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Coadministration of Melatonin and Insulin Improves Diabetes-Induced Impairment of Rat Kidney Function

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Significance of This Study

What is already known about this subject?

- It is well established that diabetes-related metabolic disorders due to hyperglycemia lead to renal complications.
- Various allopathic drugs are available for the treatment of diabetes, and they somehow control glucose metabolism. However, these drugs are unable to protect the body from other complicating sequels such as renal injury. Hence, it remains unclear whether the diabetes-induced renal damage can be prevented.

What are the new findings?

- The findings of the current study indicated that melatonin plus insulin might be an effective therapeutic combination to prevent diabetes-induced functional renal alterations as demonstrated by changes of kidney histoarchitecture, renal cortex biochemistry, serum biochemical indicators of renal function, electrolytes, and serum concentration of pro- and anti-inflammatory cytokines.
- Insulin and melatonin might act synergistically through autocrine and paracrine/endocrine pathways during the hyperglycemic condition to modulate metabolic physiology. Mitigation of diabetes-induced renal damage by melatonin and insulin involves a complex series of biochemical improvements at cellular levels. Melatonin and insulin attenuate the nephrotoxicity of diabetes through its potent antioxidant and glucose metabolizing actions, respectively, as it is believed to reinforce the antioxidant enzymes and direct free-radical scavenging at subcellular levels.

How might these results change the focus of research or clinical practice?

- The findings of the present study indicate the potential clinical importance of combined melatonin and insulin in alleviating the renal toxicity associated with diabetes. The present study should be followed by clinical placebo-controlled trials before its therapeutic significance is established.

Keywords
Cytokines · Insulin · Melatonin · MT1 and MT2 · Oxidative stress · Renal cortex · Streptozotocin

Abstract
Introduction: The present study was designed to evaluate the therapeutic efficacy of melatonin and insulin coadministration in diabetes-induced renal injury in rats. ***Research De-***

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sign and Methods: Diabetes was achieved by giving streptozotocin (15 mg/kg) for 6 consecutive days. The diabetic condition was confirmed by assessing the blood glucose level; animals having blood glucose levels above 250 mg were considered as diabetic. Following the confirmation, animals were randomly divided into different experimental groups, viz group I served as the control (CON), group II diabetic (D), group III D+melatonin (MEL), group IV D+insulin (INS), group V D+MEL+INS, group VI D+glibenclamide (GB), group VII CON+MEL, group VIII CON+INS, and group IX CON+GB. Following the completion of the experimental period, animals were sacrificed, blood was collected via a retro-orbital puncture, and kidneys were harvested. Diabetic rats exhibited a significant increment in blood glucose and biochemical indexes of renal injury (tubular disruption, swollen glomeruli with loss of glomerular spaces, and distortion of the endothelial lining) including augmented levels of serum creatinine, urea, uric acid, Na^+, and K^+, and inhibition/suppression of the activity of glutathione (GSH) peroxidase, GSH reductase, glucose-6-phosphate dehydrogenase, and GSH-S-transferase in the renal cortex. ***Results:*** By examining thiobarbiturate reactive substances, reduced GSH, superoxide dismutase activity, and catalase activity in the renal cortex of control and diabetic rats, it was documented that treatment with melatonin or insulin alone or in combination showed a significant ad integrum recovery of GSH-dependent antioxidative enzymatic activities. Melatonin and insulin coadministration caused greater reductions in circulating tumor necrosis factor-α, tumor growth factor-β1, interleukin (IL)-1β, and IL-6 levels in diabetic rats, whereas IL-10 levels increased, as compared to each treatment alone. Diabetic rats showed a significant increase in the expression of both MT1 and MT2 melatonin receptor genes. Melatonin or insulin treatment alone or in combination resulted in significant restoration of the relative expression of both melatonin receptors in the renal cortex. ***Conclusion:*** The coadministration of exogenous melatonin and insulin abolished many of the deleterious effects of type 1 diabetes on rat renal function.

Introduction

As a complex metabolic disorder, diabetes mellitus is an epidemic of foremost public health concern. The increased rate of diabetes in modern society is associated with several predisposing factors, including a sedentary lifestyle, carbohydrate-rich diet, and obesity [1]. The excessive glucose concentration in diabetes becomes autooxidized in tissues and leads to auto-oxidative glycosylation of proteins, oxidative stress, and also causes various secondary metabolic complications in different organs including the kidney [2]. Diabetic pathogenicity of the kidney includes injury not only of extrarenal small blood vessels but also of blood vessels in the renal cortex, which leads to a renal functional disability including reduced blood filtration due to mesangial cell expansion, loss of products (albumin and glucose), and tubule interstitial fibrosis. These pathophysiological complications alter the glomerular filtration rate, increase albuminuria, and disrupt blood purification, causing excess urine formation and excretion of metabolic products, and a reduced osmolality [3, 4]. Besides, diabetes induces a chronic low-grade inflammatory process that in turn reinforces renal microvascular complications [5].

Melatonin, in addition to being the chief hormone of the pineal gland associated with the regulation of sleep, the circadian system, and seasonal reproduction, is a pleiotropic molecule, exhibiting significant antioxidant, anti-inflammatory, and immunoregulatory properties [6–8]. Melatonin can reduce insulin resistance, dyslipidemia, and overweight in obese individuals [9]. It also enhances various antioxidative enzymes including glutathione (GSH) peroxidase (GPX), GSH reductase (GR), and importantly, the synthesis of GSH [10, 11]. Melatonin protects against hepatorenal damage induced by diabetic conditions [12–18]. Moreover, due to a low-level inflammatory state, diabetic patients are more susceptible to SARS-CoV-2 infection, amplifying the cytokine storm typically seen in COVID-19 patients [19].

Streptozotocin (STZ) is a diabetogenic drug synthesized by the soil bacteria, *Streptomyces achromogenes*, having a broad spectrum of antibacterial properties. This drug resembles glucose, the only difference being the presence of methyl groups; hence, it mimics glucose in terms of the glucose transporter 2 in the pancreas, targeting the insulin synthesizing β-cells and leading to a diabetic condition. Herein, diabetic animals were treated with melatonin, insulin, or a combination of both to investigate the possible impact of a single or combined therapeutic potential of melatonin and insulin to potentially reverse the histological and biochemical alterations seen during diabetic nephropathy in rats. Additionally, diabetic rats were treated with glibenclamide, a clinically employed antidiabetic molecule, to compare the therapeutic potential during either the condition alone and/or the combination of melatonin and insulin. The results obtained document the significance of melatonin as an adjuvant to insulin in preventing diabetic sequelae in insulin-deficient diabetic animals.

Table 1. Experimental design

Group	Treatment	*N*
I. CON	0.1 M sodium citrate buffer	6
II. D	STZ 15 mg/kg (6 days)	6
III. D+MEL	STZ 15 mg/kg (6 days) + melatonin 1 mg/kg (4 weeks)	6
IV. D+INS	STZ 15 mg/kg (6 days) + insulin 0.5 mL/kg (4 weeks)	6
V. D+MEL+INS	STZ 15 mg/kg(6 days) + melatonin 1 mg/kg + Insulin 0.5 mL/kg (4 weeks)	6
VI. D+GB	STZ 15 mg/kg + glibenclamide 0.5 mg/kg (4 weeks)	6
VII. CON+MEL	Melatonin 1 mg/kg (4 weeks)	6
VIII. CON+INS	Insulin 0.5 mL (20 units)/kg (4 weeks)	6
IX. CON+GB	Glibenclamide 0.5 mg/kg (4 weeks)	6

CON, control; DB, diabetic; MEL, melatonin, INS, insulin; GB, glibenclamide; STZ, streptozotocin.

