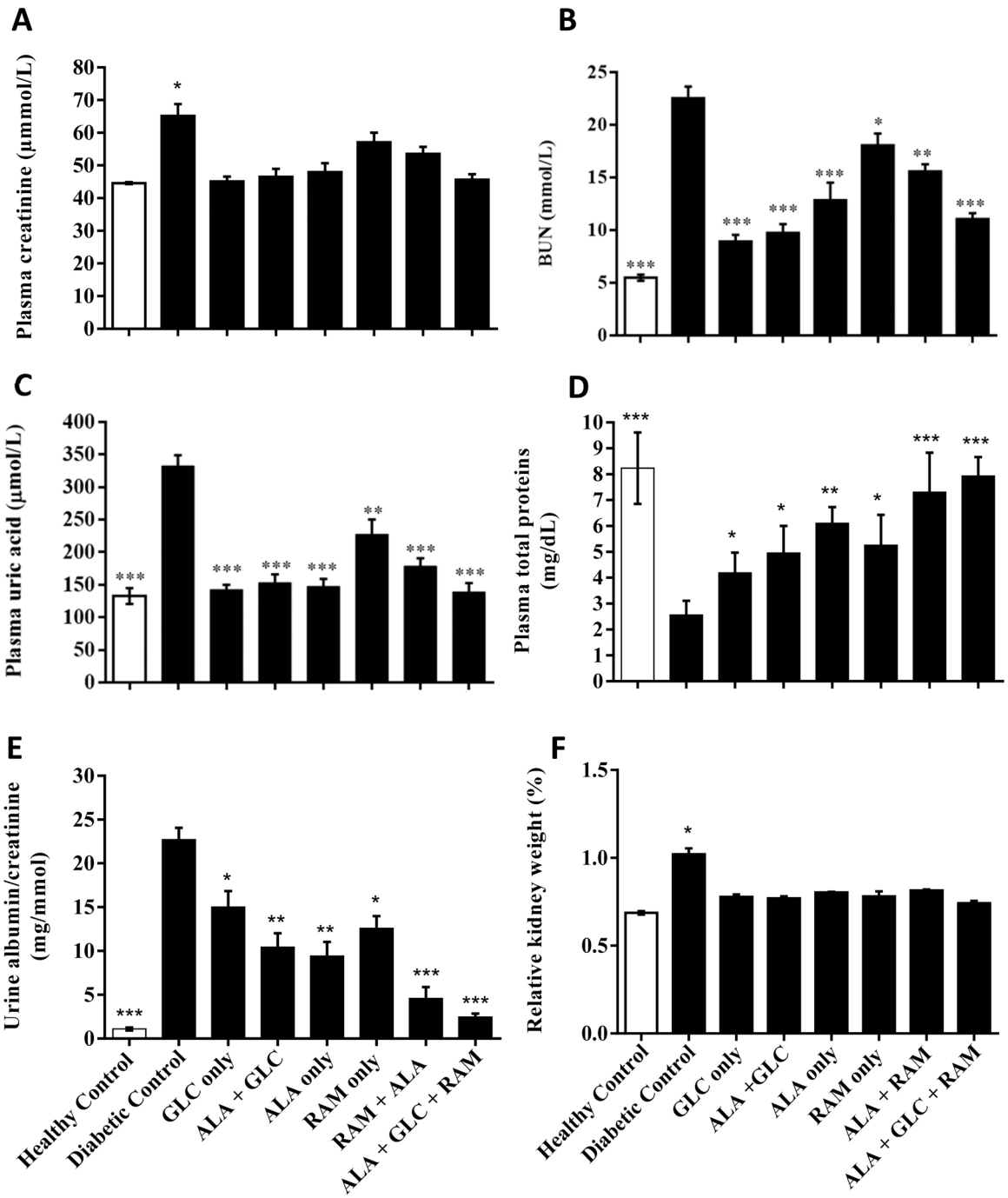

Fig. 3. Renal function parameters. Levels of (A) plasma creatinine, (B) blood urea nitrogen (BUN), (C) plasma uric acid, (D) plasma total protein, (E) urine albumin/creatinine ration and (F) relative kidney weight. Healthy control; Diabetic control; GLC = Diabetic rats treated with gliclazide; ALA+GLC = Diabetic rats treated with alpha-lipoic acid and gliclazide; ALA = Diabetic rats treated with alpha-lipoic acid; RAM = Diabetic rats treated with ramipril; ALA+RAM = Diabetic rats treated with alpha-lipoic acid and ramipril; ALA+GLC+RAM = Diabetic rats treated with alpha-lipoic acid, gliclazide and ramipril. *$p < 0.05$ vs. diabetic control, **$p < 0.01$ vs. diabetic control, ***$p < 0.001$ vs. diabetic control.

preserves pancreatic islets and maintains normoglycemia, while reducing renal injury, inflammation and renal fibrosis and improving lipid profile and renal antioxidant status in T2DM. All these effects prevented progression of diabetic nephropathy (DN) and ultimately resulted in preservation of renal function and integrity. Compared to healthy control rats, we observed a five-fold increase in glucose level and significant weight loss in our T2DM rats, which are common features associated with uncontrolled or untreated diabetes mellitus. As pharmacological management of diabetes mellitus requires continuous monitoring for optimal glycemic control, administration with gliclazide, a known second-generation sulfonylurea used together with other hypoglycemic agents in the management of T2DM, markedly reduced glucose level by half, although did not reach normoglycemic level. A similar observation was made following administration of ALA. Treatment with ramipril (an angiotensin-converting enzyme inhibitor commonly used for the treatment of patients with concomitant hypertension and diabetes mellitus with incipient or overt nephropathy) also reduced glucose level of diabetic rats albeit the level was significantly higher than monotherapies with gliclazide and ALA. However, a triple combination therapy with ALA, gliclazide and ramipril markedly

