Int. J. Morphol.,
43(6):2138-2146, 2025.

Beneficial Effect of 20-Hydroxyecdysone on Kidney Damage in Streptozotocin-Induced Diabetic Gerbil

Efecto Beneficioso de la 20-Hidroxiecdisona en el Daño Renal en Jerbos Diabéticos Inducidos por Estreptozotocina

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MALLEK, A.; SEMIANE, N.; MOKEDDEM, K.; BELLAHRECHE, Z.; SIHALI-BELOUI, O. & DAHMANI, Y. Beneficial effect of 20-hydroxyecdysone on kidney damage in streptozotocin-induced diabetic gerbil. *Int. J. Morphol., 43(6)*:2138-2146, 2025.

SUMMARY: The aim of this work was to evaluate the effects of 20-hydroxyecdysone (20E) on redox status, inflammation and renal fibrosis in streptozotocin-induced diabetic gerbil. In this study 24 gerbils were divided into 4 groups: control, diabetic gerbils induced by streptozotocin (STZ, 130 mg/kg B.W), diabetic treated with 20-hydroxyecdysone (50 mg/kg B.W) and normal gerbils received 20E (50 mg/kg B.W). Our results showed a significant decrease in plasma glucose, lipids and creatinine in diabetic group treated with 20E. We also observed a decrease in renal MDA content and an increase antioxidant activities (GSH, SOD and CAT). In addition, histomorphometric and immunhistochemical analyses revealed an improvement in renal architecture and a decrease in the percentage of collagen surface area. These results indicate that 20E has a beneficial effect on kidney damage probably through its antioxidant action.

KEY WORDS: Gerbil; Diabetes; Kidney; Oxidative stress; Histopathology; 20-Hydroxyecdysone.

INTRODUCTION

Diabetes is one of the most widespread chronic diseases in the world, affecting around 537 million adults aged between 20 and 79 years in 2021, representing 10.5 % of the world population in this age group. This number is predicted to rise to 643 million by 2030 and 783 million by 2045 (International Diabetes Federation, 2021). Diabetes is caused by a defect in the secretion and/or action of insulin or both. It is associated with numerous complications that can have deleterious end-effects on certain tissues and organs, such as eyes, brain, pancreas, kidneys and muscles. These complications are responsible for almost 2 million deaths a year worldwide.

Diabetes is one of the leading causes of diabetic nephropathy (DN). Up to 40 % of people living with diabetes develop DN (International Diabetes Federation, 2021). DN is one of the most frequent and severe complications of diabetes mellitus and is associated with increased morbidity and mortality in diabetic patients. It is characterized by increased microalbuminuria as the earliest clinical manifestation and morphological changes including thickening of the glomerular basement membrane, an expanded mesangial matrix, and tubulointerstitial fibrosis (Tervaert *et al*., 2010). Pathophysiologically, DN involves the interaction of haemodynamic and metabolic changes and genetic predisposition. The haemodynamic changes are characterized by hyperfiltration of the glomerulus and the importance of tubuloglomerular feedback control. The metabolic changes are all based on hyperglycaemia, with numerous pathways involved.

Generation of free radicals by oxidative stress pathways is increased by hyperglycaemia and lead to an excess production of cytokines and growth factors, sustaining the inflammatory phenomenon of DN and leading to an expansion of the mesangial matrix and a profibrotic state (Brosius, 2008).

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Received: 2025-06-12 Accepted: 2025-09-27

Treatment of diabetic nephropathy has been management of hyperglycaemia, blood pressure and proteinuria using hypoglycemic agents, ACE inhibitors, SGLT2 inhibitors and angiotensin receptor blockers, although the control of these therapeutic measures does not always prevent the progression of DN. Therefore, research has turned to natural therapies to develop more effective agents to control diabetes and its complications.

Ecdysteroids are a family of steroid hormones, which were initially identified in insects and crustaceans as hormones regulating molting, development, and reproduction. Analogues of these molecules, phytoecdysteroids, are also present in plants, which use them to protect themselves from phytophagous predators (insects and nematodes) by inducing molting disorders or the death of these predators. They are present in many plant species where they can reach concentrations of up to 2-3 % of the plant dry weight and where they are expected to protect plants against phytophagous insects. It is generally accepted that 20-hydroxyecdysone (20E) is the major biologically active form in insects and plants. It is known for its numerous bioactivities linked to its broad-spectrum general effects on health. 20E has a wide range of beneficial effects in mammals, such as anabolic, adaptogenic, anti-diabetic, hypolipidemic, antioxidante, hepatoprotective and nephroprotective effects, etc. (Dinan & Lafont, 2006). The purpose of this study is to assess the impact of 20E extracted from *Cyanotis vaga*, a plant of the Commelinaceae family, on renal damages in streptozotocin-induced diabetic gerbils.

MATERIAL AND METHOD

Experimental animals. Male adult gerbils (*Gerbillus gerbillus*), weighing 20-30 g were used in the present work. Gerbils were captured in Wilaya of Beni Abbes and transferred to an animal facility in Algiers. Animals were kept under standard environmental conditions (temperature 22-24 °C, humidity 50 % with an alternating 12 h light-dark cycle) with free access to food.