Table 2. Effect of exogenous melatonin and insulin alone or in combination on the weekly blood glucose level and serum random sugar level in control and diabetic rats

Groups	Weekly changes in the blood glucose level, mg/dL				
	basal	week-1	week-2	week-3	week-4
I. CON	128±112	126±8 II***	118±5 II***	120±5 II***	131±7 II***
II. D	130±13	309±14 III*, IV*, V***, VI*	315±9 III*, IV*, V***, VI*	320.33±14 III*, IV*, V***, VI*	334±21 III*, IV*, V***, VI*
III. D+MEL	119.35±1	378.99±15 IV*, V***, VI*	262.37±18 IV*, V***, VI*	243.25±15 IV*, V***, VI*	225±14 IV*, V***, VI*
IV. D+INS	119.25±13	397.45±17 III*, V***, VI*	283.25±6 III*, VII***, VIII*	224.41±18* III*, VII***, VIII*	170.3±8 III*, VII***, VIII*
V. D+MEL+INS	114±9	308±11 III*, IV*, VI*	248±20 III*, IV*, VI*	235±24 III*, IV*, VI*	220±13 III*, IV*, VI*
VI. D+GB	123.35±57	394.45±14 III*, IV*, V***	274±17 III*, IV*, V***	196.46±16 III*, IV*, V***	184.2±13 III*, IV*, V***
VII. CON+MEL	113±7	124±16 II***, III*, IV*, V**, VI*	132±8 II***, III*, IV*, V**, VI*	129.9±11 III*, IV*, V**, VI*	128±11 II***, III*, IV*, V**, VI*
VIII. CON+INS	109±11	125±15 II***, III*, IV*, V**, VI*	135±12 II***, III*, IV*, V**, VI*	128.2±9 II***, III*, IV*, V**, VI*	131.8±2 II***, III*, IV*, V**, VI*
IX. CON+GB	119.95±76	131±18 II***, III*, IV*, V*, VI*	140.75±11 III***, III*, IV*, V*, VI*	132.7±21 II***, III*, IV*, V*, VI*	123.43±4 II***, III*, IV*, V*, VI*

Shown are the means ± SEM ($n = 6$/group). ANOVA: numerals indicate the group which is significantly different, and asterisks designate the p value; for example, 126 (I) is different from 309 (II); 309 (II) is different from 378.99 (III), 397.45 (IV), 308 (V), and 394.45; (VI) and also 124 (VII), 125 (VIII), and 131 (IX) differs significantly from group 309 (II), 378.99 (III), 397.45 (IV), 308 (V), and 394.45 (VI). CON, control; DB, diabetic; MEL, melatonin; INS, insulin; GB, glibenclamide. * $p \leq 0.05$. ** $p \leq 0.01$. *** $p \leq 0.001$.

Materials and Methods

Chemicals and Reagents

Chemicals and reagents (analytical grade) were obtained from HiMedia limited, Mumbai, India, and Sisco Research limited, Mumbai, India. The source of insulin was human insulin (Actrapid, purchased from Novo Nordisk, Bagsværd, A/S-Denmark) prepared through DNA recombinant technology as mentioned in its manual.

Biochemical and ELISA Kits

Commercial biochemical kits for serum biochemistry and renal function tests (creatinine, urea, and uric acid) were purchased from ERBA Diagnostics, Germany. Kits for tumor necrosis factor (TNF)-α, tumor growth factor (TGF)-β1, interleukin (IL)-1β, IL-6, and IL-10 were purchased from Abcam, Cambridge, MA, USA.

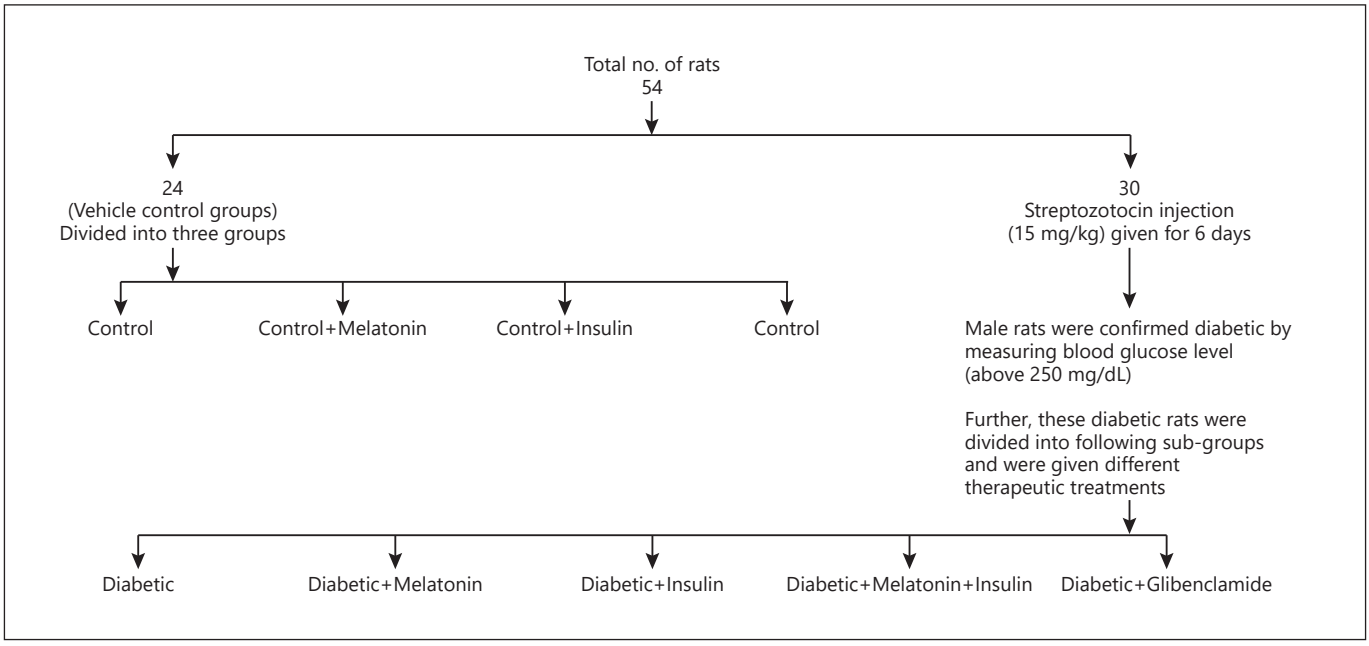


Fig. 1. Representation of the experimental design and allocation of rats into different experimental groups. CON, control; DB, diabetic; MEL, melatonin; INS, insulin; GB, glibenclamide; STZ, streptozotocin.

Animal Care

Male Wistar rats weighing 190 ± 10 g were obtained from the Defense Research and Development Establishment (DRDE) Gwalior, India. Rats were acclimatized for 2 weeks before the experiments under standard temperature, humidity, and light (12:12 h light and dark cycling with lights off at 6:00 p.m.) and with food and water ad libitum. Fifty-four rats were randomly divided into 9 groups of 6 rats each, as described in Table 1. STZ and melatonin (1 mg/kg) were administered i.p. at 10:30 a.m. and 6:30 p.m., respectively. Insulin (0.5 mL/kg or 20 units) was administered intramuscularly at 1:30 p.m. Glibenclamide (0.5 mg/kg) was given p.o. in drinking water (Table 1). STZ was acquired from HiMedia; melatonin was acquired from Sigma-Aldrich, Burlington, MA, USA; and insulin was purchased from Novo Nordisk, A/S-Denmark.

Induction and Confirmation of Diabetes

Rats were treated with an i.p. injection of STZ (15 mg/kg for 6 days) to induce a diabetic condition [14]. Blood glucose levels were monitored immediately before and at weekly intervals after the STZ injection. Animals with blood glucose levels above 250 mg/dL were considered diabetic (Table 2). The doses of melatonin, insulin, and glibenclamide employed were as described in the literature [20–22]. Rats were allocated into different experimental groups (Fig. 1).

Parameters Evaluated

At the end of the experiment (4 weeks), animals were euthanized under complete anesthesia (anesthetic ether). The renal cortex was fixed in Bouin's fluid for histological block preparation and sectioning. Renal cortex tissue was processed for lipid peroxidation (LPO), GSH, superoxide dismutase (SOD), catalase (CAT), and GSH cycle, that is, GPX, GR, and glucose-6-phosphate dehydrogenase (G6PDH). GSH-S-transferase (GST) activity was also measured. The blood was centrifuged, and the serum was stored at −20°C for analysis of renal function tests (creatinine, urea, and uric acid) and electrolytes (Na^+ and K^+).