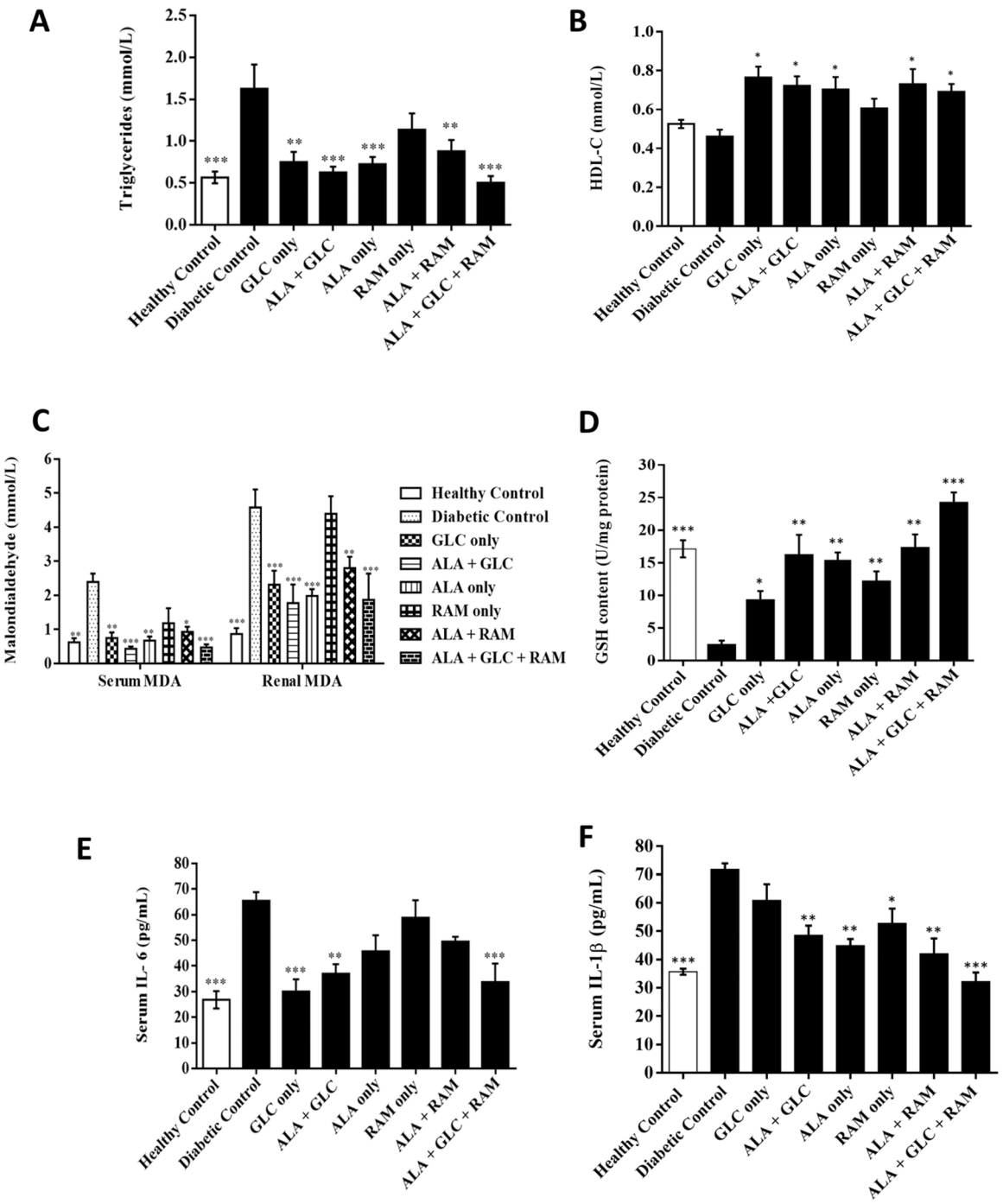

Fig. 4. Lipid profile, antioxidant and anti-inflammatory effects. Levels of (A) plasma triglycerides, (B) high-density lipoprotein-cholesterol (HDL-C), (C) serum and renal malondialdehyde (MDA) (D) renal glutathione (GSH), (E) serum IL-6 and (F) serum IL-1β. Healthy control; Diabetic control; GLC = Diabetic rats treated with gliclazide; ALA+GLC = Diabetic rats treated with alpha-lipoic acid and gliclazide; ALA = Diabetic rats treated with alpha-lipoic acid; RAM = Diabetic rats treated with ramipril; ALA+RAM = Diabetic rats treated with alpha-lipoic acid and ramipril; ALA+GLC+RAM = Diabetic rats treated with alpha-lipoic acid, gliclazide and ramipril. *$p < 0.05$ vs. diabetic control, **$p < 0.01$ vs. diabetic control, ***$p < 0.001$ vs. diabetic control.

reduced hyperglycemia to a level comparable to normoglycemia. Although we did not explore the complex interplay of metabolic and electrogenic mechanisms that regulate insulin production and secretion by β-cells of pancreatic islets of Langerhans, our observation with the triple combination therapy suggests a synergistic effect and/or activation of different mechanisms by each of these drugs, which could partly contribute to stimulating insulin release from functioning pancreatic β-cells via reduction of potassium ion permeability (a known mechanism of insulin secretion) and increasing peripheral tissue sensitivity of insulin, and thereby decreasing insulin resistance [39]. Besides, we observed adequate protection of the pancreatic islets following the triple combination therapy, which positively correlated with increased β-cell function and plasma insulin levels within the range of healthy control values, suggesting preservation of the insulin-releasing β-cells. Our observation aligns with that of Yi et al [40] who reported β-cell protection and improved islet function following ALA supplementation in a mouse model of DN. It is important to note that ALA possesses insulin-mimetic activity, enhancing the activity of insulin receptor, and