Authorization to capture animals in the desert region was given by the Ministry of Higher Education and Scientific Research (Algeria). Capture, accommodation and all experimental procedures were authorized by the Institutional Animal Protection Committee of the Algerian National Administration of Higher Education and Scientific Research (DGRSDT; http://www.dgrsdt.dz). Permits and ethical rules were respected in accordance to the guidelines of the Local Institutional Animal Care Committee of USTHB University with decision number of CEEA-USTHB-2023/11119 and were supported by the Algerian Association of Experimental Animal Sciences (Agreement number 45/DGLPAG/DVA.SDA.14).

Chemicals. Streptozotocin (STZ) was purchased from Sigma-Aldrich (Germany). 20-Hydroxyecdysone (20E; 95 % purity) was extracted from *Cyanotis vaga* and purchased from Changzou Dahua Import and Export Inc., Changzou, China. All other chemicals used were of analytical grade.

Induction of experimental diabetes. Diabetes was induced by a single intraperitoneal injection (i.p.) of 130 mg/kg body weight freshly prepared streptozotocin dissolved in 0.1 M citrate buffer (pH 4.5). Animals were considered diabetic, if their blood glucose levels were greater than 200 mg/dl 48 h after STZ injection.

Experimental protocol. After an adaptation period (two weeks), the animals were divided into four groups of six animals each:

- Group I: control gerbils received a single i.p. injection of citrate buffer.
- Group II: diabetic gerbils.
- Group III: diabetic gerbils received 20-hydroxyecdysone (50 mg/kg B.W, daily per os administration) with ground barley for 3 weeks.
- Group IV: normal gerbils received 20-hydroxyecdysone (50 mg/kg B.W, daily per os administration) with ground barley for 3 weeks.

At the end of the experimental period, animals were sacrificed and blood was collected by a retro-orbital puncture. Plasma was separated by centrifugation at 3000 rpm for 10 min and used for biochemical assays. The kidneys were immediately removed, weighed and fixed for histomorphological and histochemical studies and to evaluate oxidative stress parameters.

Biochemical assays

Determination of plasma glucose. Plasma glucose levels were determined using a colorimetric assay kit purchased from Cypress Diagnostics (Belgium) in accordance with manufacturer's instructions.

Determination of plasma lipids. Serum total cholesterol and triglycerides were assayed using reagent kits purchased from Cypress Diagnostics (Belgium).

Determination of plasma creatinine. Creatinine was measured colorimetrically with a creatinine kit (ELITechGroup) using the kinetic Jaffe reaction. The rate of formation of a colored complex between creatinine and alkaline picrate is measured.

Oxidative stress parameters.

Preparation of homogenates. Kidneys were homogenized in Tris-EDTA buffer (0.1 M. pH 7.6) and centrifuged at 1000 rpm for 5 min at 4 °C for the evaluation of malondialdehyde (MDA) content. The supernatant so obtained was further centrifuged at 9600 rpm for 20 min at 4 °C for immediate measurement of GSH content and antioxidant enzymes activities.

Determination of lipid peroxidation. The lipid peroxidation end product MDA was assayed in the kidney homogenate according to the method of Ohkawa *et al.* (1979).

Determination of GSH content. Reduced glutathione (GSH) content was estimated according to the method of Ellman (1959). The data are expressed in pmol/mg of protein.

Determination of superoxide dismutase activity. The activity of superoxide dismutase (SOD) was estimated by measuring the percentage inhibition of pyrogallol auto-oxidation by SOD according to the method of Marklund & Marklund (1974). Absorbance was measured at 420 nm. One unit of SOD was defined as the enzyme activity which inhibits the auto-oxidation of pyrogallol (20 mM in 10 mM HCl) by 50 %.

Determination of catalase activity. The activity of catalase (CAT) was estimated according to the methods of Aebi (1984). Changes in absorbance were recorded at 240 nm. The enzyme activity was calculated as millimoles of H_2O_2 consumed min^{-1} mg^{-1} protein.

Protein content

The protein content in kidney homogenate was determined according to method of Bradford (1976) using bovine serum albumin as the standard.

Histological and morphometric studies

Samples from the kidneys of control and experimental groups were fixed in 10 % neutral buffered formalin for 24 h and then dehydrated in graded ethanol series (50°, 70°, 90°, 100°). Kidneys were then cleared in two butanol baths, embedded in paraffin and cut into sections of 5 µm thickness. The slides obtained were stained with Masson's trichrome and selective staining for collagen (Sirius red). Sirius red stained sections were used to evaluate collagen using Threshold function imageJ software (2.14u-National Institutes of Health. USA). For each group of animals, the quantification of collagen was performed by measuring the percentage of stained surface selected at x400 magnification.