Assessment of Oxidative Stress

The LPO level in the renal cortical samples was evaluated by the method of Ohkawa et al. [23]. The production of the pink-colored complex thiobarbituric acid reactive oxygen species between malonaldehyde and thiobarbituric acid was estimated. Reduced GSH was measured by its reaction with 5-5′-dithiobis(2-nitrobenzoic acid) (known as DTNB or Ellman's reagent) to generate a yellow-colored product [24]. SOD activity was evaluated after centrifugation of the whole reaction mixture to split into a butanol layer containing a chromogen, and absorbance was recorded at 560 nm [25]. CAT activity was assessed by measuring the rate of decomposition of hydrogen peroxide/min [26]. G6PDH and GPX activities were assessed following the method of Ells and Kirkman and Paglia and Valentine, respectively [27, 28]. GR activity was calculated in the renal cortical samples by following a reduction in absorbance at 340 nm due to the oxidation of NADPH [29]. GST was determined by a colorimetric method [30]. The glycogen content was measured in renal cortical samples by allowing the reaction mixture to cool and then boiled and again cooled, absorbance being recorded at 625 nm [31]. The total protein content in the renal cortical samples was estimated by the method of Lowry et al. [32].

Assessment of Serum Renal Biochemical Markers

The serum was used for the assessment of creatinine, urea, uric acid, and electrolytes (Na^+ and K^+) levels by using analytic kits according to the instructions provided by the manufacturer (ERBA diagnostics Mannheim GmbH, Mallaustr, Mannheim, Germany).

Table 3. Effect of exogenous melatonin and insulin alone or in combination on renal functional tests of control and diabetic rats

Group	Renal function tests, mg/dL			Electrolytes, nmol/L	
	creatinine	urea	uric acid	Na^+	K^+
I. CON	0.65±0.10 II***	22±2.14 II***	3.82±0.27 II***	117.67±3.49 II***	2.17±0.24 II***
II. D	1.82±0.43 III**, IV*, V***, VI*	41.84±2.90 III**, IV*, V***, VI*	5.92±0.15 III**, IV*, V***, VI*	137.16±3.29 III**, IV*, V***, VI*	4.95±0.26 III**, IV*, V***, VI*
III. D+MEL	0.8±0.15 V*, VII***, VIII*	26.34±3.06 II*, V*, VII***, VIII*	4.14±0.35 V*, VII***, VIII*	125.34±2.66 V*, VII***, VIII*	3.08±0.42 V*, VII***, VIII*
IV. D+INS	0.78±0.17 III*, V***, VI*	25.84±3 III*, V***, VI*	3.95±0.25 III*, V***, VI*	126.16±2.92 III*, V***, VI*	3.34±0.38 III*, V***, VI*
V. D+MEL+INS	0.64±0.10 III*, IV*, VI*	23.34±2.58 III*, IV*, VI*	3.89±0.32 III*, IV*, VI*	121.84±2.87 III*, IV*, VI*	2.87±0.28 III*, IV*, VI*
VI. D+GB	0.89±0.10 III*, IV*, V***	26.33±3.20 III*, IV*, V***	4.05±0.38 III*, IV*, V***	125.83±2.10 III*, IV*, V***	3.62±0.28 III*, IV*, V***
VII. CON+MEL	0.63±0.14 II***, III*, IV*, V**, VI*	22.33±1.54 III***, III*, IV*, V**, VI*	3.86±0.39 II***, III*, IV*, V**, VI*	118.84±2.14 III***, III*, IV*, V**, VI*	2.85±0.17 II***, III*, IV*, V**, VI*
VIII. CON+INS	0.51±0.16 III***, III*, IV* V**, VI*	22.66±1.49 II***, III*, IV* V**, VI*	3.6±0.47 II***, III*, IV* V**, VI*	118±2.23 II***, III*, IV* V**, VI*	3±0.34 II***, III*, IV* V**, VI*
IX. CON+GB	0.64±0.09 II***, III*, IV* V**, VI*	22.34±3.00 III***, III*, IV* V**, VI*	3.76±0.18 II***, III*, IV* V**, VI*	120±2.96 II***, III*, IV* V**, VI*	2.89±0.12 II***, III*, IV* V**, VI*

Shown are the means ± SEM ($n = 6$/group). ANOVA: numerals indicate the group which is significantly different, and asterisks designate the p value; for example, 0.65 (I) is different from 1.82 (II); 1.82 (II) is different from 0.8 (III), 0.78 (IV), 0.64 (V), and 0.63 (VI); and also 0.63 (VII), 0.51 (VIII), and 0.64 (IX) differ significantly from groups 1.82 (II), 0.8 (III), 0.78 (IV), 0.64 (V), and 0.63 (VI). CON, control; DB, diabetic; MEL, melatonin; INS, insulin; GB, glibenclamide. * $p \leq 0.05$. ** $p \leq 0.01$. *** $p \leq 0.001$.

Evaluation of Circulating Cytokines

Serum cytokines (TNF-α, TGF-β1, IL-1β, IL-6, and IL-10) were measured by using ELISA assay kits, according to the protocol provided by the manufacturer (Abcam, Cambridge, MA, USA).

Receptor Assay

RT-qPCR-iScriptTM First standard cDNA synthesis kit (Bio-Rad) RT-qPCR was done for the expression assay of melatonin receptors (MT1 and MT2) by first extracting the total mRNA, followed by cDNA synthesis. The primers were purchased from Imperial Life Sciences (P) Limited, Gurugram, India.

List of Primers

Gene product	Forward	Reverse
MT1	5′-CGTTGGTGCTGATGTCG-3′	5′-AGTTTGGGTTTGCGGTC-3′
MT2	5′-CAATGCTGCGAGGCG-3′	5′-GGCGGTGGTGACGATG-3′
β-Actin	5′-GGAAATAGGGGTTAGCAC-3′	5′-CTCATGTGCGCCTACTTA-3′

RNA Isolation

Total RNA was isolated from the frozen tissue samples. Tissue samples were slowly crushed with the help of a mortar and pestle. One milliliter of RiboZol (TRIzol) was added and homogenized for 5 min. The tissue samples were homogenized in RiboZol and transferred to a fresh microtube and left for 5 min at room temperature. The pellet was washed, air-dried, and dissolved in 50 μL of DEPC water, and 5 separate aliquots were prepared and kept at −80°C for further processing. cDNA synthesis was done by using Thermo kit K1632.

Histological Preparation

The kidney cortex was washed in normal saline and fixed in Bouin's fluid. Renal cortical samples of all experimental groups were dehydrated using different graded series of ethanol. Samples were cleared using xylene and embedded in paraffin wax. Renal cortical sections of 4–5 μm thickness were cut using a rotary microtome (Leica RM2125 RT 5), stained with hematoxylin and eosin, and observed under a light microscope (Magnus, Mumbai, India).

Statistical Analysis

Results were expressed as mean ± SEM. Comparisons between experimental groups were carried out by a one-way ANOVA followed by Tukey's multiple comparison tests. SPSS (IBM 20.0 version software) was used for calculations.

Results

As shown in Tables 2 and 3, the diabetic rats displayed a considerable increment in the glucose level in the blood along with biochemical indexes of renal injury, that is, augmented creatinine, urea, uric acid, and Na^+ and K^+ levels in the serum. In every case, melatonin or insulin administration partly counteracted the effects observed, while the coadministration of melatonin plus insulin was consistently more protective toward the diabetes-induced renal cortical injury than individual administration of melatonin or insulin. Treatment of melatonin or insulin given to the control rats did not reveal any major alteration in the serum variables examined (Tables 2, 3).

