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Metformin with insulin relieves oxidative stress and confers renoprotection in type 1 diabetes in vivo

Christine Driver, Julia Achieng Hayangah, Ntsoaki Annah Nyane, Peter Mark Oroma Owira*

Molecular and Clinical Pharmacology Research Laboratory, Department of Pharmacology, Discipline of Pharmaceutical Sciences, School of Health Sciences, University of KwaZulu-Natal P.O. Box X5401, Durban, South Africa

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ABSTRACT

Background: Oxidative stress and impaired antioxidant capacity in diabetes are associated with diabetic nephropathy. Metformin, as an adjunct to insulin could decrease oxidative stress and may therefore improve renal function in type 1 diabetes (T1D).

Objectives: To investigate the effects of metformin as adds-on therapy to insulin on renal dysfunction in T1D.

Materials and Methods: Male Sprague-Dawley rats (230-250 g) were divided into 5 groups (n =7). Rats in groups A and B were orally treated with 3.0 mL/kg body weight (BW) of distilled water, while those in groups C and D were treated with insulin (4.0 U/kg BW bid) or oral metformin (250 mg/kg BW), respectively. Group E rats were similarly treated with both metformin and insulin. Groups B-E were rendered diabetic by intraperitoneal injections of 65 mg/kg BW of streptozotocin. Fasting blood glucose concentrations and glucose tolerance tests were done. The animals were sacrificed by halothane overdose after 56 days, blood taken by cardiac puncture and kidneys excised and stored at -80°C for further analysis.

Results: Untreated diabetic rats exhibited significant weight loss, increased polydipsia and polyuria, impaired glucose tolerance, electrolyte retention, reduced creatinine clearance and urea excretion and increased oxidative stress compared to controls, respectively. However, these were reversed by treatment with metformin and insulin.

Conclusions: Metformin does not improve glycemic control in TID but exerts renoprotective effects by reducing oxidative stress in the presence of insulin. Metformin should therefore be considered for adjunct therapy with insulin in TID.

Implication for health policy/practice/research/medical education:
Although metformin is known to exert no hypoglycemic effects in type 1 diabetes, our findings here suggest that it may mitigate the development of diabetic nephropathy by its anti-oxidant effects. Metformin should therefore be considered as adjunct therapy to insulin in type 1 diabetes.

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Original Article

1. Background

Type 1 diabetes (T1D) is caused by destruction of pancreatic β-cells and is routinely treated by the exogenous subcutaneous insulin injections in order to maintain normoglycemia and mitigate the development of diabetes complications (1-3). End-point micro- and macro-vascular complications such as nephropathy, retinopathy and cardiovascular disease are associated with increased morbidity and mortality in diabetic patients. Diabetic nephropathy is progressive and irreversible and is characterised by glomerular hyperfiltration, epithelial hypertrophy, microalbuminuria, glomerulus basement membrane thickening and proteinuria and is the leading cause of end-stage renal disease worldwide (4-8). Intensive glycaemic control and interventions with angiotensin converting enzyme (ACE)-inhibitors are intended to delay disease progression but are not curative (9).

*Corresponding author: Peter Mark Oroma Owira, Email: owirap@ukzn.ac.za

Hyperglycemia results in mitochondrial overproduction of reactive oxygen species (ROS), which, coupled with reduced antioxidant capacity in diabetes, are the driving forces in the development of diabetic complications (10-12). Chronic hyperglycemia increases oxidative stress by inducing a flux of electron donors (NADH and $FADH_2$) leading to electrons pile up in the mitochondrial inner membrane coenzyme Q and leakage at complexes I and III of the electron transport chain (ETC) (10,13,14). Oxidative stress causes direct damage to the glomerulus and affects tubule-interstitial structures and functions, leading to diabetic nephropathy (15). Reduction in renal function may lead to electrolytes retention and imbalance hence accelerating the development of cardiovascular disease complications (16,17).

Metformin is commonly administered as a first-line drug in the treatment of type 2 diabetes as it suppresses hepatic gluconeogenesis, decreases fatty acid oxidation and increases peripheral insulin sensitivity, leading to improved glucose uptake in the skeletal muscles and adipose tissues (7,18-20). These effects of metformin are mediated by its activation of 5'-AMP-activated protein kinase (AMPK), (a known master energy sensor that restores cellular energy balance) and also by inhibition of complex 1 of the mitochondrial ETC leading to reduced mitochondrial ROS production (21-24).

Currently, metformin is not clinically indicated for the treatment of T1D although it has been noted to reduce daily insulin requirements without improving plasma HbA1c levels (21,25-27). Metformin is not metabolized in the body and is excreted unchanged by the kidneys (28) yet chronic renal disease is very common in diabetes patients. Consequently, renal insufficiency may increase plasma metformin concentrations and the perceived risk of lactic acidosis hence glomerular filtration rate of <30 mL/min/1.73 m^2 is clinically a contraindication to its administration (29). However, despite these concerns, metformin has been shown to improve renal function in diabetic patients (30) and despite lack of supporting evidence from large scale clinical trials, the American Diabetes Association (ADA) and the European Diabetes Association have recommended administration of metformin in diabetic patients with end-stage renal disease (31). Furthermore, two petitions have been logged with the American Food and Drug Administration (FDA) to consider use of metformin in diabetic patients with moderate kidney disease (32). These appeals have been supported by experimental evidence which suggest that metformin could exert renoprotection by reducing ROS due to its antioxidant effects (33-35). Metformin has been shown to improve gentamycin-induced nephrotoxicity and also to protect renal tubules and podocytes in a diabetic state (30,32,34). Based on these findings it is conceivable to suggest that metformin can attenuate hyperglycemia-associated renal injury by decreasing ROS production and oxidative damage (36). This study therefore seeks to investigate the merits of using metformin as an adjunctive therapy to insulin in the treatment of T1D in order to prevent or delay the occurrence of renal dysfunction and aspects of diabetic nephropathy. This suggests that patients with T1D could benefit from metformin therapy to mitigate end-stage renal disease.

2. Objectives

1. To create a T1D model
2. To investigate the effects of metforminon renal dysfunction in a T1D model
3. To investigate the effects of metformin on glycemic control in T1D using insulin as a positive control
4. To investigate the effects of metformin on hyperglycemia-associated oxidative stress in a T1D rat model
5. To study the effects of metformin on hyperglycemia-associated renal dysfunction in T1D.

