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Original Article

Comparing the renoprotective effects of the antioxidants melatonin, vitamin D and vitamin E in diabetic rats

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الملخص

أهداف البحث: يرتبط داء السكري بإجهاد تأكسدي يؤدي إلى التهاب واعتلال الكلية السكري. تهدف هذه الدراسة إلى ملاحظة التأثير المحتمل لمضادات الأكسدة، الميلاتونين وفيتامين د وفيتامين هـ لحماية الكلى لفئران التجارب المصابة بالسكري.

طرق البحث: شملت الدراسة ١٠٨ فأرا قسموا إلى ١٢ مجموعة. المجموعة ١ مجموعة ضابطة من فئران طبيعية لم تتلق أي دواء. المجموعات من ٢ إلى ٤ هي لفئران طبيعية عولجت كالتالي: المجموعة ٢ بميلاتونين، والمجموعة ٣ بفيتامين هـ، والمجموعة ٤ بفيتامين د. المجموعات من ٥ إلى ١٢ هي لفئران مصابة معمليا بالسكري ثم عولجت كما يلي: المجموعة ٥ لم تتلق أي دواء، والمجموعة ٦ بالأنسولين، والمجموعة ٧ بالميلاتونين، والمجموعة ٨ بالميلاتونين والأنسولين، والمجموعة ٩ بفيتامين هـ، والمجموعة ١٠ بفيتامين هـ مع الأنسولين، والمجموعة ١١ بفيتامين د، أما المجموعة ١٢ فعولجت بفيتامين د مع الأنسولين. استمر العلاج لشهرين ثم جمعت عينات الكلى للفحص النسيجي، وعينات مصل الدم للفحص الكيميائي لقياس مستويات: سكر الدم، الإجهاد التأكسدي، وظائف الكلى، الهوموسستاين وعامل التنخر الورمي-الفا.

النتائج: أظهرت النتائج زيادة ذات دلالة إحصائية للقدرة الكلية لمضادات الأكسدة في مجموعات ٢، ٧، ٨، ١٠ و١١. زاد عامل التنخر الورمي-الفا في المجموعة ٢ ولكنه نقص في باقي المجموعات. أظهرت النتائج زيادة ذات دلالة إحصائية للكرياتينين في المجموعات ٥، ٦، ٧، ٨، ٩، ١١، ١٢. الفحص النسيجي للكلى أظهر زيادة في سمك الغشاء المبطن للأوعية الشعرية مع تصلب منتشر في الأغشية المبطنة وتصلب عقدي في الكبيبات الكلوية. العلاج

بالميلاتونين أظهر تحسن ملموس في حماية الكلى بينما لم يُحدث العلاج بفيتامين د وفيتامين هـ نفس الأثر.

الاستنتاجات: أظهر الميلاتونين سواء مع الأنسولين أو بدونه نشاطا مفيدا مضادا للأكسدة كيميائيا مع حماية لأنسجة الكلى، بينما فشل فيتامين د وفيتامين هـ في إظهار هذه التأثيرات.

الكلمات المفتاحية: السكري؛ اعتلال الكلية السكري؛ الميلاتونين؛ فيتامين د؛ وفيتامين هـ

Abstract

Objectives: Diabetes mellitus is associated with oxidative stress that leads to inflammation and diabetic nephropathy. This study aimed to determine the possible renoprotective effects of the antioxidants melatonin, vitamin D and vitamin E in diabetic rats.

Methods: We divided 108 albino rats into 12 groups. **G1** group was fed a normal diet and did not receive any medication. **G2** to **G4** consisted of non-diabetic rats that were treated as follows: **G2** with melatonin; **G3** with vitamin E; **G4** with vitamin D. Groups **G5** to **G12** consisted of diabetic rats that were treated as follows: **G5** received no medication; **G6** treated with insulin; **G7** treated with melatonin; **G8** treated with melatonin and insulin; **G9** treated with vitamin E; **G10** treated with vitamin E and insulin; **G11** treated with vitamin D and **G12** treated with vitamin D and insulin. Two months after treatment commenced, histological and biochemical examinations of glucose profile, oxidative stress status, renal function, homocysteine and TNF-α were performed.

