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Protective effects of melatonin on AT1, ACE2, and Mas genes expression in induced diabetic aortic rats

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Abstract

Diabetes mellitus (DM) is considered a threat to the vascular tone. Melatonin (MEL) exhibits broad-spectrum effects across multiple organs and displays pleiotropic characteristics. The present study aims to investigate the vascular influence of MEL administration either in vivo or in vitro on isolated thoracic aortic Mas receptor (MasR), angiotensin type 1 receptor (AT_1R), and angiotensin-converting enzyme 2 (ACE_2) gene expressions of induced diabetic rats by streptozotocin (STZ). The thoracic aortae were isolated in order to investigate the influence of MEL on MasR, AT_1R, and ACE_2 gene expression by real-time PCR (BIO-RAD), and dose-response curve (DRC) was also measured for MEL, angiotensin 1-7 (Ang 1-7) reactivity with or without MasR blocker (A779), angiotensin 1-8 (Ang 1-8) reactivity with or without AT_1R blocker, valsartan (VAL) and ACE_2 inhibitor by ADInstrument organ bath, also the present study includes the pathohistological examination of thoracic aortae tissue. Three groups of male albino rats were divided randomly into non-diabetic rats (non-DM), STZ-induced diabetes (DM), and STZ-induced diabetes treated with MEL. The DM rat's aortae exhibited a slight decrease of AT_1R gene expression and a slight increase of both Maser and ACE_2 gene expression, while in STZ-induced DM treated with MEL these targeted genes were slightly restored. Melatonin has shown positive effects on the gene expression of ACE_2, AT_1R, and MasR and has influenced the reactivity of Ang 1-7 and Ang 1-8 in the aortas of diabetic rats through distinct mechanisms.

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Introduction

Diabetes mellitus is recognized for causing significant alterations in the intracellular metabolic processes of various tissues (1). Extensive investigations have revealed that hyperglycemia has a significant role in developing microvascular complications (2). Additionally, hyperglycemia is substantial evidence indicating a decline in endothelium-dependent vasodilation in cardiovascular conditions (3). Endothelial dysfunction is considered a pivotal factor in DM (4), and it has garnered increasing attention in examining vascular disorders (5). The control of vascular tone is carefully coordinated to maintain a balance between vasodilation and vasoconstriction. Traditionally, the exploration of vascular tone regulation has centered on mechanisms specific to each of the three distinct tunica layers constituting the aortic vessel wall, including the tunica intima (T.I.), tunica media (TM), and tunica adventitia (TA) (6). The TM comprises a single layer of vascular endothelial cells (VECs), acting as a barrier that separates the circulating blood from the underlying TM layer (7). Meanwhile, TM comprises concentric layers of vascular smooth muscle cells (VSMCs) and elastic lamellae, with the number of layers varying depending on the vessel's size (8). In recent years, there has been a resurgence of interest in the vascular renin-angiotensin system (RAS) (9). It is widely recognized that the activation of RAS holds significant importance in

controlling blood pressure and the onset and persistence of vascular endothelial dysfunction (VED) (10). Dysregulation of the RAS elements' gene expression is common in diabetes, as are changes in those elements' expression (11). Angiotensin 1-8 is responsible for controlling blood pressure, maintaining electrolyte balance, and participating in the inflammatory response within the vascular wall through both angiotensin type and angiotensin type 2 receptors known as AT_1R and AT_2R, respectively (12). Indeed, evidence supports those medications targeting the RAS, such as angiotensin-converting enzyme (ACE) inhibitors and AT_1R blockers, have been shown to enhance endothelial (13). Some studies have suggested that inflammation may downregulate ACE_2 expression, potentially contributing to cardiovascular complications in diabetes (14). The regulation of AT_1R gene expression in diabetes is a complex process influenced by various factors (15). In particular, hyperglycemia may lead to increased AT_1R expression and vasoconstriction (16). On the other hand, angiotensin1-7 (Ang 1-7) is a bioactive element of the RAS that exhibits modulation of the pressure effects of Ang 1-8 (17). In particular, Ang 1-7 exerts its vasodilatory effect via G-protein-coupled receptors (GPCRs) known as Mas receptors (MasR) (18). It has been suggested that hyperglycemia may upregulate MasR expression as part of a compensatory mechanism to counteract the effects of Ang 1-8 (19). Moreover, Ang 1-7 prompts vasorelaxation by stimulating the secretion of endothelium-derived substances, including nitric oxide (NO) and prostaglandin (20). It is now clear that both A.C.E. and ACE_2 elements are established as the main Ang 1-8 and Ang 1-7 forming enzymes, respectively (21). Melatonin or N-acetyl-5-methoxytryptamine is the hormone the pineal gland produces in vertebrates (22). However, MEL plays a vital role in regulating biological rhythms (23). Melatonin has been shown to improve VECs function, essential for maintaining healthy blood vessels via NO production (24). It has been suggested that activating vascular melatonin receptors (MT_1R and MT_2R) leads to a series of intracellular events that ultimately relax VSMCs (25). Besides, MT_2R in VECs maintains proper endothelial function (26). Multiple studies suggested that MEL has been recognized for its potent antioxidant properties and significantly protects vascular tissues from oxidative stress (27).