3. Materials and Methods

3.1. Chemicals and reagents

Streptozotocin, D-glucose, citrate buffer, phosphate buffer, thiobarbituric acid (TBA), metaphosphoric acid chips, butylated hydroxytoluene and phosphoric acid were all purchased from Sigma-Aldrich Pty. Ltd, Johannesburg South Africa. Metformin (Accord, Sandton, South Africa), insulin (Novo Nordisk, Norway), portable glucometers and glucose test strips (OneTouch Select, Zug, Switzerland) were purchased from a local pharmacy. Superoxide dismutase (SOD) assay and glutathione (GSH) assay kits were purchased from Cayman Chemicals (Michigan, USA). Halothane used to euthanize the animals were provided by the Biomedical Resource Unit (BRU) of the University of KwaZulu-Natal, Durban, South Africa.

3.2. Animal treatment

Male Sprague-Dawley rats (230-300 g) were housed 7 rats per cage and had free access to food and drinking water for the duration of the study (56 days). The rats were divided into five groups (n = 7), (Table 1) and maintained on a 12-hour dark/light cycle (08:00-20:00) in an air-controlled room (temperature 25°C ± 2°C, humidity 55 ± - 5%). Rats were fed normal chow

containing; protein, fibre, starch and calcium.

3.3. Induction of experimental diabetes

Fasting blood glucose (FBG) levels were determined then diabetes was induced in groups B-E via a single intraperitoneal injection of streptozotocin (65 mg/kg body weight [BW]) from a stock solution of 36 mg/mL in 0.1 M citrate buffer of pH 4.5 (Table 1). Diabetes was confirmed 48 hours after the administration of streptozotocin on venous blood obtained via tail pricks using glucometer strips and a hand-held glucometer (Bayer Acensia®). Rats with FBG above of 7.5 mmol/L were considered diabetic and included in the study.

The rats were weighed daily, water consumption similarly recorded and FBG tests done every 14 days. On day 51, 24-hour urine samples were collected from the animals in metabolic cages and kept at -80°C for further analysis. GTTs were done prior to sacrificing by halothane overdose. Blood samples were collected by cardiac puncture and kidneys excised and washed in phosphate-buffered saline before being snap-frozen in liquid nitrogen and stored at -80°C until further analysis.

3.4. Methods

3.4.1. Glucose tolerance tests

All animals were fasted overnight and FBG concentrations determined before intraperitoneal injections of 3.0 g/kg BW of glucose in normal saline. Blood glucose concentrations were determined at 0, 15, 30, 60, 90 and 120 minutes, respectively. Areas-under- the-curve (AUC) were calculated from blood glucose concentrations versus time (min) plots and expressed as mM × min or UC units.

3.4.2. Urea and electrolytes

Serum and urine Na and K concentrations were measured by Beckman Coulter Synchron Aqua CAL® system using chloride, potassium and sodium diagnostic kits, respectively. The reactions were done by mixing 40 µL of sample with 1.32 mL, of buffer solution (constituted with Tris buffer) in a ratio of 1:33.

Table 1. Animal treatment groups[a]

Groups	Distilled H_2O	Insulin	Metformin	Metformin + Insulin
A	3.0			
B	3.0			
C		4.0		
D			250	
E				250 + 4.0

[a]Metformin (mg/kg body weight [BW], orally), insulin (IU/kg BW, SC bd) and distilled water (mL/kg BW, orally) were administered daily.

Creatinine concentrations in urine and serum samples were measured by a Beckman Coulter diagnostic kit, according to the manufacturer's instructions as previously described (37). Creatinine clearance was hence calculated using the following formula:

$$\mathrm{CrCl} = \frac{\text{Creatinine urine } (\mu\text{mol/L})}{\text{Creatinine serum } (\mu\text{mol/L})} \times \frac{\text{urine volume (mL)}}{\text{time (mins)}}$$

An enzymatic reaction rate was used to determine the concentrations of urea in urine samples by a Beckman Coulter diagnostic kit as per the reactions:

$$Urea + H_2O \xrightarrow{Urease} 2NH_3 + CO_2$$

$$NH_3 + \alpha\text{-}Ketoglutarate + NADH + H^+ \xrightarrow{GLDH} Glutamate + NAD^+ + H_2$$

$$(GLDH = Glutamate\ Dehydrogenase)$$

3.5. Oxidative stress

3.5.1. Superoxide dismutase assay

Both MnSOD and CuZnSOD were measured using SOD assay kit as per the manufacturer's instructions. Kidney tissues were homogenised 1:7 w/v in ice cold buffer, pH 7.2 (containing 1.0 M EGTA, 210 mM mannitol, 70 nM sucrose and 20 mM HEPES) then centrifuged at 1500 g for 5 minutes.

CuZnSOD was measured by adding 200 µL radical detector (tetrazolium salt solution) and 10 µL of sample (supernatant) or standard into the microtiter plate wells before initiating the reaction by adding 20 µL of diluted xanthine oxidase. MnSOD, was measured by adding 190 µL of radical detector and 10 µL of 3 mM potassium cyanide to the sample and xanthine oxidase. The plates were then covered and incubated at room temperature on a plate shaker for 30 minutes. Absorbance was read at 450 nm using a microplate reader (Biochron®, EZ Read 400, Cambridge, UK). SOD concentrations were calculated by extrapolation from the standard curve and values expressed in units of enzyme normalized to cellular mg of protein.

3.5.2. Glutathione concentrations

Kidney tissues were prepared by homogenisation in 1:7 w/v cold buffer, pH 7 (containing 50 mM MES and 1.0 mM EDTA). The samples were then centrifuged at 10 000 g for 15 minutes at 4°C before the supernatant was removed and stored at -20°C. Deproteination was carried out by mixing equal volumes of sample homogenate supernatant with metaphosphoric acid solution and vortexing. The mixture was then centrifuged at 2000 g for 2 minutes and the supernatant collected. A solution of 4.0 M triethanolamine was then mixed with the supernatant

in a ratio of 50:1.

The assay was carried out by adding 150 µL of Cayman's glutathione assay cocktail (prepared as per the manufacturer's instructions) to each well containing 50 µL of sample and standards then incubating in the dark on an orbital shaker for 25 mins and measuring the absorbance at 410 nm using a microplate reader (Biochron®, EZ Read 400, Cambridge, UK). Glutathione concentrations (µM/mg protein) were then calculated by extrapolation from the standard curve.

3.5.3. Lipid peroxidation-thiobarbituric acid reactive substances (TBARS) assay

Lipid peroxidation was determined by measuring the concentrations of malondialdehyde (MDA) in samples according to the modified methods of Phulukdaree et al (38) and Hermes-Lima et al (39). In brief, a solution was prepared using TBA (1% w/v)/ 0.1 mM butylated hydroxytoluene (BHT). In a set of clean tubes 200 uL of plasma samples were added to a solution of 500 µL of 2% phosphoric acid (H_3PO_4), 400 µL of 7% H_3PO_4, 400 µL of the TBA/BHT and 200 µL of 1.0 M HCl, respectively. The tubes were then incubated in boiling water (100°C) for 15 minutes before cooling to room temperature. To each test tube was then added 1.5 mL of butanol and vortexed then 200 µL of the top phase transferred into a 96-well µL plate and absorbance read at 532 nm and 600 nm, respectively using a microplate reader (Biochron®, EZ Read 400, Cambridge, UK). The plasma MDA concentrations were then determined by using extinction coefficient 156 mM^{-1}.