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Results: Total antioxidant capacity (TAC) increased significantly in groups G2, 7, 8, 10 and 11. TNF-α significantly increased in G2, but decreased in all other groups. Creatinine increased significantly in groups G5, 6, 7, 8, 9, 11 and 12. In the kidneys of the diabetic rats, thickened capillary basement membrane, diffuse mesangial sclerosis and nodular glomerulosclerosis was observed. Rats treated with melatonin showed marked improvement in these symptoms. However, in those treated with vitamin D and E, thickened capillary basement membrane and mesangial sclerosis was still present.

Conclusions: Melatonin, administered either with or without insulin had a significant biochemical antioxidant effect and histological renoprotective effect. Conversely, vitamin D and E did not appear to have any effects on the parameters measured.

Keywords: Diabetes; Diabetic nephropathy; Melatonin; Vitamin D; Vitamin E

Introduction

Diabetes mellitus is a group of detrimental disorders that can cause multiorgan failure.[1] It is increasing in prevalence worldwide, largely due to the rising incidence of type 2 diabetes (T2D).[2] It has deleterious effects on the kidneys and can lead to diabetic nephropathy. This significantly impairs kidney function and their ability to maintain body fluids by filtering waste and toxic products, and regulate blood pressure.

Diabetic nephropathy causes glomerulosclerosis, which results in the thickened glomerular basement membrane, diffusion of the mesangial sclerosis, microaneurysms and interstitial inflammation.[3] The primary mechanisms for the development of diabetic nephropathy remain unknown. However, multiple factors have been suggested to contribute to its pathogenesis, such as oxidative stress, metabolic disturbance and hemodynamic changes.[4] It has also been proposed that hyperglycaemia-induced dyslipidaemia in diabetes, particularly in T2D, is an important risk factor for the progression of diabetic nephropathy into glomerulosclerosis and tubulointerstitial injury.[5−7] Furthermore, hyperglycaemia is associated with the abnormal activation of several metabolic pathways such as polyol, protein kinase C, the hexosamines pathway, the formation of advanced glycation end products (AGEs), and the activation of NADPH-oxidases and angiotensin II, which can lead to over-induction of oxidative and nitrosative radicals, as well as the stimulation of inflammatory cytokines.[8]

Under normal physiological conditions, reactive oxygen species (ROS) regulate cell signalling, proliferation, differentiation and apoptosis in the renal system.[4] However, there is mounting evidence that over-induction of ROS in diabetes plays a key role in the distortion of metabolic pathways in the kidneys and the development of diabetic nephropathy.[9] Such upregulation triggers inflammation, fibrosis, and endothelial dysfunction in the kidneys.[10] The use of antioxidants to combat the harmful effects of oxidative stress in diabetic nephropathy has been reported in a number of experimental models. For example, extract from ginkgo biloba and allicin improved glomerular hypertrophy in diabetic rats,[11,12] and administration of *Lindera strychnifolia* decreased the development of diabetic nephropathy in mice.[13] In addition, rosmarinic acid, aster koraiensis, vitamin C, alpha lipoic acid, ferulic acid and coenzyme Q10 have been shown to treat glomerulosclerosis.[14] These reports demonstrate the importance of identifying the most effective antioxidants for treating diabetic nephropathy.

Melatonin has powerful antioxidative and protective properties,[15] and is the only substance which can be used to treat all aspects of the 'devil's triangle' (increased free-radical production, decreased antioxidant capacity, increased and excessive inflammation).[16] It is also thought to have renoprotective properties as it can reduce symptoms during the early stages of glomerulopathy.[17−19] Diabetic neuropathy, retinopathy and vasculopathy have also been associated with vitamin D and E status.[20,21] Patients with diabetic nephropathy were found to have reduced serum levels of vitamin D.[22] However, whether vitamin D supplements are beneficial for combating diabetes-mediated kidney dysfunction remains controversial.[23,24] Similarly, the role of vitamin E in protecting or treating diabetic nephropathy has not been fully established.