Histochemical study

Slides were deparaffinized and rehydrated progressively then incubated in warm citrate buffer for 15 min. After cooling, they were rinsed in Tris buffer (3X5min). Endogenous peroxidase activity was inhibited with hydrogen peroxide (3 %) for 15 min and nonspecific sites were blocked by incubation with goat serum. Sections were incubated overnight at 4 °C with mouse monoclonal anti-CD45 antibody (Cell Marque, USA). The sections were then incubated with a biotinylated anti-rabbit IgG secondary antibody (Dako, Denmark), followed by streptavidin-peroxidase (Dako, Denmark). The primary antibody-secondary antibody antigen complex was detected using DAB. The appearance of a brown coloration is observed under a light microscope. The sections are counterstained using hematoxylin.

Statistical analysis

The results were expressed as mean ± SEM and analyzed using GraphPad Prism 6 software (Graph Pad Software, Inc., San Diego, CA, USA). For statistical analyses, Kruskal-Walis H test followed by Mann-Witney-U test were used. Differences were considered statistically significant if $p < 0.05$.

RESULTS

Effects of 20-hydroxyecdysone on plasma glucose. The blood glucose level in diabetic gerbils was higher than that of the control groups, and the difference was significant ($P< 0.01$). Treatment of diabetic animals with 20E resulted in a significant decrease in plasma glucose levels and statistical analysis was significant ($P< 0.01$) (Table I). Normal gerbils treated with 20E showed no significant changes in plasma glucose when compared to control gerbils.

Table I. Biochemical parameters in control and experimental gerbils.

Groups	Glucose (mg/dl)	Triglycerides (mg/dl)	Cholesterol (mg/dl)	Creatinine (mg/dl)
Control	104.3 ± 6.7	118.6 ± 14.6	88.6 ± 8.9	0,29 ± 0,062
DC	462.8 ± 38.4**	247.9 ± 46.9*	294.0 ± 42.9**	1,2 ± 0,3**
DC+20E	121.2 ± 22.7++	137.8 ± 19.5	169.7 ± 70.4+	0,35 ± 0,13+
+20E	112.0 ± 11.5	110.6 ± 18.2	78.3 ± 6.1	0,28 ± 0,08

Values are mean ± SEM. *: control *vs* diabetic (DC). +: DC *vs* diabetic treated with 20E (DC+20E). Values are considered significantly different at $P < 0.05$.

Effects of 20-hydroxyecdysone on plasma lipid profile. Diabetic gerbils exhibited significant increase in plasmatic triglycerides (P< 0.05) and total cholesterol (P< 0.01) compared to that of controls. Conversely, in diabetic gerbils that received 20E, a decrease in plasma triglycerides (44 %) and total cholesterol (P< 0.05) levels were observed when compared with diabetic group (Table I). Furthermore, no significant variation was observed between control and normal gerbils treated with 20E.

Effects of 20-hydroxyecdysone on plasma creatinine. Plasma creatinine was increased in diabetic group when compared to the control (P < 0.01). 20-hydroxyecdysone administration significantly reduced blood creatinine in diabetic gerbils (P < 0.05). Furthermore, no significant variation was observed between control and normal gerbils treated with 20E.

Effects of 20-hydroxyecdysone on oxidative stress. Figure 1 summarizes effects of 20E on lipid peroxidation and antioxidant activities in the kidneys of control and experimental gerbils. The kidney of diabetic gerbils showed markedly (P< 0.01) increased MDA content as compared to the control group. Diabetic gerbils receiving 20E showed a significant (P < 0.05) decrease in MDA as compared to the diabetic control (Fig. 1A). Normal gerbils treated with 20E showed no changes in MDA content when compared to control gerbils. The levels of GSH and the activity of SOD and CAT were significantly (P< 0.01, P< 0.01, P < 0.05 respectively) decreased in the kidney of diabetic gerbils as compared to the control group. By contrast, the administration of 20E positively affected the levels of GSH (Fig. 1B) and enzymatic antioxidants SOD (P< 0.01), CAT (P< 0.05) (Figs. 1C and 1D). No significant variation was observed between control and normal gerbils treated with 20E.