Tissue TBARS assays were carried out according to the modified method of Hermes-Lima et al.[40] Frozen kidney tissues were homogenised in a 1:10 (w/v) ratio with cold 1.1% phosphoric acid. A solution containing 1% TBA, 50 mM NaOH and 0.1 mM BHT was made for the assay. Into clean sets of test tubes 400 µL of sample homogenates were added to 400 µL TBA/BHT solution and 200 µL of 7% phosphoric acid (final pH of 1.6) and similarly processed as plasma samples.

3.6. Histological examinations

The excised kidneys were fixed in 10% buffered formalin and routinely processed for paraffin embedding. From each sample, 2 µm- thick sections were obtained and mounted on the microscope slides. Slides were stained with hematoxylin and eosin (H&E) and Masson's trichrome stain (MTS) for evaluation and differentiated in acid alcohol then washed with water to remove excess stain. The stained slides were viewed using The Nikon compound light microscope equipped with a camera for image capturing. The images were then analysed by a pathologist.

3.7. Ethical issues

The research followed the tenets of the Declaration of Helsinki. This project and protocols were confirmed to be in accordance with the guidelines and approved by the Animal Ethics Committee of the University of the KwaZulu-Natal (Ethics reference number# 078/14/Animal).

3.8. Statistical analysis

Data was presented as mean ± SD and analysed using the statistical software, GraphPad Prism® (San Diego, USA) version 5.0. Student *t* tests or one-way analysis of variance (ANOVA) followed by Student-Newman–Keuls test were carried out where appropriate. A *P* value of <0.005 was considered statistically significant.

4. Results

4.1. Natural growth

Untreated diabetic animals experienced retarded

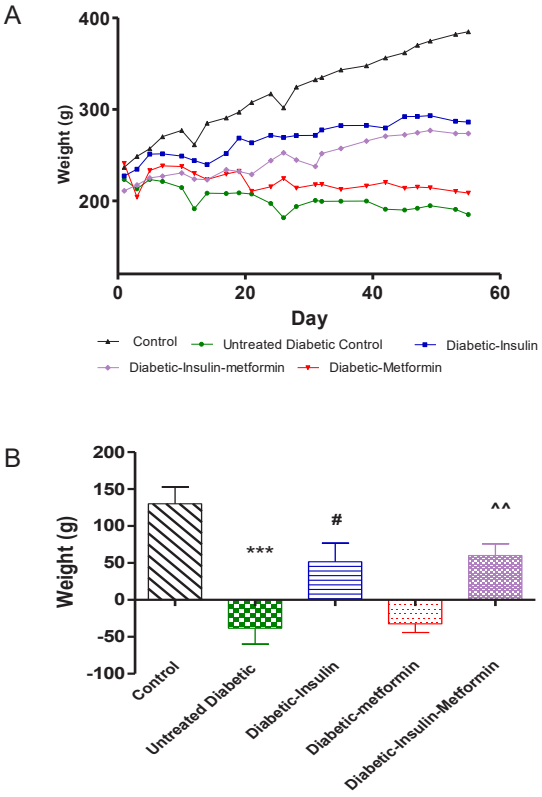

Figure 1. (**A**) Natural growth of animals presented as live weights. Sudden drops in weights coincided with the days when the animals were starved overnight for the determination of FBG and (**B**) Changes in live weights measured as the difference in animal weights before and after treatment. *** $P < 0.001$ compared to control group; #, ^^ $P < 0.05$ compared to untreated diabetic group.

weight gain compared to controls (Figure 1A). However, treatment with insulin with or without metformin resulted in increased weight gain compared to untreated diabetic rats, respectively. Metformin alone did not have significant effects on animal weights compared to the untreated diabetic controls. Consequently, weight gain measured as change in weight between the beginning and the end of the study showed that untreated diabetic animals experienced significantly ($P<0.001$) negative weight gain compared to the normal control group (Figure 1B). Insulin with or without metformin significantly ($P<0.05$) improved weight loss compared to the untreated diabetic animals. However, metformin alone did not significantly improve weight loss in diabetic rats compared to the untreated diabetic ones (Figure 1B).

4.2. Fasting blood glucose concentrations

Diabetic animals had significantly ($P<0.001$) higher FBG levels compared to the normal controls. Treatment with metformin, insulin or in combination did not significantly improve FBG compared to the untreated diabetic rats (Figure 2).

4.3. Glucose tolerance tests

Blood glucose concentration-time plots showed that diabetic animals were severely glucose intolerant compared to controls (Figure 3A). Calculated AUCs suggested that untreated diabetic rats were significantly ($P<0.001$) glucose intolerant compared to controls and that treatment with insulin with or without metformin significantly ($P<0.05$) improved glucose intolerance in diabetic rats compared to non-treated diabetic group (Figure 3B). Treatment with metformin alone did not significantly improve glucose intolerance in diabetic rats compared to the untreated controls.

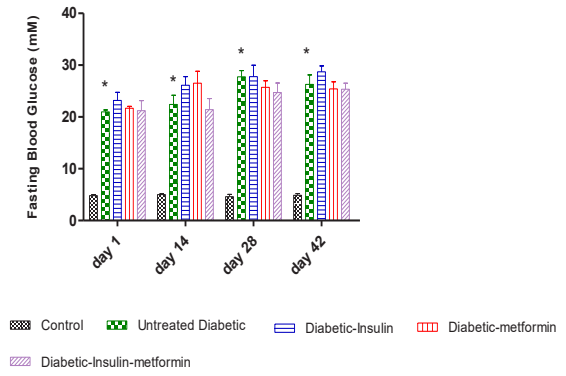

Figure 2. FBG concentrations during treatment. Blood was obtained by tail pricking then mounted on strips and glucose levels measured using a hand held glucometer. Insulin or metformin treatment was withheld prior to the tests. * $P<0.001$ for untreated control versus the control group.

4.4. Water intake and urine output

Untreated diabetic animals drank significantly ($P<0.001$) more water than controls while treatment with metformin and insulin alone or in combination significantly ($P<0.05$) reduced water intake corrected to body weights compared to untreated diabetic rats (Figure 4A), suggesting reduction of polydipsia in diabetic animals. Furthermore, untreated diabetic animals showed significantly ($P<0.0001$) increased urine output compared to controls but treatment with either metformin, insulin or both did not significantly reduce urine output in diabetics rats compared to non-treated diabetic ones (Figure 4B), suggesting increased polyuria in diabetic rats.