Despite advances in ROS pathophysiology, there are currently no therapeutic options available for reducing the oxidative stress-mediated progression of diabetic nephropathy. Furthermore, as there is insufficient evidence regarding the renoprotective effects of vitamin D and E on diabetic nephropathy, it is important to explore their therapeutic potentials. In this study, we investigated the renoprotective potential of melatonin, vitamin D and vitamin E in rats with streptozotocin (STZ)-induced diabetes. We assessed parameters such as glucose profile, lipid profile, kidney function biomarkers, tumour necrosis factor-α, homocysteine and total antioxidant capacity. We also conducted histopathological examinations to determine the structural integrity of the kidneys. Our findings will provide insights for the potential development of new therapies to control diabetes-mediated renal complications.

Materials and Methods

Experiments were conducted on 120 male albino rats, of which 108 survived till the end of the study period. To induce diabetes in the rats, nicotinamide (230 mg/kg) was

injected, followed by an intraperitoneal injection of STZ (65 mg/kg) 15 min later.[25] In control rats, equal volume of normal saline was injected. The STZ was prepared by dissolving STZ powder in saline buffered at pH 4.0 with sodium citrate. The establishment of diabetes was determined by monitoring the blood glucose levels, and only rats with high glucose levels (more than 110 mg/dl) were used for experiments.

Treatments

Rats (200−250 g) were divided into 12 groups. **G1** (*n = 10*) was the control group, in which rats were fed normal diets and received no medication. **G2** to **G4** (*n = 10* per group) consisted of non-diabetic rats treated orally as follows: **G2** treated with melatonin (0.3 mg/kg); **G3** treated with vit-E (40 mg/kg); **G4** treated with vit-D (40 mg/kg). **G5** to **G12** (*n = 8* to 10 per group) was comprised of diabetic rats treated orally as follows: **G5** received no medication; **G6** treated with insulin only; **G7** treated with melatonin (0.3 mg/kg); **G8** treated with melatonin (0.3 mg/kg) and insulin; **G9** treated with vit-E (40 mg/kg); **G10** treated with vit-E (40 mg/kg) and insulin; **G11** treated with vit-D (40 mg/kg) and **G12** treated with vit-D (40 mg/kg) and insulin.

Initially, the diabetic groups included more than 10 rats per group. However, the variation in the sample size of groups was due to death of some diabetic rats during the study. Insulin dose was calculated according to the blood glucose level and the weight of each rat. Each treatment lasted for eight weeks.

Biochemical analysis

Rats were killed after eight weeks of treatment, and blood was collected for biochemical measurements. Glycosylated haemoglobin (HbA1c) was measured on the same day from fresh blood samples collected in EDTA vacutainer tubes. For other biochemical measurements, blood was collected in plain tubes and kept for 30 min to coagulate; blood was then was centrifuged at 1.000−2000 x g for 15 min to separate the serum. Serum samples were then frozen (at −20 °C) in five Eppendorf tubes (1 ml each) for the measurement of fasting blood sugar (FBS), fructosamine (FA), total antioxidant capacity (TAC), oxidized low density lipoprotein (Ox-LDL), homocysteine (HC), and tumour necrosis factor-α (TNF-α). Creatinine, blood urea nitrogen (BUN), uric acid and albumin were also determined to assess kidney function. Microalbuminuria can also be used to assess kidney function. However, difficulties in collecting urine samples from the rats meant that it was not possible to assess this. After the measurements had been taken, the serum was kept at −80 °C for future examinations.

HbA1c was assessed using the ion exchange resin technique (Human Gesellschaft fur Biochemica und Diagnostia mbH, Wiesbaden, Germany). Measurements of FBS, creatinine, BUN, uric acid and albumin were assessed using automated systems (COBAS® Integra 400 Plus, Roche Diagnostics, Hoffmann-LaRoche, Rotkreuz, Switzerland) and were calibrated by using a calibrator obtained from Roche Diagnostics. Fructosamine levels were measured using a fructosamine kit from POINTE SCIENTIFIC, Inc. (Canton, MI, USA). Ox-LDL was assessed using a competitive ELISA kit (Mercodia AB, Uppsala, Sweden), and TAC and TNF-α levels were measured with ELISA Kits (rat) obtained from MyBioSource, Inc. (San Diego, CA, USA). Homocysteine levels were determined using a reagent set from SCIENTI-FIC, Inc., (Orlando,FL, USA). All commercial kits were evaluated for their sensitivity, linearity and precision by the manufacturers.