Assessment of the renal cortex for histopathology in the different experimental groups of rats is represented in Figure 1a, b and summarized in Table 4. The diabetic rats showed degeneration of the tubular structure, swallowing in glomeruli with loss of glomerular spaces, and distortion of the endothelial lining. This contrasted with the normal cellular glomeruli, regular and uniform glomerular space, preserved lining of the endothelium, well-structured Bowman's capsule, and proper tubular alignment seen in controls. The combined administration of melatonin and insulin to diabetic rats achieved ad integrum repair of renal histoarchitecture (Fig. 2a, b; Table 4). Diabetic rats showed a significant reduction in body weight. The combined treatment of exogenous melatonin and insulin given to the diabetic rats resulted in significant restoration in body weight (Fig. 3).

Glucose which remains unmetabolized gets oxidized during the diabetic condition and leads to free radical production as well as a subsequently increased rate of LPO in the renal cortex. Table 5 summarizes the impact of exogenously administered melatonin and insulin alone or co-treatment on thiobarbiturate reactive substances, reduced GSH, SOD, CAT, and protein content in the renal cortex of control as well as in diabetic rats. The coadministration of insulin and melatonin reduces diabetes-induced renal cortical oxidative stress bioindicators more effectively than either treatment alone (Table 5). Although the individual administration of melatonin or insulin did not affect the total protein or glycogen content, the co-treatment of melatonin and insulin showed a noteworthy increment in the total cellular protein and glycogen content nearly to the control values (Table 5; Fig. 4).

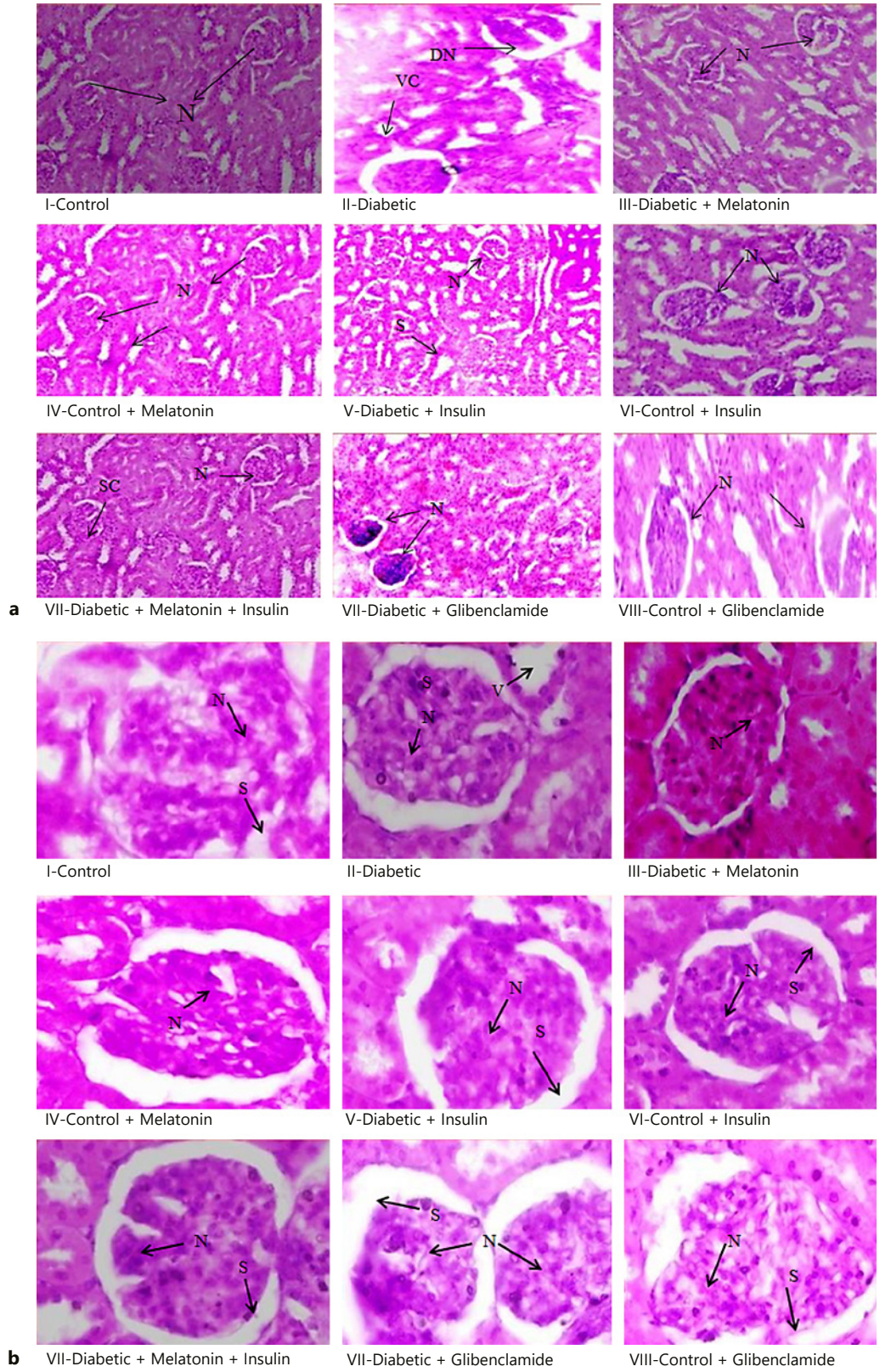
Table 4. Histopathological alterations (–) no damage, (+) slight damage, (++) moderate damage, and (+++) severe damage in the renal cortex of control and diabetic rats are shown

Histological remarks	I. CON	II. D	III. D+MEL	IV. D+INS	V. D+MEL+INS	VI. D+GB	VII. CON+MEL	VIII. CON+INS	IX. CON+GB
Regeneration of tubules	–	+++	–	+	–	–	–	–	–
Rate of glomerular damage	–	+++	++	++	–	+	–	–	–
Vacuolation	–	+++	–	++	–	–	–	–	–
Glomerular space status	–	+++	++	–	–	–	–	+	–

CON, control; DB, diabetic; MEL, melatonin; INS, insulin; GB, glibenclamide.

Fig. 2. a, **b** Histomicrograph of the kidney cortex showing the effect of melatonin and insulin alone or in combination. I. CON showing normal glomeruli and basement membrane, normal glomerular space, and normal tubular epithelium. II. D: STZ-induced diabetes caused thickening of the basement membrane, reduced glomerular space, glomerular damage, degeneration of tubular epithelium, and congestion of blood vessels. III. D+MEL treatment shows reduced thickening of the basement membrane and restored damage in glomeruli. IV. D+INS: insulin administration recovered cellular damage, maintained the normal cellularity of glomeruli, normal glomerular space, and normal blood vessels in STZ-treated rats. V. D+MEL+INS: combined administration of melatonin and insulin to diabetic rats demonstrated almost total restoration from toxic manifestations as shown by healthy Bowman's capsule, normal glomeruli, and tubules. VI. D+GB: a standard antidiabetic drug restored the STZ-induced cellular damage in glomeruli. VII. CON+MEL: melatonin treatment of the control maintained the normal cellularity of glomeruli, normal glomerular space, and normal blood vessels. VIII. CON+INS: insulin administration did not modify normal cellular architecture; magnification ×10 and ×40. IX. CON+GB: glibenclamide administration did not modify the normal architecture of the kidney; magnification ×10 and ×40. CON, control; DB, diabetic; MEL, melatonin, INS, insulin; GB, glibenclamide; STZ, streptozotocin.