4.5. Urea and electrolytes

Urinary Na^+ and K^+ output were significantly ($P<0.001$) reduced in untreated diabetic animals compared to normal controls (Figure 5). Treatment with metformin and insulin alone or in combination significantly ($P<0.05$) improved urinary Na^+ and K^+ excretion compared to untreated diabetic controls (Figure 5).

Serum creatinine corrected to body weight were significantly ($P<0.001$) higher in the untreated

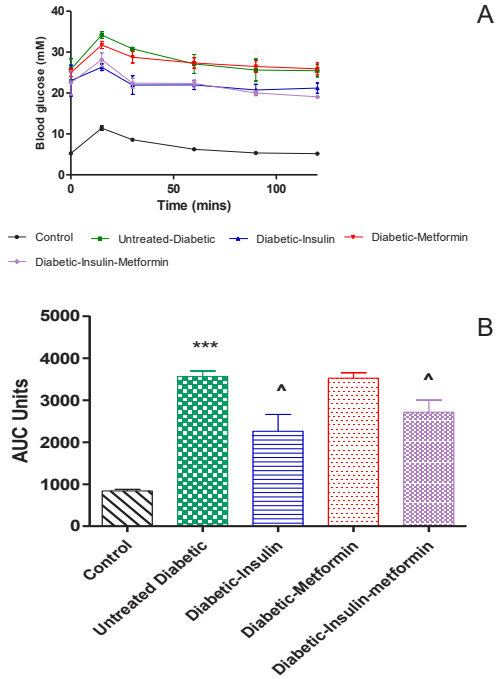

Figure 3. (**A**) Blood glucose concentrations measured at 30-minute intervals for 2 h and plotted against time after the fasted animals were challenged with intraperitoneal 3.0 g/kg of glucose in normal saline in GTT. (**B**) Calculated AUC from blood glucose concentrations/time plots. *** $P<0.001$ compared to control and ^ $P<0.05$ compared to the untreated diabetic group.

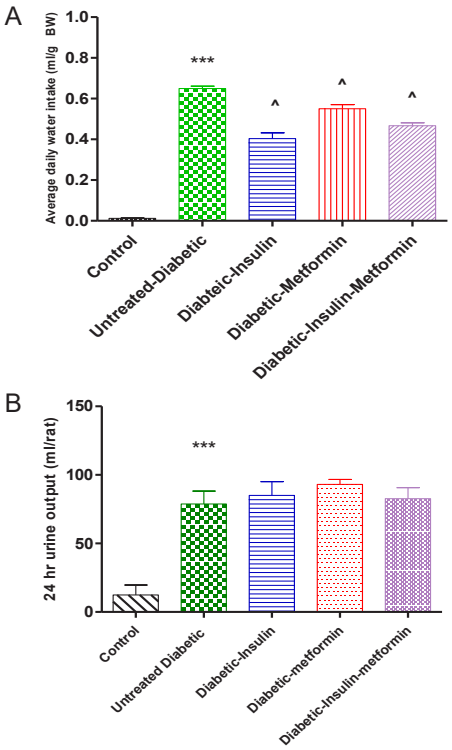

Figure 4. (A) Average daily water consumption during the treatment period expressed as mL/g body weight (BW). (B) Average 24-h urine output when the animals were put in metabolic cages and the urine collected. *** $P<0.001$ compared to controls; ^ $P<0.05$ compared to untreated diabetic group.

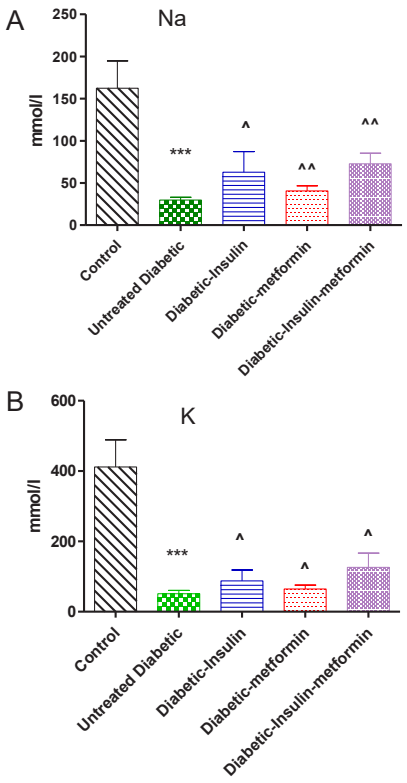

Figure 5. Electrolyte concentrations in 24-hr urine samples collected when the animals were put in metabolic cages. *** $P<0.001$ compared to control group. ^, ^^ $P<0.05$ compared to untreated diabetic group.

diabetic rats compared to controls (Figure 6A). However, treatment with metformin and insulin but not either alone significantly ($P<0.05$) reduced serum creatinine concentrations compared to untreated diabetic animals (Figure 6A).

Creatinine excretion was significantly ($P<0.001$) reduced in untreated diabetic animals compared to the control group. However, treatment with insulin and metformin but not either alone similarly significantly ($P<0.01$) increased urinary creatinine excretion in diabetic animals compared to untreated diabetic animals (Figure 6B).

Consequently, calculated creatinine clearance suggested that non-treated diabetic animals had significantly ($P<0.05$) reduced creatinine clearance compared to the control rats but treatment with metformin and insulin in combination significantly ($P<0.01$) increased creatinine clearance compared to non-treated diabetic rats (Figure 7).

Untreated diabetic rats had significantly ($P<0.001$) reduced urine urea output compared to the control group (Figure 8). However, treatment with metformin and insulin significantly ($P<0.005$) increased urea excretion compared to the untreated diabetic rats (Figure 8). Treatment with insulin or metformin alone did not significantly increase urea concentrations compared to untreated diabetic animals.

4.6. Biomarkers of renal oxidative stress

4.6.1. Superoxide dismutase

MnSOD activity was significantly ($P<0.005$) increased in the kidneys of untreated diabetic rats compared to the control group (Figure 9A). Treatment with metformin, with or without insulin, significantly ($P<0.001$) reduced MnSOD activity compared to untreated diabetic rats. Similarly, the activity of CuZnSOD was significantly ($P<0.05$) increased in the kidneys of untreated diabetic compared to the control groups and treatment with metformin alone or with insulin significantly ($P<0.05$) reduced CuZnSOD activity in diabetic rats compared to the untreated diabetic group (Figure 9B).