Histopathological examination

Kidneys from all rats which were killed at the end of treatment period were collected for histopathological examination. Tissue sections were immediately fixed in formalin (10%), then were immersed in ethanol of increasing concentration until pure water-free alcohol was obtained. The tissue sections were then immersed in xylene to completely remove the alcohol, and were embedded in blocks of paraffin wax. The blocks were then clinched to a microtome and cut into sections, which were loaded onto microscopic slides and deparaffinized by running them through alcohol to water. The slides were stained using haematoxylin and eosin (H&E) stain and thin glass covers were placed on top.

Statistical analysis

Data analysis was conducted using SPSS (V20, SPSS Inc.). The levels of metabolic parameters in the different groups were compared using descriptive statistics and one-way ANOVAs. Statistical significance was determined at $p < 0.05$.

Results

The results are illustrated in Table 1. When compared to the results of the control group[1]: glucose was significantly higher ($p < 0.05$) in all groups except group 2. HbA1c was significantly higher ($p < 0.05$) in groups 4, 5, 6 and 7. Fructosamine was significantly higher ($p < 0.05$) in all groups except group 2 and 9. TAC was higher ($p < 0.05$) in groups 2, 7, 8, 10 and 11. However, there were no significant differences in Ox-LDL for all groups. Homocysteine was significantly higher ($p < 0.05$) in group 11 only. TNF-α was significantly higher ($p < 0.05$) in group 2 but was lower than the control ($p < 0.05$) in all other groups. In all groups apart from group 12, which had significantly lower levels, there were no differences in albumin across all groups when compared with the control. BUN was significantly higher ($p < 0.05$) in group 9 but was significantly lower ($p < 0.05$) in group 12. There were no significant differences in uric acid across groups, except for group 4, which was significantly lower. Creatinine was significantly higher ($p < 0.05$) in groups 5, 6, 7, 8, 9, 11 and 12. There were no significant changes in BUN/creatinine ratios, except for in group 12, where the ratio was significantly lower than that of the control.

Histopathological findings

Compared to the non-diabetic control (Figure 1-A), diabetic kidneys had thickened capillary basement

Table 1: Effects of melatonin, vitamin E and D in diabetic rats.