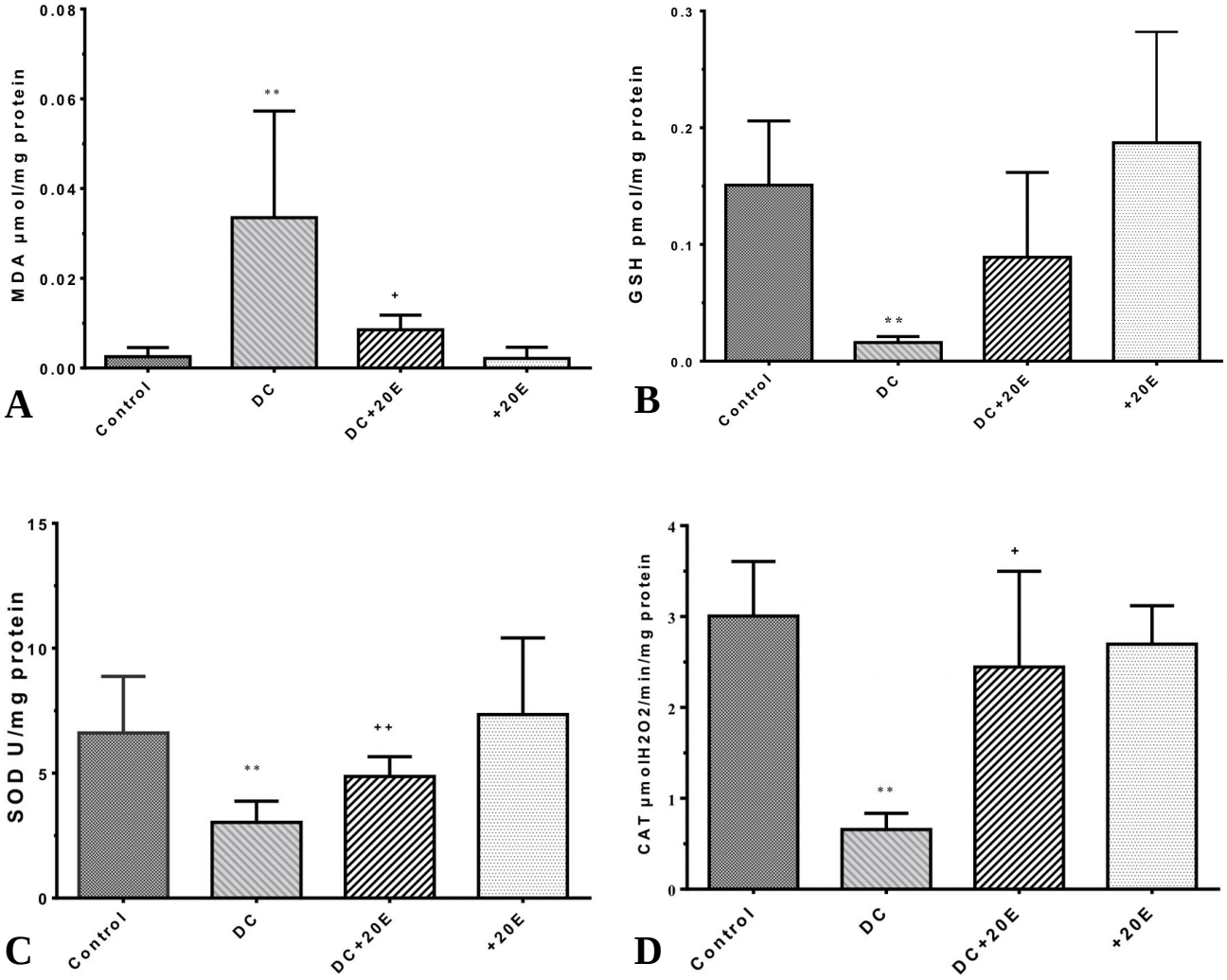


Fig.1 Oxidative stress parameters in control and experimental gerbils. A: MDA; B: GSH; C: SOD; D: CAT. Values are mean ± SEM. *: control vs diabetic gerbils (DC). +: DC *vs* diabetic treated with 20E (DC+20E). Values are considered significantly different at P < 0.05.

Effects of 20-hydroxyecdysone on renal histomorphometry. The microscopic examination of sections in the kidney stained with Masson's trichrome of control and normal gerbils receiving 20E revealed (Fig. 2 a,b) normal renal cortex and parenchyma with normal glomeruli and renal tubules. In contrast, sections in the kidney of diabetic gerbils showed cellular and matrix alterations. Mesangial matrix expansion and basement membrane thickening in