4.6.2. Lipid peroxidation measured by TBARS assay

Plasma and renal tissue homogenate MDA concentrations were significantly ($P<0.05$) elevated in untreated diabetic rats compared to the control groups, respectively but treatment of diabetic rats with insulin, metformin alone or in combination with insulin significantly ($P<0.05$) reduced plasma and

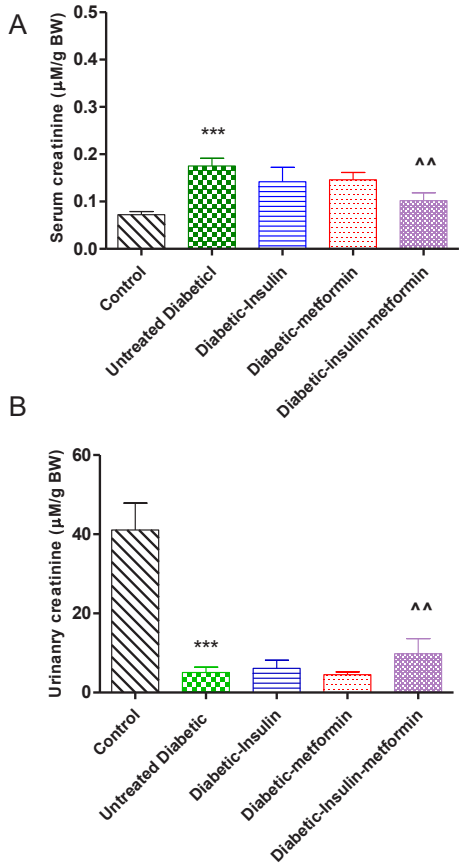

Figure 6. (A) Serum creatinine concentrations corrected to live body weights and B: Creatinine excretion in 24-h urine samples. Creatinine concentrations were expressed as µM/g body weight (BW). *** $P<0.001$ compared to the control group. ^^ $P<0.05$ compared to untreated diabetic group, respectively

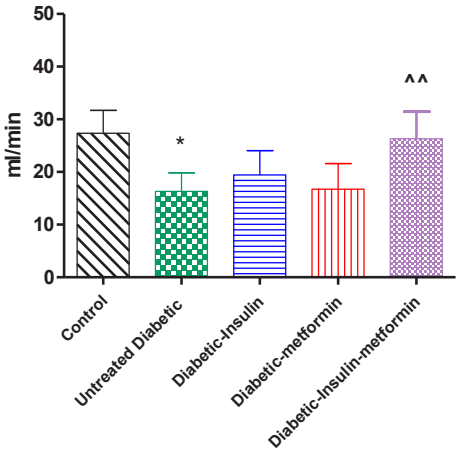

Figure 7. Calculated creatinine clearance using the formula; [Creatinine concentrations in urine/ Creatinine concentrations in serum) × urine volume (mL)/ time (min). *$P<0.05$ compared to control rats and ^^ $P<0.01$ compared to untreated diabetic rats.

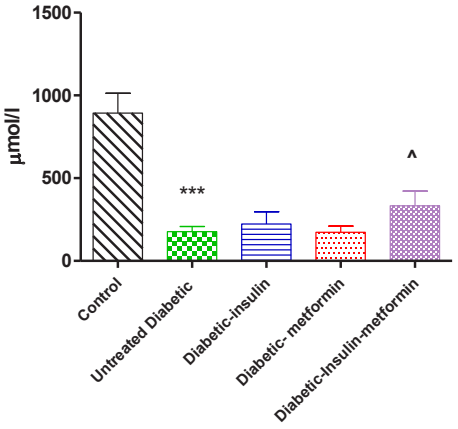

Figure 8. Urea concentrations measured in urine collected after the animals were confined in metabolic cages for 24 h. *** $P<0.001$ compared to control rats and ^$P<0.05$ compared to untreated diabetic rats.

renal MDA concentrations compared to untreated diabetic groups, respectively (Figure 10A and B).

4.6.3. Glutathione

Glutathione concentrations measured in renal tissue homogenates were significantly ($P<0.05$) reduced in untreated diabetic rats compared to controls (Figure 11). However, treatment with metformin alone or in combination with insulin significantly ($P<0.05$) increased glutathione concentrations in diabetic rats compared to non-treated diabetic rats.

4.7. Histopathology of the kidneys

H&E stains of kidney sections viewed under light microscope showed mesangial hypercellularity and enlarged glomeruli basement membranes in untreated- and metformin only treated-diabetic rats (Figure 12). Metformin and insulin treatment reduced mesangial expansion and reduced enlargement of glomeruli basement membrane similarly to controls.

5. Discussion

T1D was induced by intraperitoneal injections of 65 mg/kg BW of streptozotocin, a cytotoxic glucose analogue which completely destroys the pancreatic β-cells and obliterates insulin secretion leading to hyperglycemia (40). Diabetic rats presented with classic symptoms of T1D such as weight loss (Figure 1), impaired glucose tolerance (Figures 2 and 3), polyuria and polydipsia (Figure 4). Treatment with metformin only, did not show significant improvement in weight gain while insulin alone or in combination with metformin as expected, significantly improved weight loss compared to untreated diabetic groups, respectively (Figure 1). Since insulin treatment was withheld on the day of GTT, it was therefore not surprising that FBG or GTT did not improve in insulin only-treated animals compared to the untreated diabetic groups (Figures 2 and 3). It is

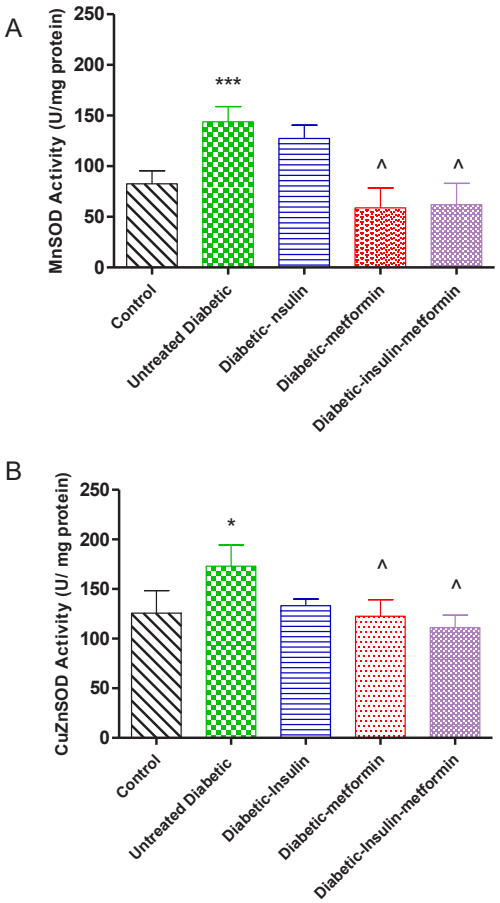

Figure 9. Renal tissue homogenates SOD activity expressed U/mg of protein. A: MnSOD and B: CuZnSOD. ***, * $P<0.005$ compared to the control group, ^ $P<0.001$ compared to untreated diabetic group.