Group	G1	G2	G3	G4	G5	G6	G7	G8	G9	G10	G11	G12
FBS (mg/dl)	98.3 ± 8.2	113 ± 23.7	126* ± 11.2	125* ± 14.9	146* ± 7.2	146* ± 9.1	143* ± 9.9	144* ± 6.0	148* ± 2.3	143* ± 6.7	148* ± 0.7	130* ± 16.4
HbA1c (%)	4.1 ± 0.4	4.0 ± 0.6	4.8* ± 1.1	4.6* ± 0.5	4.8* ± 0.6	4.9* ± 0.5	4.6* ± 0.5	4.3 ± 0.3	4.8 ± 0.8	4.2 ± 0.5	4.6 ± 0.01	4.2 ± 0.6
Fructosamine (mmol/l)	0.49 ± 0.04	0.46 ± 0.2	0.7* ± 0.2	0.87* ± 0.2	0.9* ± 0.3	1.16 ± 0.4	1.0* ± 0.3	0.95* ± 0.2	0.75 ± 0.4	0.9* ± 0.3	0.9* ± 0.01	0.7* ± 0.1
TAC (ng/ml)	7.25 ± 0.4	7.92* ± 0.6	7.23 ± 0.4	7.19 ± 0.6	7.43 ± 0.3	7.43 ± 0.8	7.8* ± 0.5	7.8* ± 0.4	7.2 ± 0.2	8.1* ± 0.5	8.7* ± 0.4	7.7 ± 0.8
Ox-LDL (ng/ml)	48.1 ± 3.1	63.1 ± 16.1	49.2 ± 5.6	52.5 ± 9.5	61.1 ± 15.3	54 ± 6.7	46.9 ± 2.8	49.7 ± 4.6	54.8 ± 5.6	45 ± 3.6	52.5 ± 2.1	45.7 ± 4.7
Homocysteine (nmol/ml)	13.9 ± 1.6	14.4 ± 1.9	13.4 ± 1.9	12.5 ± 2.2	14.2 ± 3.4	15.1 ± 4.1	14.8 ± 2.5	15.1 ± 6.2	13.5 ± 2.96	13.2 ± 4.9	11* ± 2.8	13 ± 2.1
TNF-α (pg/ml)	20.3 ± 3.2	33.6* ± 6.1	12.7* ± 3.1	16.3* ± 2.96	9.3* ± 5.1	13.9* ± 4.2	8.8* ± 5.5	11.1* ± 7.9	12.0* ± 4.1	10.0* ± 1.7	7.0* ± 0.1	7.0* ± 2.7
Albumin (g/dl)	4.58 ± 0.3	4.85 ± 1.3	4.62 ± 0.3	4.38 ± 0.2	4.6 ± 0.6	4.74 ± 0.4	4.67 ± 0.4	4.34 ± 0.2	4.28 ± 0.2	4.81 ± 0.4	4.01 ± 0.6	3.56 ± 0.3
BUN (mmol/l)	6.96 ± 0.95	7.0 ± 0.4	6.46 ± 1.3	7.0 ± 1.3	8.1 ± 1.6	8.9 ± 2.5	9.86 ± 3.4	10.4 ± 4.0	13.8* ± 2.9	6.9 ± 0.6	8.5 ± 0.3	4.73* ± 0.9
Uric Acid (µmol/l)	85 ± 24.4	86 ± 35.1	74.2 ± 41.5	57* ± 8.5	78.6 ± 32.5	91.2 ± 62.1	85.2 ± 25.9	75.8 ± 34.2	79.4 ± 29.9	56 ± 7.0	62.5 ± 14.9	76.3 ± 2.3
Creatinine (µmol/l)	17.4 ± 4.0	25.8 ± 8.0	18.2 ± 7.4	17.6 ± 1.7	24.6* ± 3.7	28.6* ± 8.1	25.6* ± 6.9	27* ± 5.8	30* ± 3.2	19.7 ± 0.6	26* ± 1.4	24* ± 2.0
Bun/Creatinine ratio	0.43 ± 0.15	0.29 ± 0.07	0.39 ± 0.12	0.39 ± 0.07	0.33 ± 0.06	0.32 ± 0.09	0.38 ± 0.07	0.39 ± 0.15	0.46 ± 0.10	0.35 ± 0.02	0.33 ± 0.01	0.2* ± 0.06

* indicates $P < 0.05$ as compared to G1. The values are represented as mean ± SD.

membrane, diffuse mesangial sclerosis, and nodular glomerulosclerosis (Figure 1-B). The glomerular capillary basement membranes of diabetic kidneys had thickened throughout their entire length (Figure 1-B). In addition, in some kidneys there was evidence of severe pyelonephritis and localized abscess formation (Figure 1-C and -D). Interestingly, these symptoms in the kidneys of diabetic rats treated with melatonin, either with (Figure 1-E) or without insulin (Figure 1-F) had improved significantly. In contrast, in the kidneys of rats treated with vitamin D, either with (Figure 1-G) or without insulin (Figure 1-H) showed thickened capillary basement membrane and mesangial sclerosis. Similarly, the kidneys of diabetic rats treated with vitamin E, either with (Figure 1-I) or without insulin (Figure 1-J) also showed thickened capillary basement membrane and mesangial sclerosis.

Discussion

In our study, melatonin appeared to have a beneficial antioxidant effect on both non-diabetic and diabetic rats, when administered alone and with insulin. In particular, treatment with melatonin resulted in increased TAC, decreased levels of ox-LDL, homocysteine and TNF-α, when compared to non-diabetic control rats. In terms of histopathology, the kidneys of diabetic rats had obvious damage and changes in the structure of renal tissues were evident. Remarkably, administration of melatonin, both with and without insulin, induced marked improvements in renal tissues.

Biochemically, based on the levels of TAC, ox-LDL and homocysteine in diabetic rats treated with vitamin E, compared to non-diabetic rates, vitamin E did not have renal protective effects. However, using insulin to control blood sugar level in the presence of vitamin E increased TAC to levels comparable to those of non-diabetic rats. In terms of histopathological effects, vitamin E administered both with or without insulin in diabetic rats also did not have a renal protective effect. The administration of vitamin D to diabetic rats increased their TAC levels to those of non-diabetic rats. However, it did not have a positive effect on any other antioxidant parameters. Similar to vitamin E, vitamin D did not appear to infer any histopathological renal protection. The results also demonstrated that all diabetic rats taking the three antioxidants, both with and without insulin, had increased serum levels of creatinine compared to that of non-diabetic rats. Although this increase was within the normal range in rats, and there were no significant changes in other renal functions.