(For figure see next page.)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III-Diabetic + Melatonin
IV-Control + Melatonin
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VI-Control + Insulin
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I-Control
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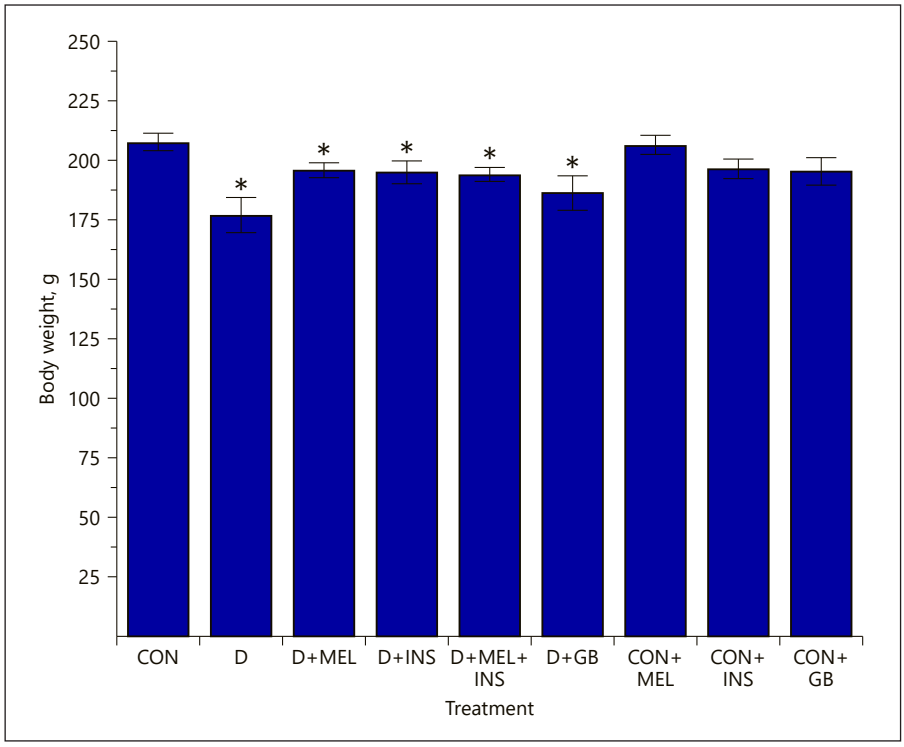

Fig. 3. Effect of exogenous melatonin and insulin on the body weight of STZ-induced diabetic rats. Histogram represents mean ± SE; $n = 6$; CON, control; DB, diabetic; MEL, melatonin, INS, insulin; GB, glibenclamide; STZ, streptozotocin. $^{**}p < 0.01$ and $^{***}p < 0.001$. Group I (CON) versus group II (D) group II (D) versus group III (D+MEL); group II (D) versus group IV (D+INS); group II (D) versus group V (D+MEL+INS) group II (D) versus group VI (D+GB).

Diabetic rats exhibited a significant inhibition/suppression of GPX, GR, G6PDH, and GST in the renal cortex (Table 6). Administration of melatonin and insulin (alone or in combination) showed ad integrum recovery in GSH-dependent antioxidative enzymatic activities.

Table 7 summarizes the impact of melatonin and insulin treatment on circulating serum cytokines in diabetic rats. A considerable elevation occurred in TNF-α, TGF-β1, IL-1β, and IL-6 blood levels in diabetic rats in comparison to the normal controls, whereas IL-10 levels decreased. Melatonin and insulin coadministration showed a significantly higher reduction in circulating TNF-α, TGF-β1, IL-1β, and IL-6 and a significantly higher augmentation in serum IL-10, as compared to each treatment alone (Table 7). Figure 3 depicts the expression of MT1 and MT2 melatonin receptor RNA in the kidney cortex. In diabetic rats, a considerable amplification in expression was observed for both receptor subtypes, and melatonin and insulin treatment alone or in combination counteracted the effect (Fig. 5). Treatment of control rats with insulin or glibenclamide maintained the expression of MT2 receptor. The finding of overexpression of MT1 following the insulin and glibenclamide treatment (CON+INS and CON+GB groups) suggests that because of the low circulating levels of melatonin, the melatonin receptor was overexpressed possibly to compensate for the low melatonin levels.

Discussion

The current study focused on the efficacy of the co-treatment with melatonin and insulin to improve deteriorated renal function in a rat model of type 1 diabetes mellitus. In agreement with previous studies [33], the diabetic condition induces nephrotoxicity, as shown by the significant increase of urea, uric acid, and creatinine in the serum. Hyperuricemia and creatinine are indices of renal dysfunction and more precise markers of kidney function than urea alone. In an organism, the fundamental end product of protein catabolism is urea, so that its rise indicates an abnormal protein glycation rate during diabetes. This is due to decreased muscles and the elevated release of purine because of the increased activity of xanthine oxidase [34].

Coadministration of melatonin and insulin restored the urea, uric acid, and creatinine levels to the control value. The therapeutic role of melatonin and insulin is probably based on the neutralization of free radicals, hence preventing renal cortical damage [35, 36]. Exogenous insulin normalizes the nitrogen level by metabolizing urea and restoring glucose metabolism and its subsequent conversion to glycogen.

Elevated levels of Na^+ and K^+ are observed in diabetic nephropathy [37–39]. Diabetic hyperkalemia reduces

Table 5. Effect of exogenous melatonin and insulin alone or in combination on TBARS reduced GSH, SOD, CAT, and protein content in the renal cortex of control and diabetic rats

Groups	TBARS, nmol/mg protein	GSH, μmoles/mg protein	SOD, units/min/mg protein	CAT, units/min/mg protein	Protein, mg/mg tissue
I. CON	43.6±3.04 II***	7.15±0.72 II***	43.09±2.18 II***	606.65±25.69 II***	33.05±0.74 II***
II. D	23.77±1.98 III**, IV*, V***, V*	3.82±0.65 III**, IV*, V***, V*	21.07±2.62 III**, IV*, V***, V*	350.88±30.55 III**, IV*, V***, V*	15.57±0.82 III**, IV*, V***, V*
III. D+MEL	38.34±1.92 IV*, V***, VI*	4.95±0.39 IV*, V***, VI*	38.81±1.39 IV*, V***, VI*	554.9±15.03 IV*, V***, VI*	30.14±1.16 IV*, V***, VI*
IV. D+INS	39.24±1.69 III**, V***, VI*	8.1±0.41 III**, V***, IV*	32.88±1.47 IIII**, V***, IV*	548.9±21.96 IIII**, V***, IV*	29.105±1.50 IIII**, V***,I V*
V. D+MEL+INS	40.65±176 III*, IV*, V***, VI*	7.05±0.96 III*, IV*, V***, VI*	43±1.76 III*, IV*, V***, VI*	598.9±28.10 III*, IV*, V***, VI*	31.67±0.55 III*, IV*, V***, VI*
VI. D+GB	30.87±1.97 IIII*, IV*, V***	5.0±0.58 IIII*, IV*, V***	36.97±2.93 IIII*, IV*, V***	499.97±15.19 IIII*, IV*, V***	28.59±0.79 IIII*, IV*, V***
VII. CON+MEL	42.97±1.89 II***, III*, IV*, IV**	6.69±0.94 II***, III*, IV*, V**	47.84±2.19 III***, III*, IV*, V**, V*	614.97±16.98 III***, III*, IV*, V**, V*	32.72±2.64 INS III***, III*, IV*, V**, V*
VIII. CON+INS	42.23±2.12 II***, III*, IV*, V**	6.9±0.75 II***, III*, IV*, V**, V*	43.19±2.12 II***, III*, IV*, V**, V*	598.33±29.32 II***, III*, IV*, V**, V*	32.86±3.16 II***, III*, IV*, V**, V*
IX. CON+GB	44.48±2.23 II***, III*, IV*, V**, V*	6.67±0.89 II***, III*, IV*, V**, V*	43.86±2.20 II***, III*, IV*, V**, V*	596.03±21.56 II***, III*, IV*, V**, V*	33.1±2.50 II***, III*, IV*, V**, V*

Shown are the means ± SEM (n = 6/group). ANOVA: numerals indicate the group which mean is significantly different, and asterisks designate the p value; for example, 43.6 (I) is different from 23.77 (II); 23.77 (II) is different from 38.34 (III), 39.24 (IV), 40.65 (V), and 30.87 (VI); and also 42.97 (VII), 42.23 (VIII), and 44.48 (IX) differ significantly from groups 23.77 (II), 38.34 (III), 39.24 (IV), 40.65 (V), and 30.87 (VI). GSH, glutathione; TBARS, thiobarbituric acid reactive oxygen species; SOD, superoxide dismutase; CAT, catalase; CON, control; DB, diabetic; MEL, melatonin; INS, insulin; GB, glibenclamide. * $p \leq 0.05$. ** $p \leq 0.01$. *** $p \leq 0.001$.

glomerular filtration, and hypernatremia and hyperosmolality are related to disruption of insulin-mediated glucose metabolism as well as glucagon-dependent glucose release [40]. The coadministration of insulin and melatonin significantly restored Na^+ and K^+ concentrations nearly to normal levels. Insulin activates the Na^+-K^+ ATPase pump and reduces the generation of free radicals [41].