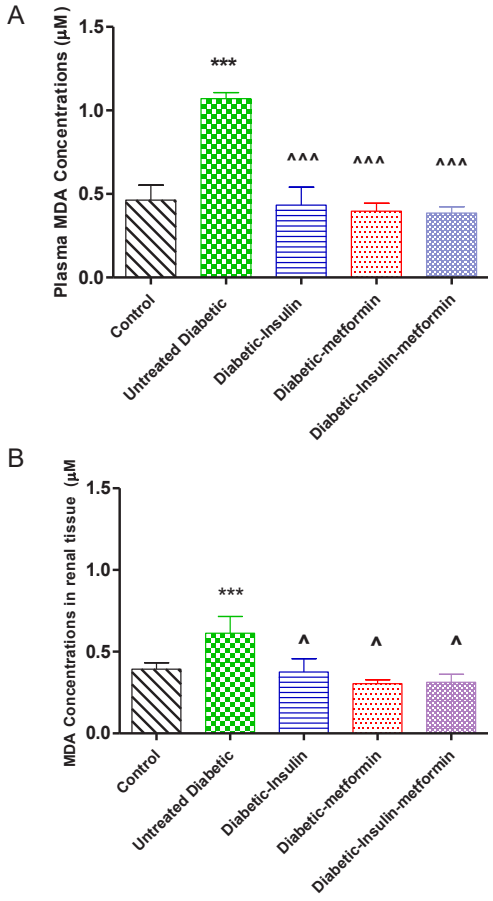

Figure 10. Lipid peroxidation measured as MDA concentrations by TBARS assay in: A:- Plasma and B:- Renal tissue homogenates. *** $P<$ 0.001 compared to untreated normal control, ^,^^$P<$ 0.05 compared to untreated diabetic control.

therefore apparent in our model that anabolic effects of insulin reduced protein and lipid catabolism while metformin, a known anti-obesity agent in type 2 diabetes did not improve weight loss in diabetic rats. Consequently, metformin had no significant effects on FBG but in the presence of insulin, glucose intolerance was significantly improved compared to the untreated diabetic rats (Figures 2 and 3) similarly to previous studies that have shown that despite its improvement of insulin sensitivity, metformin does not lower HBA1c is T1D (21,25,41).

Diabetes is the most common cause of impairment of renal function leading to end-stage renal disease and increased risk of morbidity and mortality (42-44). Deteriorated renal function in diabetes is characterised by microalbuminuria, increased plasma and reduced urine creatinine due to low creatinine clearance, reduced urea excretion, salt and water retention (45). Although we did not measure microalbuminuria, our diabetic model presented with reduced urea excretion and creatinine clearance, low urine output, Na and K retention (Figures 6-8) suggesting impairment of renal functions. In the development of diabetic nephropathy, microalbuminuria is not apparent at stage 2 where there is normal glomerular filtration rate, steady serum creatinine levels, elevated blood pressure and glomerulosclerosis (36,46). Consequently, kidney sections stained with H&E showed mesangial hypercellularity and enlarged glomeruli basement membranes in metformin only-treated or untreated diabetic rats (Figure 12). Treatment with metformin and insulin in combination but not separately significantly improved creatinine clearance, urea excretion, and urine output compared to non-treated diabetic animals (Figures 6 to 8). However, metformin and insulin in combination or separately significantly improved Na and K excretion in diabetic rats (Figure 5). These results therefore suggest that the development of diabetic nephropathy in the untreated diabetic rats was at or beyond stage 3 (normally characterised by microalbuminuria, decline in glomerular filtration rate and glomerulosclerosis) (46) and that treatment with metformin and insulin delayed/prevented the onset of diabetic nephropathy and potentially cardiovascular

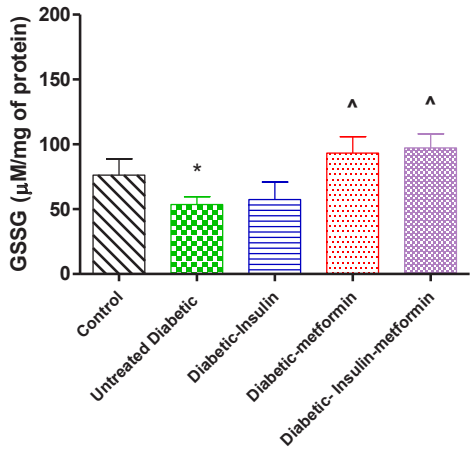

Figure 11. Glutathione concentrations measured in homogenised renal tissues. *$P<0.05$ compared to control group and ^$P<0.05$ compared to untreated diabetic control.

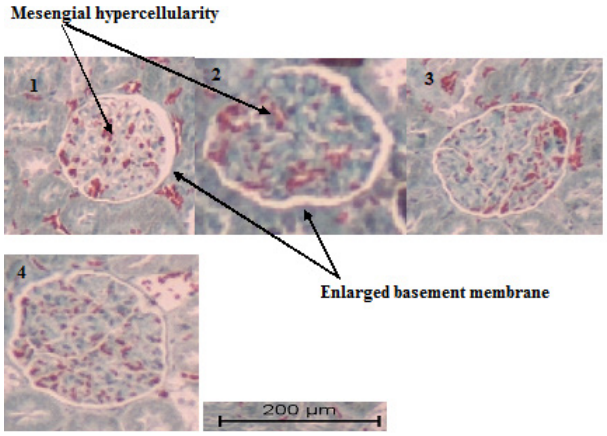

Figure 12. Kidney sections stained with H&E as visualised by light microscopy showing glomeruli with enlarged basement membrane and mesangial expansion. 1. Untreated diabetic 2. Metformin-treated diabetic 3. Metformin and insulin.

complications in diabetic animals.

Hyperglycemia has been shown to decrease the body's total antioxidant capacity and increases oxidative stress, which accelerates diabetic complications (13,18,47). SOD is a powerful antioxidant that is induced by increased oxidative stress (3, 11). In our study, untreated diabetic rats had significantly elevated mitochondrial MnSOD or cytosolic CuZnSOD levels, compared to controls (Figure 9). Metformin alone or in combination with insulin significantly reduced SOD activities compared to the untreated diabetic rats (Figure 9). Similarly, the highly inducible potent antioxidant, GSH, was significantly depleted in the kidneys of untreated diabetic rats compared to controls but treatment of diabetic rats with metformin alone or in combination with insulin significantly reversed this (Figure 11). Oxidative stress resulting from hyperglycemia depletes GSH through diversion of NADPH by aldolase reductase to produce sorbitol in the polyol pathway (11, 47). It is therefore conceivable that metformin decreased oxidative stress in the mitochondria by activating AMPK and inhibiting complex 1 of the ETC leading to reduced ROS production (23,5,48), hence reduced SOD activity (34,49) and also increased GSH salvage. These observations are further supported by our results which show that metformin effects were more enhanced on MnSOD than CuZnSOD (Figure 10). Additionally, metformin may directly quench ROS in the mitochondria, or alternatively induce MnSOD and promote mitochondrial biogenesis by activating the PGC-1alpha pathway as previously reported (50). The effects of metformin on other nuclear transcription/response factors (Nrf) or antioxidant response elements which are known to boost antioxidant capacity are currently under investigation by our group but for now the molecular mechanisms of the apparent metformin effects remain speculative.