thereby leading to cytoprotection via inhibition of apoptotic pathway [41]. It also increases insulin sensitivity in skeletal muscles by enhancing plasma translocation of glucose transporter protein subtype 4 (GLUT 4) and stimulating AMP-activated protein kinase (AMPK) signalling pathways [42,43] as well as phosphoinositide-3-kinase (PI3K), promoting tyrosine phosphorylation in the insulin receptor and improving PI3K-dependent glucose uptake [41,44,45], all of which are important glucose-regulating mechanisms. Since skeletal muscle serves as a major sink in the body for glucose after a meal, pharmacological agents that facilitate glucose uptake by skeletal muscle are potentially useful in the management or treatment of T2DM in the long-term. These mechanisms of ALA together with the well-established closure of ATP-sensitive potassium channels (in β-cell plasma membrane) resulting in Ca^{2+} influx and Ca^{2+}-dependent insulin granule exocytosis by sulfonylureas such as gliclazide [46–48] and the mild glucose-lowering effect of ramipril, may account for the restoration to normoglycemic level observed in the triple combination therapy in our study. Although this synergistic effect or activation of different mechanisms with the triple combination therapy may raise the risk of hypoglycemia, further studies will be necessary to investigate this possibility and to determine whether dose adjustment would be required for long-term glycemic control.

Consistent with the hyperglycemia in untreated diabetic control rats, induction of T2DM over 6 weeks resulted in substantial pathological changes in the glomerular and tubular compartments of the kidney as well as renal fibrosis as revealed by PAS, KIM-1, α-SMA, TUNEL and Masson's trichrome stains. The renal pathological changes were also observed in the levels of markers of renal function (plasma creatinine, BUN and uric acid) as well as relative kidney weight, all of which indicate the occurrence of DN. KIM-1 (kidney injury molecule-1) is a transmembrane protein that is abundantly expressed in damaged proximal tubule epithelial cells but absent in normal healthy kidneys, and facilitates early diagnosis of kidney diseases. Our study revealed pronounced increase in renal KIM-1 expression in untreated diabetic control rats, which indicates extensive proximal tubule epithelial cell injury induced by T2DM. While mono- and dual therapies reduced renal KIM-1 expression in the diabetic animals, signifying some protection against DN progression, triple combination therapy produced the most renoprotective effect. This finding suggests that co-administration of antidiabetic drug and blockade of the renin-angiotensin-aldosterone system (RAAS) with an ACE inhibitor (e.g. ramipril) along with ALA supplementation protects the kidney against early development and progression of DN beyond that offered by conventional therapy. Interestingly, whereas some studies reported renal protection by ramipril in diabetic rats beyond its antihypertensive effect [49], we observed no significant difference in renal KIM-1 expression as well as plasma creatinine level between untreated diabetic control and ramipril-treated diabetic rats. This difference could be due to the difference in duration of treatment, as we administered ramipril for 6 weeks while they administered it for 9 weeks [49]. As we did not measure blood pressure in our T2DM rats, it is possible that these rats were not hypertensive, hence monotherapy with ramipril had no effect on kidney morphology. However, we observed significant decrease in BUN and plasma uric acid in ramipril-treated diabetic rats compared to untreated diabetic control rats, which is consistent with the report of Thangaraju et al [49].