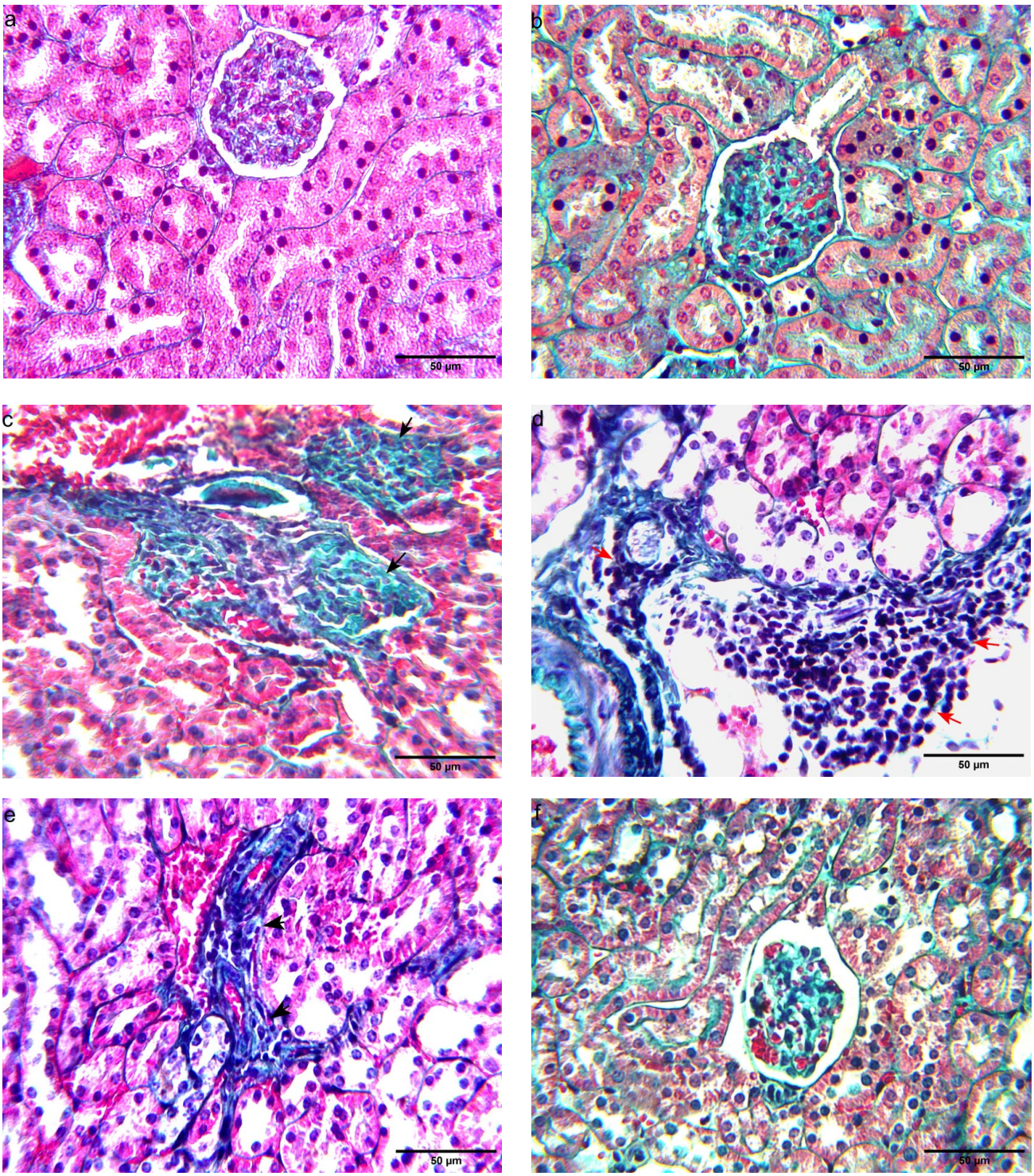
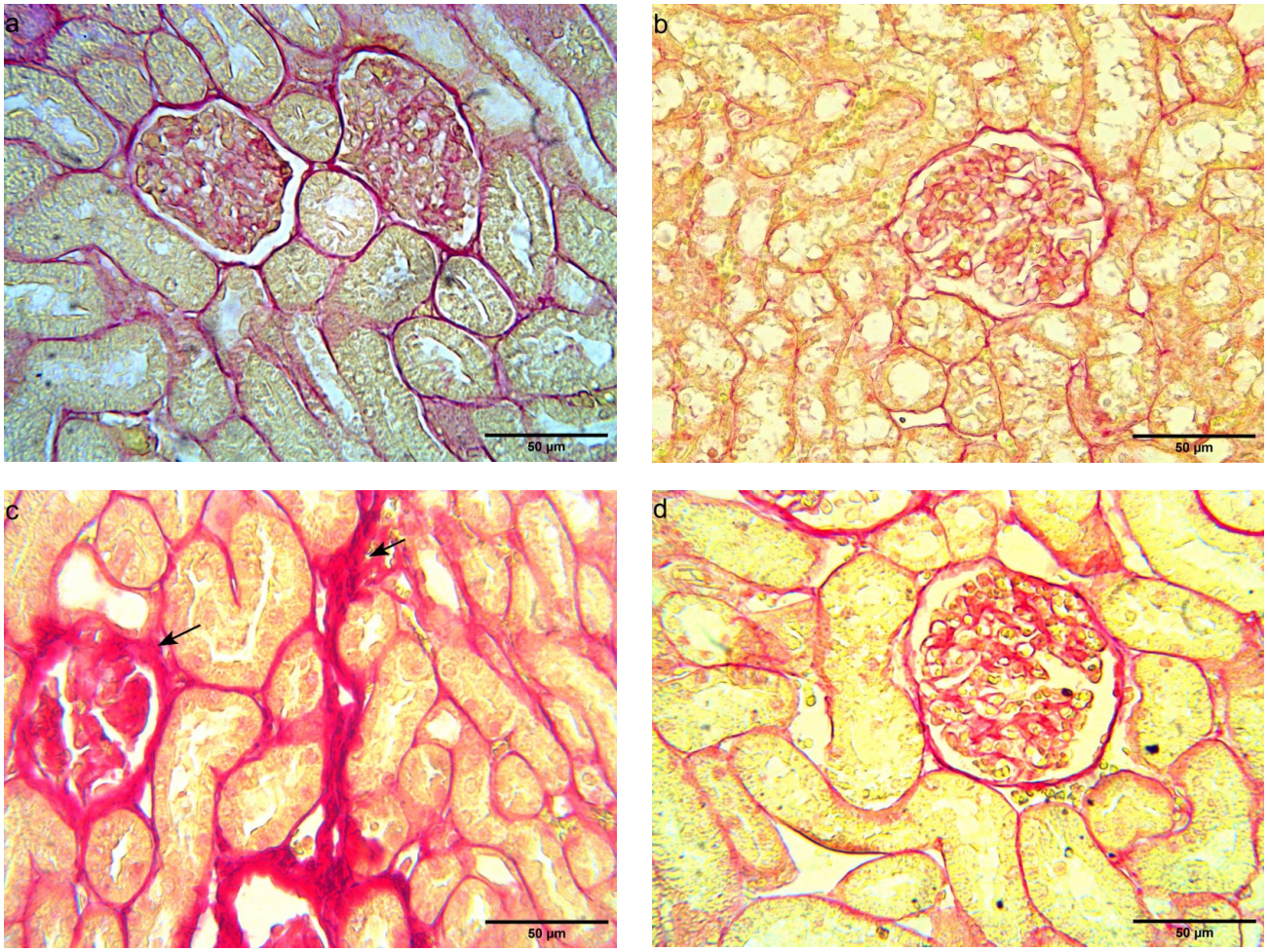