A significant fall in the body weight and muscle protein content, presumably resulting from an elevation in the wasting of muscles and fat and protein catabolism, were found in diabetic rats. In the current study, the co-treatment of melatonin plus insulin was given to diabetic animals for 4 weeks and resulted in a complete restoration of the body weight and protein content, presumably by inhibiting lipolysis in adipose tissue [42, 43]. Uncontrolled glucose metabolism resulted in a severe decrease in body weight despite increased appetite, predominantly due to a decrease of muscles and fatty tissue because of excessive catabolism of proteins [44]. Tian et al. [45] reported that the glucose-lowering potential of jointly administered melatonin and insulin influenced the enhancement of activation of β-cell degranulation. Melatonin could have a stimulatory impact on the insulin-producing β-cells by causing their regeneration as well as preventing their degeneration, whereas insulin stimulates glucose storage into glycogen. Previous results supported are consistent with the findings of the current study which reported that melatonin enhances insulin secretion through melatonin-dependent IP3 release and may contribute to the short-term support of the IP3-releasing agent such as acetylcholine [46]. Furthermore, during the diabetic condition, glycogen synthetase activity is inhibited, affecting the glycogen storage and its synthesis in the hepatocytes and skeletal muscles [10]. Hence, deprivation of glycogen in the kidney cortex ensues. Diabetic rats receiving co-treatment of insulin as well as melatonin revealed a considerable elevation in quantity of

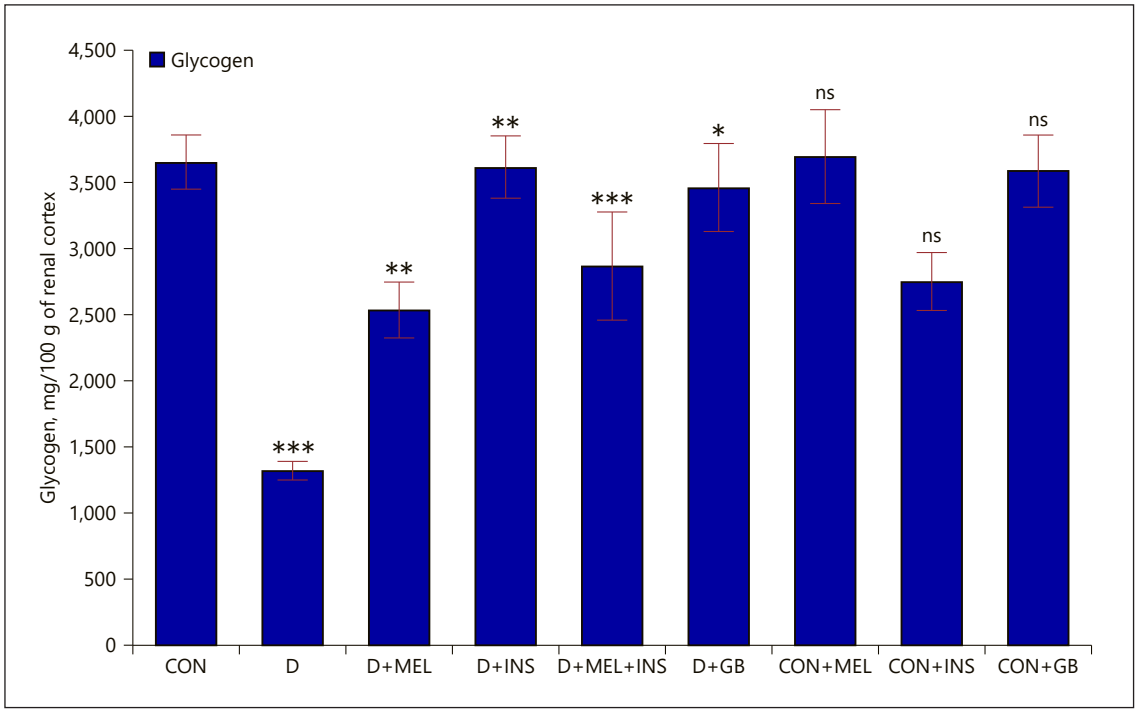

Fig. 4. Effect of exogenous melatonin and insulin on the glycogen content in the renal cortex in STZ-induced diabetic rats. Histogram represents mean ± SE; $n = 6$; CON, control; DB, diabetic; MEL, melatonin; INS, insulin; GB, glibenclamide; STZ, streptozotocin. $^{**}p < 0.01$ and $^{***}p < 0.001$. Group I (CON) versus group II (D) group II (D) versus group III (D+MEL) group II (D) versus group IV (D+INS) group II (D) versus group V (D+MEL+INS) group II (D) versus group VI (D+GB).

glycogen in the kidney cortex. The likely cause may be the normalization of the glycogen synthase by exogenous insulin.

In the current study, LPO was elevated considerably in diabetic rats; treatment of melatonin and insulin given to the diabetic rats induced a noteworthy decrease in LPO back to control values. The present findings agree with previous studies reporting that melatonin neutralizes free radicals, acting as a scavenger of the hydroxyl radicals as well as peroxyl radicals [47]. Melatonin is a lipophilic as well as a fairly hydrophobic molecule that can reach tissue and cellular compartments through a nonreceptor pathway [48]. Exogenous insulin might have reactivated glucose metabolism and hence caused the increased efficiency of melatonin. The results were comparable to those of the standard hypoglycemic drug glibenclamide as far as the restoration of LPO levels.

Diabetic rats revealed a considerable reduction in GSH, SOD, CAT, GSH cycle enzymes (GPX, G6PDH, and GR) and GST activities in the renal cortex, all the effects being counteracted by the coadministration of melatonin and insulin. These results agreed with earlier findings [6, 48, 49]. GST levels were restored by melatonin and might catalyze GSH conjugation reactions, leading to an alteration in the intracellular GSH level of the renal cortex and shields cells against free-radical-generating agents. Reduced GSH constitutes 98% of the GSH in the cells, whereas GSSG constitutes only 2% [48, 50–54]. Thus, the current data indicate that the conjoint treatment of melatonin and insulin stimulated the antioxidative enzymatic activities and overturned the entire distorted activities of enzymes.

Renal diseases entail vascular complications, loss of podocytes, and epithelial dysfunctions, which lead to pathogenesis via inflammation, cell hypertrophy, and dedifferentiation through the activation of classic pathways of regeneration [55]. Inflammatory cytokines are related to the initiation of renal complications such as fibrosis in diabetes. Excessive glucose elevates the expression of TNF-α, IL-6, and monocyte chemoattractant protein-1. In diabetic rats, inflammatory cytokines (TNF-α, IL-6, IL-1β, and TGF-β1) levels in the serum were elevated, while those of the anti-inflammatory IL-10 decreased, presumably because of the alteration in K^+ [56]. Elevation of serum TNF-α, TGF-β1, IL-1β, and IL-6 and the decrement of IL-10 in diabetic rats indicate an altered innate

Table 6. Effect of exogenous melatonin and insulin alone or in combination on GSH cycle enzymes (GR, GPX, and G6PDH) and GST in the renal cortex of control and diabetic rats