There is accumulating evidence that, due to its antioxidant properties, melatonin may play a role in ameliorating diabetes and attenuating diabetic nephropathy.[26,27] In vivo studies have demonstrated that melatonin have antioxidant and anti-inflammatory effects in subjects with diabetic nephropathy.[19,27,28] Furthermore, in an in vitro model of diabetic nephropathy, Ji and Xu revealed that melatonin has anti-apoptotic effects.[29] Our results are in agreement with those findings and support other studies which have shown that melatonin ameliorates diabetic complications such as retinopathy and nephropathy.[28,30]
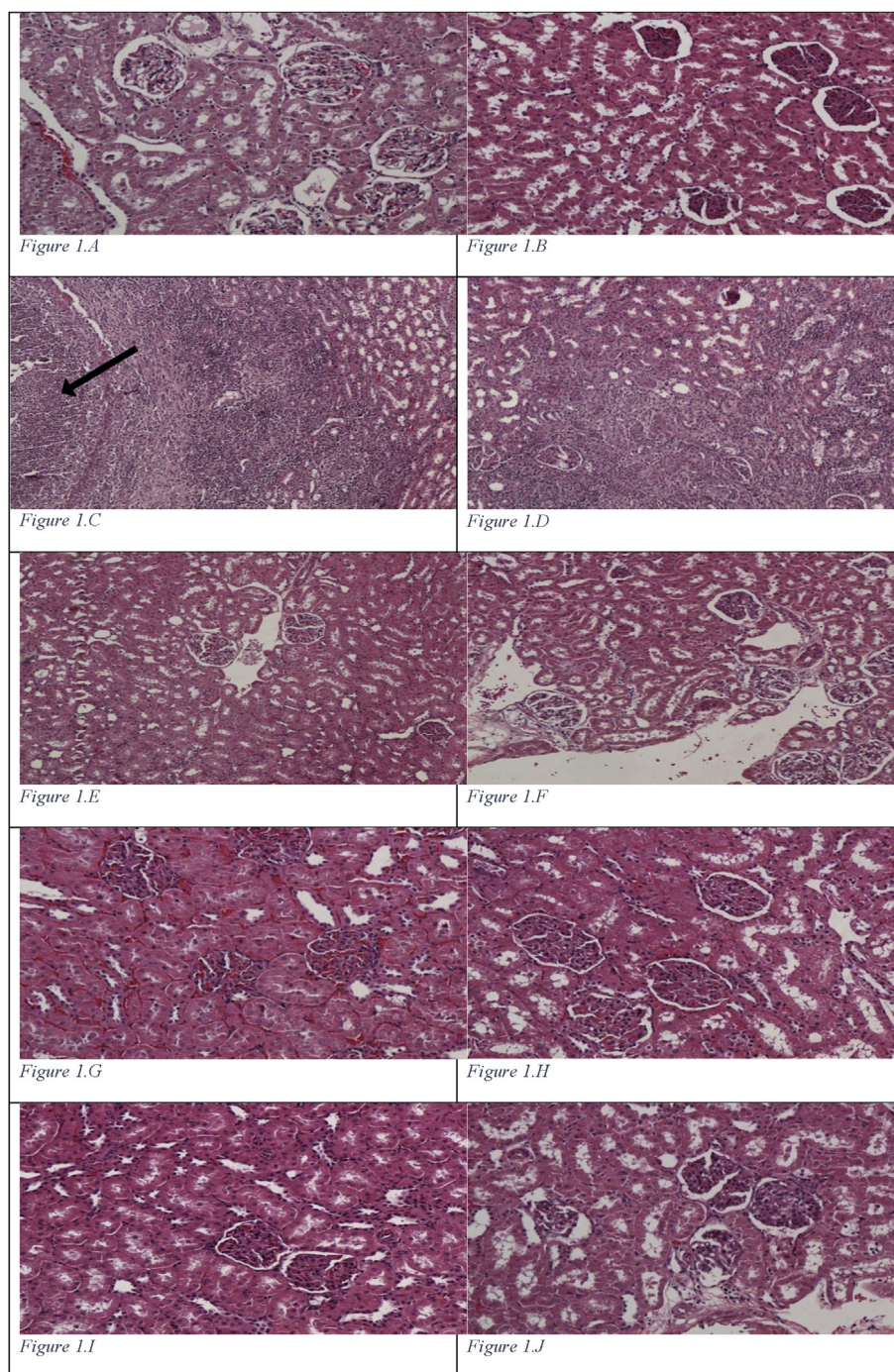
Figure 1: Histopathological examination by light microscope of diabetic rats treated with melatonin, vitamin D or vitamin E with or without insulin. Groups are shown as follows: Normal rats (A), diabetic rats (B, C and D), diabetic rats treated with melatonin and insulin (E), diabetic rats treated with melatonin only (F), diabetic rats treated with vitamin D and insulin (G), diabetic rats treated with vitamin D only (H), diabetic rats treated with vitamin E and insulin (I), and diabetic rats treated with vitamin E only (J).