Fig. 2.Representative images of kidneys of control and experimental gerbils. Masson's trichrome staining, original magnification: 400x. Control (a) and normal gerbil treated with 20E (b) showing normal architecture. Diabetic gerbils showing glomerulosclerosis (c, black arrow); inflammation (d, red arrow) and fibrosis (e, black arrow). Diabetic gerbils treated with 20E showing an improved structure (f).

glomeruli and tubules (Fig. 2c), inflammatory cell infiltration (Fig. 2d) and matrix alterations (Fig. 2e) were observed. Diabetic gerbils treated with 50 mg/kg 20E showed an improvement in the structure of kidney (Fig. 2f). Furthermore, histochemical analysis using Sirius red staining indicated fibrosis installation materialized by an accumulation of collagen both at the perivascular and in interstitial level in diabetic gerbils (Fig. 3A). The morphometric study (Fig. 3B) showed a significant increase ($P< 0.0001$) of collagen in diabetic gerbils as compared to the control group. 20E administration drastically reduced collagen accumulation in diabetic gerbils ($P< 0.0001$). No significant variation was observed between control and normal gerbils treated with 20E.



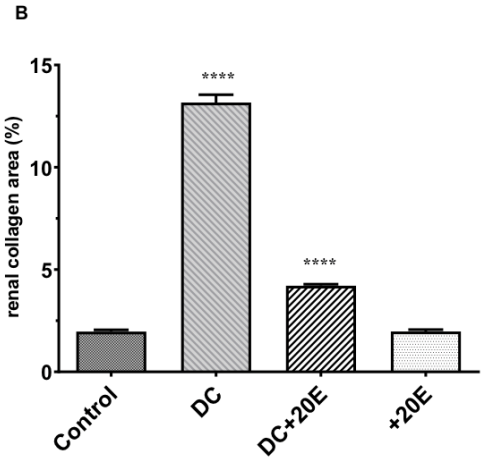


Fig. 3. A: Representative images of kidney of control and experimental gerbils. Sirius red staining, original magnification: 400x. Control (a) and normal gerbil treated with 20E (b); diabetic gerbils (c); diabetic gerbils treated with 20E (d). B: Quantification of renal collagen area of control and experimental gerbils. Sirius red staining, original magnification: 400x. Values are mean ± SEM. *: control vs diabetic gerbils (DC). +: DC *vs* diabetic treated with 20E (DC+20E). Values are considered significantly different at $P < 0.05$.

Effects of 20-hydroxyecdysone on renal inflammation. In the control and normal gerbils receiving 20E, no immunostaining of CD45 was reported (Fig. 4a,b). In diabetic animals, in accordance with the histological study inflammation was confirmed by a positive staining with CD45 compared to controls (Fig. 4c). After 20E treatment, a weak immunostaining of CD45 in renal tissue was observed (Fig. 4d).