Groups	GSH cycle enzymes (unit/min/mg protein)			GST (unit/min/mg protein)
	GR	GPX	G6PDH	
I. CON	45.7±4.05 II**	9.14±0.72 II**	47.10±2.18 II**	610.73±25.69 II**
II. D	20.55±2.02 III*, IV*, V***, VI*	4.94±0.65 III*, IV*, V***, VI*	24.09±3.71 III*, IV*, V***, VI*	350.88±30.55 III*, IV*, V***, VI*
III. D+MEL	40.34±1.92 IV*, V***, VI*	6.95±0.39 IV*, V***, VI*	35.73±2.01 IV*, V***, VI*	554.9±53.54 IV*, V***, VI*
IV. D+INS	39.24±1.69 III*, V***, VI*	8.1±0.41 III*, V***, VI*	35.92±2.81 III*, V***, VI*	548.9±21.96 III*, V***, VI*
V. D+MEL+INS	43.86±176 III*, IV*, VI*	9.05±0.96 III*, IV*, VI*	46±1.65 III*, IV*, VI*	609.10±34.12 III*, IV*, VI*
VI. D+GB	34.87±1.97 III*, IV*, V***	9.0±0.58 III*, IV*, V***	38.92±1.99 III*, IV*, V***	520.76±18.21 III*, IV*, V***
VII. CON+MEL	44.81±2.04 II***, III*, IV*, V***, VI*	8.03±0.83 II***, III*, IV*, V***, VI*	46.94±3.02 II***, III*, IV*, V***, VI*	607.82±21.91 II***, III*, IV*, V***, VI*
VIII. CON+INS	42.23±2.12 II***, III*, IV*, V***, VI*	6.9±0.75 II***, III*, IV*, V***, VI*	44.32±3.21 II***, III*, IV*, V***, VI*	602.41±32.21 II***, III*, IV*, V***, VI*
IX. CON+GB	44.48±2.23 II***, III*, IV*, V***, VI*	6.67±0.89 II***, III*, IV*, V***, VI*	46.76±1.92 II***, III*, IV*, V***, VI*	607.04±31.67 II***, III*, IV*, V***, VI*

Shown are the means ± SEM (n = 6/group). ANOVA: numerals indicate the group which mean is significantly different, and asterisks designate the p value; for example, 45.7 (I) is different from 20.55 (II); 20.55 (II) is different from 40.34 (III), 39.24 (IV), 43.86 (V), and 34.87 (VI); and also 44.81 (VII), 42.23 (VIII), and 44.48 (IX) differ significantly from groups 20.55 (II), 40.34 (III), 39.24 (IV), 43.86 (V), and 34.87 (VI). CON, control; DB, diabetic; MEL, melatonin; INS, insulin; GB, glibenclamide; GSH, glutathione; GPX, glutathione peroxidase; GR, glutathione reductase; G6PDH, glucose-6-phosphate dehydrogenase; GST, glutathione-S-transferase. * $p \leq 0.05$. ** $p \leq 0.01$. *** $p \leq 0.001$.

immunity and chronic inflammation strongly associated with insulin resistance, as reported earlier [57]. The co-treatment of melatonin and insulin when given to diabetic animals showed considerably greater changes in all the cytokines examined in comparison to each treatment alone. It can be concluded that the combined treatment of a potent immune modulator like melatonin and of an immunocompetence enhancer like insulin normalizes the quantity of pro-inflammatory cytokines (TNF, TGF-β1, IL-1β, and IL-6) and improves the levels of IL-10 in circulation. Melatonin decreases the level of pro-inflammatory cytokines by reducing the free-radical-mediated damage; hence, it contributes to the reduction of pro-inflammatory cytokines and increases anti-inflammatory cytokines [58]. Moreover, the anti-inflammatory effect of insulin can be explained through the activation of cytokine expression because insulin affects the differentiation and survival of cells [59].

Chronic inflammation triggers the activation of intrinsic renal immune cells [60]. The augmented TGF-β1 levels due to inflammatory stimuli in diabetes lead to the elevated synthesis of collagen and its deposition as the extracellular matrix, resulting in glomerulosclerosis [61]. Experimental studies reported that melatonin is a master regulator of inflammation, regulating pro- and anti-inflammatory cytokines in different adverse physiological conditions [62–64]. Insulin, in addition to its role in reducing the detrimental effects of hyperglycemia, also directly regulates the production of pro- as well as anti-inflammatory cytokines and acts on the cells of the immune system to increase. Therefore, melatonin and insulin combined therapy may be an excellent approach to prevent chronic inflammation during diabetes and renal damage [65].

The fibrosis and congestion of blood vessels and deformations in Bowman's capsule are the main pathological

Table 7. Effect of exogenous melatonin and insulin alone or in combination on serum TNF-α, IL-6, IL-1β, TGF-β1, and IL-10 in control and diabetic rats

Serum cytokines, MIU/mL					
groups	TNF-α	IL-10	IL-6	IL-1β	TGF-β1
I. CON	68.07±2.30 II***	74.54±0.67 II***	21.7±0.03 II***	104.05±1.78 II***	100.7±2.60 II***
II. D	130.45±3.70 III***, IV*, V***, VI*	56.43±3.40 III***, IV*, V***, VI*	34.4±0.76 III***, IV*, V***, VI*	116.76±1.21 III***, IV*, V***, VI*	370.27±7.33 III***, IV*, V***, VI*
III. D+MEL	100.67±4.20 IV*, V***, VI*	68.23±0.53 IV*, V***, VI*	26.2±0.54 IV*, V***, VI*	109.63±1.34 IV*, V***, VI*	204.65±6.9 IV*, V***, VI*
IV. D+INS	113.02±1.9 III***, V***, VI*	67.67±0.42 III***, V***, VI*	25.1±0.56 III***, V***, VI*	107.65±1.98 III***, V***, VI*	240.7±7.40 III***, V***, VI*
V. D+MEL+INS	71.8±1.1 III***, IV*, VI*	73.56±0.72 III***, IV*, VI*	22.09±0.21 III***, IV*, VI*	107.54±1.34 III***, IV*, VI*	110.8±6.30 III***, IV*, VI*
VI. D+GB	115.05±1.00 III***, IV*, V***	65.99±0.82 III***, IV*, V***	27.8±0.34 III***, IV*, V***	111.45±1.27 III***, IV*, V***	150.4±7.90 III***, IV*, V***
VII. CON+MEL	67.09±2.10 II***, III*, IV*, V***, VI*	74.31±0.65 II***, III*, IV*, V***, VI*	22.2±0.21 II***, III*, IV*, V***, VI*	105.76±1.87 II***, III*, IV*, V***, VI*	102.5±5.80 II***, III*, IV*, V***, VI*
VIII. CON+INS	68.9±1.3 II*, III*, IV*, V**, VI*	66.51±0.54 II*, III*, IV*, V**, VI*	23.9±0.67 II*, III*, IV*, V**, VI*	103.91±1.45 II*, III*, IV*, V**, VI*	106.8±8.9 II*, III*, IV*, V**, VI*
IX. CON+GB	68.09±0.6 II***, III*, IV*, V***, VI*	73.01±0.65 II***, III*, IV*, V***, VI*	23.09±0.87 II***, III*, IV*, V***, VI*	105.68±1.67 II***, III*, IV*, V***, VI*	115.5±7.70 II***, III*, IV*, V***, VI*

Shown are the means ± SEM (n = 6/group). ANOVA: numerals indicate the group which mean is significantly different, and asterisks designate the p value; for example, 68.07 (I) is different from 130.45 (II); 130.45 (II) differs from 100.67 (III), 113 (IV), 115.05 (V), and 71.8 (VI); and also 67.09 (VII), 68.9 (VIII), and 68.09 (IX) differ significantly from groups 130.45 (II), 100.67 (III), 113 (IV), 115.05 (V), and 71.8 (VI). TNF-α, tumor necrosis factor-α; IL, interleukin; TGF-β1, tumor growth factor-β1; CON, control; DB, diabetic; MEL, melatonin; INS, insulin; GB, glibenclamide. * $p \leq 0.05$. ** $p \leq 0.01$. *** $p \leq 0.001$.

alterations induced in the kidney during diabetic conditions and were also observed in the present study as histopathological examination indicated an increased mesangial matrix in the glomerulus, basement membrane thickening, and interstitial fibrosis. The deteriorative nephrotoxic effect of diabetes can be monitored by the notable changes in the cellular architecture of the renal cortex. In the present study, degeneration of glomeruli, distinct tubular artifacts, permeation of interstitial mononuclear cells, fibrosis, congested blood vessels, and deformation of Bowman's capsule were seen in the kidney of diabetic rats. These findings agree with previous reports in the literature [66]. Coadministration of melatonin and insulin to the diabetic rats brought about an almost total restoration, from toxic morphological manifestations to healthy Bowman's capsule, normal glomeruli, and tubules. The free-radical-scavenging potential of melatonin makes it able to prevent the cellular damage in the kidney, and also, insulin lowers the cellular destruction by normalizing glucose metabolism and preventing auto-oxidation of glucose. Moreover, insulin also delays cellular damages [67, 68]. Melatonin is a potent antioxidant, partially due to its molecular structure, which is both lipophilic and hydrophilic. Therefore, it crosses all major barriers easily and is accumulated in higher amounts within subcellular organelles like mitochondria which are the main sites for reactive oxygen species production. Therefore, the administration of melatonin and insulin plays 2 major functions which are neutralization of free radicals and inhibition of oxidants, while insulin normalizes the glucose metabolism in the cells by converting it into glycogen. Hence, conversion of excess glucose into glycogen by insulin simultaneously with inhibition of oxidants by melatonin decreases the oxidative stress in cells. Both these activities prevent the histological damage in renal tissues.