Consequently, MDA concentrations measured by TBARS assay in both plasma and kidney homogenates were significantly elevated in non-treated diabetic rats compared to controls but treatment of diabetic rats with metformin either alone or in combination with insulin normalized this (Figure 10). This further suggests that whereas insulin boosts the capacity of endogenous enzymatic antioxidant systems by its known anabolic effects, metformin has direct or indirect antioxidant effects that reduced ROS hence preserved or regenerated endogenous antioxidants.

6. Conclusions

The data presented here indicates that metformin does not improve glycaemic control in TID but does have protective effect against diabetic renal dysfunction. This suggests that in combination with insulin, metformin can mitigate the development of diabetic nephropathy.

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Conflicts of interest

All author declared no conflict of interest.

Authors' contribution

CD; participated in the performance of the research. JAN; contributed to all aspect of the study. CD and

JAN; achieved statics, data analysis and collected the data. NAN and PMOO participated in research design and the writing of the paper.

Ethical considerations

Ethical issues (including plagiarism, data fabrication, double publication) have been completely observed by the authors.

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References

1. Satirapoj B, Adler SG. Comprehensive approach to diabetic nephropathy. Kidney Res Clin Pract. 2014;33(3):121-31. doi: 10.1016/j.krcp.2014.08.001.
2. International Diabetes Federation. IDF Diabetes Atlas. 6th ed. IDF; 2013:160.
3. Forbes JM, Cooper ME. Mechanisms of diabetic complications. Physiol Rev. 2013;93(1):137-88. doi: 10.1152/physrev.00045.2011.
4. Sun YM, Su Y, Li J, Wang LF. Recent advances in understanding the biochemical and molecular mechanism of diabetic nephropathy. Biochem Biophys Res Commun. 2013;433(4):359-61. doi: 10.1016/j.bbrc.2013.02.120.
5. Roshan B, Stanton RC. A story of microalbuminuria and diabetic nephropathy. J Nephropathol. 2013;2(4):234-40. doi: 10.12860/JNP.2013.37.
6. Tavafi M. Diabetic nephropathy and antioxidants. J Nephropathol. 2013;2(1):20-7. doi: 10.5812/nephropathol.9093.
7. Susztak K, Raff AC, Schiffer M, Böttinger EP. Glucose-induced reactive oxygen species cause apoptosis of podocytes and podocyte depletion at the onset of diabetic nephropathy. Diabetes. 2006;55(1):225-33. doi: 10.2337/diabetes.55.01.06.db05-0894.
8. Satirapoj B. Nephropathy in diabetes. Adv Exp Med Biol. 2012;771:107-122.
9. Ahmad J. Management of diabetic nephropathy: recent progress and future perspective. Diabetes Metab Synd. 2015; 9(4):343–358. doi: 10.1016/j.dsx.2015.02.008.
10. Giacco F, Brownlee M. Oxidative stress and diabetic complications. Circ Res. 2010;107(9):1058-70. doi: 10.1161/CIRCRESAHA.110.223545.
11. Niedowicz DM, Daleke DL. The role of oxidative stress in diabetic complications. Cell Biochem Biophys. 2005;43(2):289-330. doi: 10.1385/CBB:43:2:289.
12. Maritim A, Sanders R, Watkins JB. Diabetes, oxidative stress, and antioxidants: a review. J Biochem Mol Toxicol. 2003;17(1):24-38. doi: 10.1002/jbt.10058.
13. Stadler K. Oxidative stress in diabetes. Adv Exp Med Biol. 2012;771:272-87.
14. de M Bandeira S, da Fonseca LJ, da S Guedes G, Rabelo LA, Goulart MO, Vasconcelos SM. Oxidative stress as an underlying contributor in the development of chronic complications in diabetes mellitus. Int J Mol Sci. 2013;14(2):3265-84. doi: 10.3390/ijms14023265.
15. Louro TM, Matafome PN, Nunes EC, da Cunha FX, Seiça RM. Insulin and metformin may prevent renal injury in young type 2 diabetic Goto–Kakizaki rats. Eur J Pharmacol. 2011;653(1-3):89-94. doi: 10.1016/j.ejphar.2010.11.029.
16. Karmarkar S, Macnab R. Fluid and electrolyte problems in renal dysfunction. Anaesth and Int Care Med. 2012;13(7):332-5. doi: 10.1016/j.mpaic.2012.04.011.
17. Potpara TS, Jokic V, Dagres N, Marin F, Prostran MS, Blomstrom-Lundqvist C, et al. Cardiac arrhythmias in patients with chronic kidney disease: implications of renal failure for antiarrhythmic drug therapy. Curr Med Chem. 2016;23(19):2070-83.
18. Khan AS, McLoughney CR, Ahmed A. The effect of metformin on blood glucose control in overweight patients with Type 1 diabetes. Diabet Med. 2006;23(10):1079-84. doi: 10.1111/j.1464-5491.2006.01966.x.
19. Jacobsen IB, Henriksen JE, Beck-Nielsen H. The effect of metformin in overweight patients with type 1 diabetes and poor metabolic control. Basic Clin Pharmacol Toxicol. 2009;105(3):145-9. doi: 10.1111/j.1742-7843.2009.00380.x.
20. Sarnblad S, Kroon M, Aman J. Metformin as additional therapy in adolescents with poorly controlled type 1 diabetes: randomised placebo-controlled trial with aspects on insulin sensitivity. Eur J Endocrinol. 2003;149(4):323-9.
21. Pernicova I, Korbonits M. Metformin mode of action and clinical implications for diabetes and cancer. Nat Rev Endocrinol. 2014;10(3):143-56. doi: 10.1038/nrendo.2013.256.
22. Rena G, Pearson, ER, Sakamoto K. Molecular mechanism of action of metformin: old or new insights? Diabetologia. 2013;56(9):1898-906. doi: 10.1007/s00125-013-2991-0.
23. Viollet B, Guigas B, Sanz Garcia N, Leclerc J, Foretz M, Andreelli F. Cellular and molecular mechanisms of metformin: an overview. Clin Sci. 2012;122(6):253-70. doi: 10.1042/CS20110386.
24. Whittington HJ, Hall AR, McLaughlin CP, Hausenloy DJ, Yellon DM, Mocanu MM. Chronic metformin associated cardioprotection against infarction: not just a glucose lowering phenomenon. Cardiovasc Drugs Ther. 2013;27(1):5-16. doi: 10.1007/s10557-012-6425-x.
25. Moon R, Bascombe LA, Holt R. The addition of metformin in type 1 diabetes improves insulin sensitivity, diabetic control, body composition and patient well-being. Diabetes Obes Metab. 2007;9(1):143-5. doi: 10.1111/j.1463-1326.2006.00599.x.
26. Lund SS, Tarnow L, Astrup AS, Hovind P, Jacobsen PK, Alibegovic AC, et al. Effect of adjunct metformin