In addition to the significant renal KIM-1 expression observed in our study, the interstitium of kidneys of untreated diabetic control rats also showed excessive collagen deposition and markedly increased expression of alpha-smooth muscle actin (α-SMA; a common marker protein of smooth muscle cells and myofibroblasts), both of which characterize renal interstitial fibrosis [50]. Importantly, we observed substantial reduction in collagen deposition and α-SMA expression in the renal interstitium following triple combination therapy, which is comparable to that in kidneys from healthy control group while mono- and dual therapies did not achieve such result. A previous study in a mouse model of DN also showed that upregulation of α-SMA expression was associated with mesangial cell activation in the glomerulus, leading to increased deposition of extracellular matrix and ultimately resulting in glomerulosclerosis [51]. Although we did not observe α-SMA expression in the glomeruli of kidneys of any of the groups, and also did not explore the mechanism that underlies substantial downregulation of α-SMA expression in the tubulointerstitial compartment by the triple combination therapy, our observation suggests that inhibition of α-SMA expression along with reduced formation and deposition of collagen and possibly other proteins in the fibrotic pathway partly contributes to protection against early DN development and progression by preserving renal structure and function. Our observation further suggests that activation of fibrotic pathway plays an important role in the early development and progression of DN, and therefore further investigations are needed to unravel the mechanisms that downregulate collagen and α-SMA expression and possibly other proteins in the fibrotic pathway following the triple combination therapy under diabetic condition such as in T2DM. As the most anti-fibrotic effect was observed following the triple combination therapy, it is noteworthy that ALA possesses anti-fibrotic property, as was shown in a rat model of obstructive nephropathy (a kidney condition characterized by structural or functional hindrance to normal urine flow), in which ALA markedly downregulated renal expression of transforming growth factor-1 (TGF-β1; a multifunctional cytokine involved in glomerulosclerosis and tubulointerstitial fibrosis) and attenuated renal injury [52]. Ramipril was also reported to protect against DN by suppressing matrix metalloproteinase 2 (MM2; a protein that plays a profibrogenic role) [53] while gliclazide also protects against mesangial matrix expansion [11]. Therefore, it is possible that the individual anti-fibrotic properties of these drugs produced a synergistic effect or may have inhibited several different fibrotic proteins in the fibrotic pathway, leading to the substantial α-SMA suppression and reduction in collagen deposition and the consequent renoprotection in our study. Contrary to these reports including ours, Grdović et al [54] recently reported that ALA did not downregulate α-SMA and TGF-β1 expressions in the kidneys of T2DM rats after 8 weeks of treatment. This discrepancy could be attributed to the dose of ALA and the route of administration, since Grdović et al [54] administered a low dose (10 mg/kg) intraperitoneally while we administered 60 mg/kg ALA orally, and therefore, would result in different bioavailability of ALA with different effects in the two studies. Therefore, the dose of ALA and its route of administration should be considered while evaluating its therapeutic effects. Our TUNEL staining, a method which detects apoptotic cells that undergo DNA fragmentation in the last phase of apoptosis, revealed significant degree of apoptosis of renal tubular cells of untreated T2DM rats. However, our triple combination therapy strongly protected the renal tubular cells from apoptosis compared to mono- and dual therapies.

Another marker of renal function that we measured is proteinuria, particularly albuminuria, which is generally acknowledged as glomerular damage marker and widely considered as the earliest index of DN and other kidney diseases. Thus, the level of albuminuria corresponds with the prognosis for renal function. Therefore, pharmacological interventions that retard DN progression also attenuate albuminuria. It is worth noting that during its renal passage, albumin is returned intact to the blood after glomerular filtration in the retrieval pathway under physiological conditions. However, a very tiny fraction of albumin may escape the retrieval pathway and may remain in the glomerular filtrate, where it is subjected to endocytosis and lysosomal degradation in the degradation pathway along the nephron and subsequently excreted in the urine [55–57]. Our assessment of renal function shows high urine albumin/creatinine ratio and low levels of total protein in the plasma of the untreated diabetic group, which reflect damage to the glomerular filtration barrier and compromised reabsorptive function of renal proximal tubules, all of which may indicate the occurrence of DN in these rats. This was reduced using the various treatment regimen, with the triple combination therapy having a better effect than mono- and dual therapies. Other rodent models and clinical studies of DN have also reported a quantitative regression of proteinuria or albuminuria to

normoalbuminuria, which correlated with renal protection against DN following monotherapies with ALA and ramipril [40, 58–62].