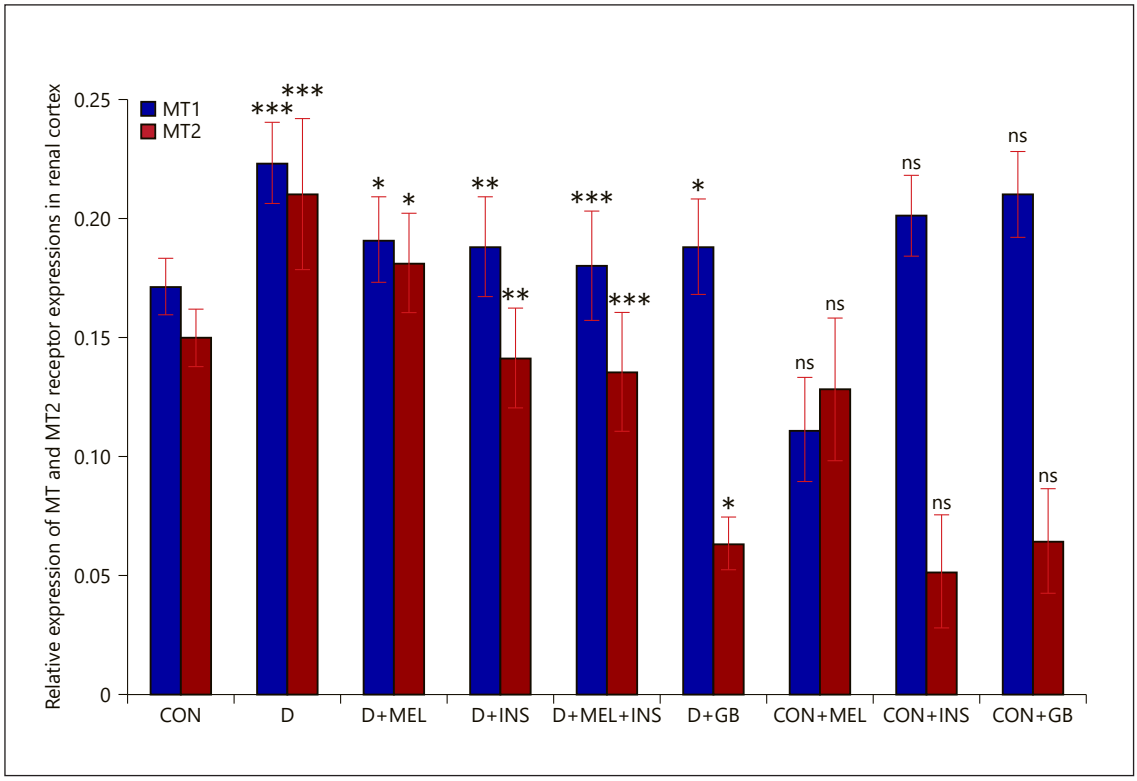

Fig. 5. Effect of exogenous and insulin alone and in combination on MT1 and MT2 relative receptor expression in the renal cortex of STZ-induced diabetic rats. Histogram represents mean + SE; $n = 6$; CON, control; DB, diabetic; MEL, melatonin; INS, insulin; GB, glibenclamide; STZ, streptozotocin. $^{**}p < 0.01$ and $^{***}p < 0.001$. Group I (CON) versus group II (D) group II (D) versus group III (D+MEL) group II (D) versus group IV (D+INS) group II (D) versus group V (D+MEL+INS) group II (D) versus group VI (D+GB).

Melatonin receptors (MT1 and MT2) are distributed in the peripheral tissues such as the liver and kidney [69–71]. During induction of the diabetic state, pathogenic changes in the expression of the melatonin receptors MT1 and MT2 were noted. The renal cortex of diabetic rats revealed considerable upregulation of those receptors, presumably because of the low circulating levels of melatonin occurring in diabetes [71–73]. Melatonin and insulin treatment alone or in combination resulted in a momentous reversal in the relative expression of MT1 as well as MT2 receptors in the renal cortex. Treatment of control rats with insulin or glibenclamide brought about a significant depression in MT2 receptor expression. Overexpression of MT1 following the insulin and glibenclamide treatment (CON+INS and CON+GB groups) treatment established that low circulating levels of melatonin produced overexpression of the melatonin receptor.

Summarizing, the results of the current study allow the conclusion that melatonin plus insulin might be an effective therapeutic combination to prevent diabetes-induced functional renal alterations, as demonstrated by changes in kidney histoarchitecture, renal cortex biochemistry (LPO, GSH, SOD, CAT, GPX, GR, G6PDH, GST, glycogen, and total cellular protein content), serum biochemical indicators of renal function (creatinine, urea, and uric acid), electrolytes (Na^+ and K^+), and serum concentration of pro- and anti-inflammatory cytokines (TNF, TGF-β1, IL-1β, IL-6, and IL-10), regulating the innate immune system. The favorably safe nature and nephroprotective property of melatonin and insulin suggest it to be a pharmacological adjunct to increase the therapeutic window of important nephrotoxic drugs in clinical trials. Mitigation of diabetes-induced renal damages by melatonin and insulin involves a complex series of biochemical improvements at cellular levels. Melatonin and insulin attenuate the nephrotoxicity of diabetes through their potent antioxidant and glucose metabolizing actions, respectively, as it is believed to reinforce the antioxidant enzymes and direct free-radical scavenging at subcellular levels. Apart

from its antioxidant actions, melatonin and insulin modulate various inflammatory cytokines that are also associated with its ability to restore histological and cell survival. Melatonin displays direct antioxidant and anti-inflammatory action without any interventions of melatonin receptors (MT1 and MT2), found on the kidney, in the correction of drug-induced nephrotoxicity. Therefore, investigation regarding melatonin receptors and insulin involved in the mitigation of diabetes-induced renal injury may further justify the use of melatonin for moderation of glucose metabolism, neutralization of free radicals and inflammatory cytokines, and stimulation of antioxidant enzymes.

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Statement of Ethics

All the experimental protocols were approved by the Animal Committee for the Control and Supervision of Experiments on Animals (CPCSEA) under the Institutional Animal Ethics Committee and were conducted in accordance with the guidelines of the Institutional Animal Ethics Committee (IAEC) at SLT Institute of Pharmaceutical Sciences, Guru Ghasidas Vishwavidyalaya, Bilaspur, Chhattisgarh, India (registration no.: 994/Go/ERe/S/06/CPCSEA), and within the framework of the revised Animals (Specific Procedure) Act of 2002 of Govt. of India on animal welfare. During the experimental procedure, all efforts were made to minimize the suffering of the animals.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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The authors have not received any funding for this study.

Author Contributions

Y.A.H., S.R., S.R.P.P., G.M.B., R.J.R., and D.P.C. contributed equally regarding the concept development and study design; data analysis, interpretation, and preparation; as well as critical revision of the manuscript. All the authors read and approved the final version of the manuscript.

Data Availability Statement

All data generated or analyzed during this study are included in this article. No supplementary file has been submitted along with the de-identified dataset used and/or analyzed during the current study.

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