treatment in patients with type-1 diabetes and persistent inadequate glycaemic control. A randomized study. PLoS One. 2008;3(10):e3363. doi: 10.1371/journal.pone.0003363.
27. Connelly P, McKay G, Petrie JR. Metformin in type 1 diabetes. Pract Diabet. 2015;32(5):186-7.
28. Scheen AJ. Clinical pharmacokinetics of metformin. Clin Pharmacokinet. 1996;30:359-71
29. National Kidney Foundation. KDOQI Clinical Practice Guideline for Diabetes and CKD: 2012 update. Am J Kidney Dis. 2012;60(5):850-86.
30. Hung AM, Roumie CL, Greevy RA, Liu X, Grijalva CG, Murff HJ, et al. Comparative effectiveness of incident oral antidiabetic drugs on kidney function. Kidney Int. 2012;81(7):698-706. doi: 10.1038/ki.2011.444.
31. Inzucchi SE, Bergenstal RM, Buse JB, Diamant M, Ferrannini E, Nauck M, et al. American Diabetes A, European Association for the Study of D: Management of hyperglycemia in type 2 diabetes: a patient-centred approach: position statement of the American Diabetes Association (ADA) and the European Association for the Study of Diabetes (EASD). Diabetes Care. 2012;35(6):1364-1379. doi: 10.2337/dc12-0413.
32. Inzucchi SE, Lipska KJ, Mayo H, Bailey CJ, McGuire DK. Metformin in patients with type 2 diabetes and kidney disease: a systematic review. JAMA. 2014;312:2668-75. doi: 10.1001/jama.2014.15298.
33. Herrington WG, Levy JB. Metformin: effective and safe in renal disease? Int Urol Nephrol. 2008;40(2):411-7. doi: 10.1007/s11255-008-9371-6.
34. DeFronzo R, Fleming GA, Chen K, Bicsak TA. Metformin-associated lactic acidosis: current perspectives on causes and risk. Metabolism. 2016;65(2):20-9. doi: 10.1016/j.metabol.2015.10.014.
35. Kim SA, Choi HC. Metformin inhibits inflammatory response via AMPK–PTEN pathway in vascular smooth muscle cells. Biochem Biophys Res Commun. 2012;425(4):866-72. doi: 10.1016/j.bbrc.2012.07
36. Anabtawi A, Miles JM. Metformin: nonglycemic effects and potential novel indications. Endocr Pract. 2016;22(8):999-1007. doi: 10.4158/EP151145.RA.
37. Heinegard D, Tiderstrom G. Determination of serum creatinine by a direct colorimetric method. Clin Chim Acta. 1973;43(3):305-10.
38. Phulukdaree A, Moodley D, Chuturgoon AA. The effects of *Sutherlandia frutescens* extracts in cultured renal proximal and distal tubule epithelial cells. S Afr J Sci. 2010;106(1-2):54-8.
39. Hermes-Lima M, Willmore WG, Storey KB. Quantification of lipid peroxidation in tissue extracts based on Fe (III) xylenol orange complex formation. Free Radic Biol Med. 1995;19(3):271-80. doi: 10.1016/0891-5849(95)00020-X.
40. Lenzen S. The mechanisms of alloxan- and streptozotocin-induced diabetes. Diabetologia. 2008;51(2):216-26. doi: 10.1007/s00125-007-0886-7.
41. Vella S, Buetow L, Royle P, Livingstone S, Colhoun HM, Petrie JR. The use of metformin in type 1 diabetes: a systematic review of efficacy. Diabetologia. 2010;53(5):809-20. doi: 10.1007/s00125-009-1636-9.
42. Collins AJ, Foley RN, Herzog C, Chavers BM, Gilbertson D, Ishani A, et al. Excerpts from the US renal data system 2009 annual data report. Am J Kidney Dis. 2010;55(1 Suppl 1):S1-420,. doi: 10.1053/j.ajkd.2009.10.009.
43. Go AS, Chertow GM, Fan D, McCulloch CE, Hsu CY. Chronic kidney disease and the risks of death, cardiovascular events, and hospitalization. N Engl J Med. 2004;351:1296-305. doi: 10.1056/NEJMoa041031.
44. Hallan SI, Dahl K, Oien CM, Diana C, Grootendorst DC, Arne Aasberg A, et al. Screening strategies for chronic kidney disease in the general population: follow-up of cross sectional health survey. BMJ. 2006;333:1047. doi: 10.1136/bmj.39001.657755.BE.
45. Wasung ME, Chawla LS, Madero M. Biomarkers of renal function, which and when? Clin Chim Acta. 2015;438:350-7. doi: 10.1016/j.cca.2014.08.039
46. Thomas C, Thomas L. Renal failure-measuring the glomerular filtration rate. Dtsch Arztebl Int. 2009;106(51-52):849-54. doi: 10.3238/arztebl.2009.0849.
47. Tang WH, Martin KA, Hwa J. Aldose reductase, oxidative stress, and diabetic mellitus. Front Pharmacol. 2012;3:87. doi: 10.3389/fphar.2012.00087.
48. Ansley DM, Wang B. Oxidative stress and myocardial injury in the diabetic heart. J Pathol. 2013;229(2):232-241. doi: 10.1002/path.4113.
49. Bułdak Ł, Łabuzek K, Bułdak RJ, Kozłowski M, Machnik G, Liber S, et al. Metformin affects macrophages' phenotype and improves the activity of glutathione peroxidase, superoxide dismutase, catalase and decreases malondialdehyde concentration in a partially AMPK-independent manner in LPS-stimulated human monocytes/macrophages. Pharmacol Rep. 2014;66(3):418-29. doi: 10.1016/j.pharep.2013.11.008.
50. Fujisawa K, Nishikawa T, Kukidome D, Imoto K, Yamashiro T, Motoshima H, et al. TZDs reduce mitochondrial ROS production and enhance mitochondrial biogenesis. Biochem Biophys Res Commun. 2009;379(1):43-8. doi: 10.1016/j.bbrc.2008.11.141