Akin to the effect on proteinuria, we also investigated additional mechanisms of prevention of early DN progression by the triple combination therapy. It is well-documented that hyperlipidemia, reactive oxygen species (ROS)-induced oxidative stress and inflammation are major pathological factors in DN development and progression [10–14, 24,40]. While these pathological pathways were activated following T2DM induction in the present study, mono- and combination therapies with ALA, gliclazide and ramipril for 6 weeks inhibited their effects as characterized by reduced levels of plasma triglycerides, serum IL-β, IL-6 and serum and renal malondialdehyde (MDA; a by-product of lipid peroxidation and indication of ROS production), while increasing the levels of plasma high-density lipoprotein cholesterol and renal glutathione (GSH; the most abundant naturally-occurring antioxidant in mammalian cells). Among these three factors, ROS-induced oxidative stress is considered the unifying pathogenetic factor and a major molecular pathway in the pathogenesis and progression of diabetic complications including DN. It arises due to overproduction of ROS via hyperglycemia, protein kinase C activation, formation of advanced glycation end-product, flux of polyol and hexosamine pathways, activation of nuclear factor kappa B (NF-κB; an inflammation-relevant transcription factor) and many more. Importantly, each of these pathways has been reported to contribute, at least in part, to cellular toxicity in DN through increased synthesis of extracellular matrix [63–66]. ROS-induced oxidative stress has also been implicated in the induction of inflammation, resulting in tissue damage in DN and other diabetic complications [67], as genetic inhibition of NADPH oxidase (NOX2; the primary source of ROS production in phagocytes) blocked secretion of IL-1β and other pro-inflammatory cytokines [66–68]. In line with these reports, we observed normalization in the levels of serum IL-1β and IL-6 following the triple combination therapy. A previous study also reported pharmacological inhibition of NOX2 with gliclazide via its azabicyclo-octyl ring structure, leading to supressed intercellular adhesion molecule-1 (ICAM-1) and ROS-induced oxidative stress, and enhanced production of manganese superoxide dismutase (MnSOD; a mitochondrial antioxidant) as well as increased nitric oxide bioavailability in the kidneys of diabetic rats fed with high-cholesterol diet, and thereby resulting in renal protection [11]. Specifically, the mitochondria in proximal tubules were identified as the principal site of ROS production during DN in ALA-deficient mice [40]. Hence, ALA administration significantly increased mitochondrial membrane potential (an indication of improved mitochondrial bioenergetics) and suppressed mitochondrial ROS production in the kidney of diabetic rats [10]. This suggests that by acting as a cofactor for mitochondrial multienzyme complexes, ALA can influence the mitochondrial antioxidant status through its ROS-scavenging action, and may increase cellular defence by activating other antioxidants (e.g. GSH) as observed in the present study. All of these mechanisms may contribute to attenuation of ROS-induced oxidative stress, and thus providing a greater resistance to renal cell injury. In a recent DN study in rats, Zhang et al [69] also reported that gliclazide administration inhibited endoplasmic reticulum (ER) stress via downregulation of the renal ER stress proteins GRP78 and sXBP1 and mRNA expression, which contributed to renal protection. Therefore, it is likely that the significant renal protection by the triple combination therapy observed in the present study is via the same mechanisms including lipid control mechanisms and possibly via other unidentified protective pathways that restored renal antioxidant status as was also observed in other preclinical and clinical studies [24,40,58, 69–71]. These pieces of empirical evidence suggest that interrupting the overproduction of ROS would be a promising approach to normalize these pathological pathways in the treatment of DN and other diabetic complications.

In summary, we have shown that early pharmacological intervention in DN in addition to ALA supplementation of conventional anti-diabetic therapy prevents early development and progression of DN in T2DM. Our findings suggest that ALA supplementation in DN is a more efficient pharmacotherapeutic approach that provides renal protection beyond that offered by conventional anti-diabetic therapy. Thus, we have provided an additional theoretical option for clinical treatment of DN in T2DM patients.

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CRediT authorship contribution statement

GJD conceived and designed the experiment; GJD obtained funding; GJD, KKA and SA performed the experiment; SA and SDM provided technical assistance; GJD provided the overall direction of the study; GJD and KKA analysed the data; GJD and KKA prepared the figures; GJD drafted the manuscript. GJD, BBN and SA revised the manuscript. All authors have read and approved the final manuscript. There is no conflict of interest.

Conflict of interest statement

None.

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