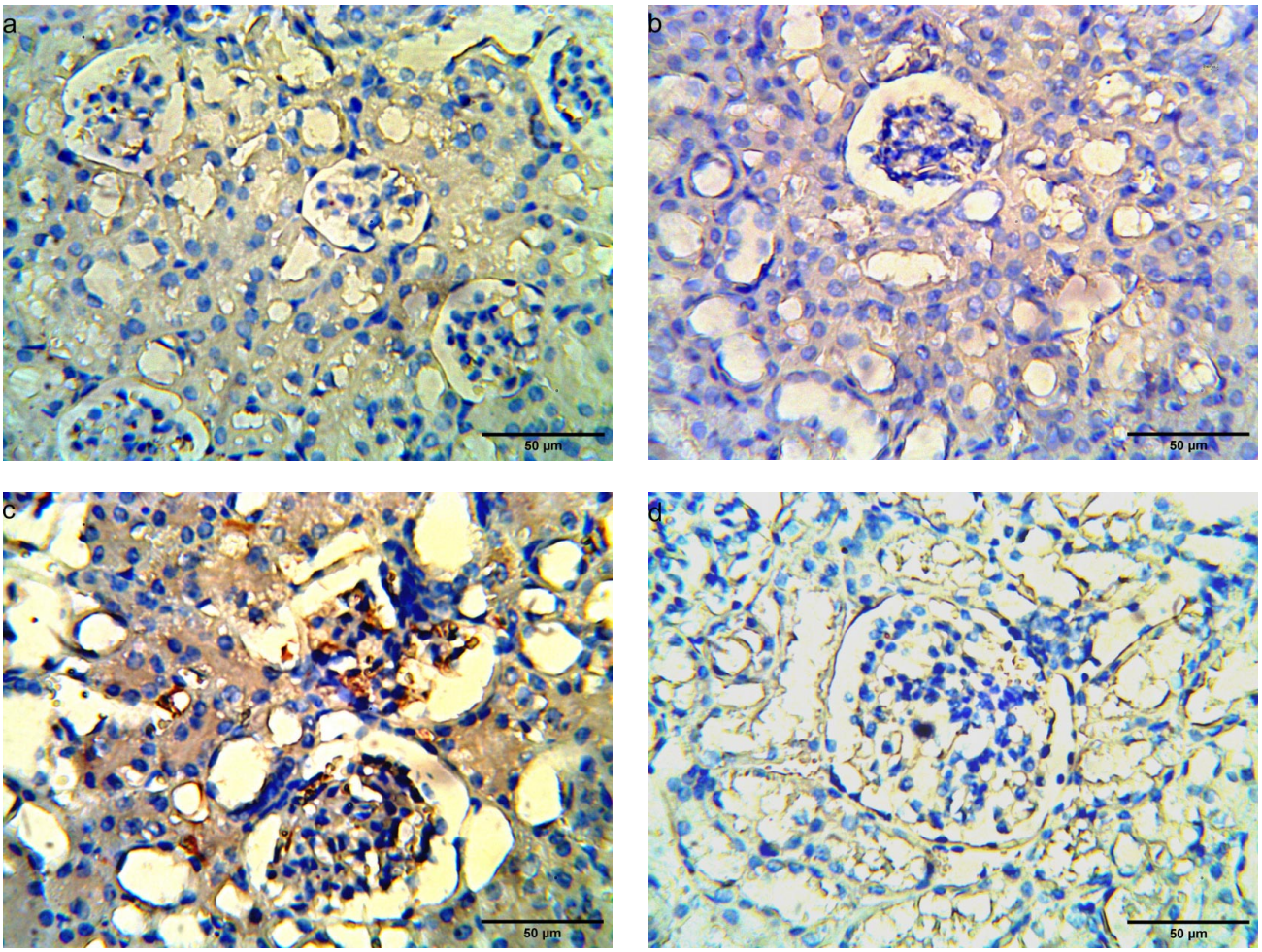


Fig. 4. Representative images of CD45-stained kidney sections of control and experimental gerbils, original magnification: 400x. Control (a) and normal gerbil treated with 20E (b); diabetic gerbils (c): CD45 antibody highlights influx of immune cells (brown indicates positive CD45); diabetic gerbils treated with 20E (d).

DISCUSSION

The experimental model used in the present study is a nocturnal wild desert rodent of the Gerbillinae subfamily. Previous work by our team has shown the value of using this model to study obesity (Semiane *et al*., 2017) and diabetes (Mallek *et al*., 2018) as well as the effect of 20E-based phytotherapy on several organs (Mallek *et al*., 2018; Bellahreche *et al*., 2023).

In this study we investigated the effects of 20E, one of the most common and abundant phytoecdysteroids known for its beneficial effects in mammals, on oxidative stress, as well as inflammation and fibrosis of kidneys in STZ-induced diabetic gerbils.

The present work showed increased of plasma glucose in diabetic gerbils when compared to controls. However, administration of 20E showed significant improvement in blood glucose level. The anti-diabetic properties of phytoecdysteroids have been reported in several studies. When 20E is administered to hyperglycaemic rodents, it has beneficial effects on carbohydrate homeostasis by significantly reducing glycaemia and stimulating glycogen synthesis (Yoshida *et al*., 1971). 20E is also thought to promote insulin sensitivity (Chen *et al*., 2009).

It is well established that diabetes is also associated with hyperlipidaemia. Injection of STZ resulted in a disturbance of the lipid profile showing high triglycerides and total cholesterol levels in diabetic gerbils. Treatment with 20E brought plasma triglycerides and cholesterol. Our results are in agreement with Hamden *et al*. (2008). Several studies have shown that the hypocholesterolaemic effect of phytoecdysteroids results from an increase in the conversion of cholesterol into bile acids and a reduction in its intestinal absorption (Syrov *et al*., 1983).