Hyperglycaemia-induced oxidative stress plays an important role linking diabetes and its complications.[31,32] The pathogenesis of diabetic kidney disease is related to the over induction of ROS, which leads to considerable alterations in the morphology and physiology of various tissue proteins and lipids in the kidney.[33−35] Oxidative stress stimulates inflammatory mediators such as TNF-α, macrophage chemotactic proteins, and IL-1β and IL-6.[33]

Oxidative stress induces several pathophysiological pathways, such as those involved in the formation AGEs; the polyol pathway; and growth factor, cytokine, hexosamine and protein kinase C pathways, leading to diabetic nephropathy[10,36]

Generally, antioxidants have several physiological roles such as scavenging and diminishing the damaging effects of free radicals; and improving the body's natural defence

systems by prompting antioxidant enzymes and proteins that are involved in antioxidant pathways. The protective effect of any antioxidant compound depends on its type, structure, concentration, stage and the severity of the disease.[37,38] Vitamin E has several physiological roles, such as stimulation of the immune system and prevention of genetic changes through inhibition of DNA destruction. Additionally, vitamin E directly detoxifies free radicals and interacts with recycling processes to create reduced forms of vitamin A and C.[39]

Previous clinical studies have reported improvements in the symptoms of diabetic nephropathy following treatment with vitamin D. These benefits were due to the reversal of the progression of diabetic nephropathy, following improvements in glucose metabolism, and reductions in the activity of the renin angiotensin system and fibrosis. There is a reciprocal relationship between serum levels of vitamin D and the prevalence of diabetes and its complications.[40] Therefore, the negative effect of both vitamin E and D in our study in protecting the kidneys of diabetic rats, even when administered in combination with insulin, may be due to inappropriate dosage and/or duration of treatment.

Conclusion

Melatonin administration in both non-diabetic and diabetic rats treated with or without insulin, had beneficial antioxidant effects biochemically, and renoprotective effects histologically. Treatment with vitamin E and D did not appear to have such beneficial effects. However, this may be due to the low dosage administered and/or the short duration of treatment. Further studies are encouraged where vitamins are administered at higher doses over a longer period of time to determine whether they confer renoprotective benefits in diabetic rats.

Source of funding

Conflict of interest

The authors have no conflict of interest to declare.

Ethical approval

The experimental protocols were approved by the University of Umm Al-Qura Ethics Board, and followed the guidelines of the NIH institute and its policy on the usage of laboratory animals. All experiments followed the code of ethics of the World Medical Association (Declaration of Helsinki) for animal experiments.

Authors contributions

AAA conceived and designed the study, provided research materials, conducted research, interpreted data, provided logistical support and wrote the first and final draft of the manuscript. EEM designed the study, performed the biochemical experiments, collected, organized, analysed and interpreted data and wrote the initial and final draft of the manuscript. SHH designed and revised the study, analysed and interpreted data and wrote the first and final draft of the manuscript. GEE participated in article preparation, participated in the experimental work and performed the histopathological examination, and revised the manuscript. All authors have critically reviewed and approved the final draft and are responsible for the content and similarity index of the manuscript.

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