Extensive evidence suggests that oxidative stress is one of the major risk factors involved in the pathogenesis of diabetic kidney disease. Our findings showed that MDA levels were increased in the kidney of the diabetic groups compared with controls. Lipid peroxidation results in membrane destruction, and increase the production of reactive products that themselves can react with and damage proteins and DNA. In addition to the increase in lipid peroxidation, there was a significant decrease in GSH levels, SOD and CAT activities. In diabetic animals, we observed an effect of 20E treatment, manifested by a restoration of oxidative status in the kidney. As in the gerbil, Hamden *et al*. (2008) reported an increase in renal SOD and GSH concentrations in alloxane-induced diabetic rats treated with an extract of Ajuga iva. Our results also corroborate those of Sundaram *et al*. (2012) who reported antioxidant effects of 20E on the kidney of diabetic rats. The hypoglycaemic effect of phytoecdysteroids prevents oxidative stress in the kidney by reducing ROS production, lipid autoxidation and protein glycation.

Histopathological and histochemical examinations of kidney sections of diabetic gerbils showed basement membrane thickening, inflammatory cells infiltration and fibrosis. It is well established that hyperglycaemia-induced oxidative stress in STZ diabetes contributes to multiple physiological events including cell damage and inflammation, particularly in kidney.

Fibrosis is a process marked by excessive extracellular matrix deposits that contribute to functional parenchyma damage by fibrotic tissue. Morphometric analysis of kidney sections from diabetic gerbils stained with Sirius red revealed a significant increase in percentage area of collagen compared with controls. TGF-β-1 is a pro-fibrotic cytokine that has been identified as a critical regulator of ECM protein synthesis in DN. TGF-β1 stimulates the synthesis of fibronectin and collagen I and IV (Ziyadeh *et al*., 2004). It also contributes to excessive matrix accumulation by inhibiting metalloproteinases-1. It has been confirmed that TGF-β1/pSmad2/3 signalling is highly activated in kidneys of DN (Li *et al*., 2013).

Our results showed that 20E treatment mitigated kidney damage in the diabetic gerbils. Similar observations were reported in STZ-induced diabetic rats treated with 20E (Sundaram *et al*., 2012). *In vitro*, 20E was shown to have beneficial effects on fibrosis in the proximal tubule of the kidney. According to this study, 20E could act by suppressing TGF-β1 post-receptor signalling and restoring the epithelial character of the tubules by inhibiting Snail protein expression (Hung *et al*., 2012). *In vivo*, 20E improved renal function in STZ-treated rats by reducing renal TGF-b1content (Gui *et al*., 2014) and prevented the onset of hepatic fibrosis in obese gerbils (Agoun *et al*., 2019).

Mas receptor is expressed in many tissues, such as kidney. Activation of Mas by 20E could explain the antifibrotic effect on kidney of diabetic gerbils through its antioxidant action and its ability to inhibit TGF-β1. A decrease in creatinine and oxidative stress markers (Zou *et al*., 2010a), as well as a decrease in renal collagen and CTGF were reported in STZ-induced diabetic rats treated with ecdysterone suggesting that ecdysterone could protect renal function by improving oxidative stress and inhibiting CTGF and collagen expressions (Zou *et al*., 2010b).

In gerbils, 20E is more bioavailable than in other laboratory rodents. This bioavailability is probably due to better intestinal absorption and/or lower elimination. 20E also has a higher half-life than in mice when administered pers os and intra-peritoneally (Bellahreche & Dahmani, 2023). These pharmacokinetic properties may contribute to the significant effects of 20E treatment on renal function in the diabetic gerbil.

CONCLUSION

The present study demonstrates that 20E improved plasmatic glucose, lipid profile, creatinine and redox status as well as histopathological changes in the kidney of STZ-diabetic gerbils. Thus, 20E has a beneficial effect on kidney probably through its antioxidant action. Furthermore, more potential mechanisms need to be explored relating to the nephroprotective effect of 20E in the future.

ACKNOWLEDGEMENTS. We thank Pr. René Lafont for reading and commenting on the manuscript.

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RESUMEN: El objetivo de este trabajo fue evaluar los efectos de la 20-hidroxiecdisona (20E) en el estado redox, la

inflamación y la fibrosis renal en jerbos diabéticos inducidos por estreptozotocina. En este estudio, 24 jerbos se dividieron en 4 grupos: control, jerbos diabéticos inducidos con estreptozotocina (STZ, 130 mg/kg de peso corporal), jerbos diabéticos tratados con 20-hidroxiecdisona (50 mg/kg de peso corporal) y jerbos normales que recibieron 20E (50 mg/kg de peso corporal). Nuestros resultados mostraron una disminución significativa de la glucosa plasmática, los lípidos y la creatinina en el grupo diabético tratado con 20E. También se observó una disminución del contenido renal de MDA y un aumento de las actividades antioxidantes (GSH, SOD y CAT). Además, los análisis histomorfométricos e inmunohistoquímicos revelaron una mejora en la arquitectura renal y una disminución del porcentaje de superficie de colágeno. Estos resultados indican que el 20E tiene un efecto beneficioso sobre el daño renal, probablemente debido a su acción antioxidante.

PALABRAS CLAVE: Jerbo; Diabetes; Riñón; Estrés oxidativo; Histopatología; 20-Hidroxiecdisona.

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