Given the identified gap in research, it is clear that there is a significant deficiency in comprehensive studies that thoroughly explore how MEL affects the expression and regulation of AT_1R, MasR, and ACE_2 within the RAS diabetic rat's aortae. Expanding on these findings, our primary goal was to investigate whether MEL could mitigate the progression of VED, impact targeted gene expression, and influence aortic reactivity through Ang 1-7 and Ang 1-8 in a situation where diabetes was induced using STZ injection.

Materials and methods

Laboratory animals

Male albino rats (*Rattus norvegicus*) weighing about 250-300 g was conducted. Animals were acclimatized at standard conditions, including 23 ± 2 °C on a 12 h/12 h light/dark cycle (06:00 - 18:00 light), regular diet, and free access to tap water. The Animal Research Ethic Committee confirmed that the current study belonged to the College of Science, Salahaddin University-Erbil, Erbil, Iraq (Reference number 4190 and Date of Issue: October 19, 2023).

Experimental design

The current study included three sets of experiments to investigate the influence of MEL on a vascular Ang 1-7 receptor (MasR), Ang 1-8 type1 receptor (AT_1R), and ACE_2 genes in isolated thoracic aorta in STZ-induced DM rats. In experiment I, 24 isolated thoracic aortas from 24 rats were used in the molecular study to investigate MasR, AT_1R, and ACE_2 gene expression of non-DM, STZ-induced DM, and STZ-induced DM treated with MEL. The experiment II included three sub-experiments. Sub-experiment I encompassed the DRC of MEL in non-DM (n=6) and STZ-induced DM (n=5). Sub-experiment II encompassed the DRC of Ang 1-7 in non-DM with Mas blocker (A777) (n=9) or without Mas blocker (A777) (n=16), the DRC of Ang 1-7 in STZ-induced-DM with Mas blocker (A777) (n=8) or without Mas blocker (A777) (n=11), and the DRC of Ang 1-7 in STZ-induced-DM treated with MEL, with Mas blocker (A777) (n=8) or without Mas blocker (A777) (n=13). Sub-experiment III encompassed the DRC of Ang 1-8 in non-DM with AT_1R blocker (VAL) (n=5) or without AT_1R blocker (VAL) (n=8), the DRC of Ang 1-8 in STZ-induced DM with AT_1R blocker (VAL) (n=9) and (VAL+MEL) (n=4) or without AT_1R blocker (VAL) and MEL (n=8), and the DRC of Ang 1-8 in non-DM with ACE_2 inhibitor (n=4) and (ACE_2 inhibitor +MEL) (n=6) or without AT_1R blocker (VAL) and ACE_2 inhibitor (n=8). Experiment III included the histological and morphometric changes of the thoracic aorta in non-DM (n=17), STZ-induced DM (n=13), and STZ-induced DM treated with MEL (n=19).

Chemicals

Angiotensin 1-8, Ang 1-7, ACE_2 inhibitor, A779 (MasR blocker), phenylephrine, Acetylcholine, and streptozotocin were acquired from Sigma Aldrich (USA). Valsartan (AT_1R blocker) was procured from Awamedica (Iraq), and MEL was obtained from MELAPLAN (PLANTE PHARMA, Poland). Ethanol, xylene, and paraffin wax were sourced from Scharlau (Spain).

Diabetes mellitus type-1 induction

Diabetes mellitus was induced as a model of vascular impairment. Streptozotocin was dissolved in a cooled sodium citrate buffer (pH 4.5), then 50 mg/kg/body weight

of STZ intraperitoneal (i.p.) was injected (28). After that, the rats were fed 5% Dextrose for 24 hours. The confirmed diabetic rat's condition was tested by a tail blood glucose measurement. The values represent diabetes when higher than 250 mg/dL about 48 h after STZ injection.

Melatonin dose preparation

Melatonin tablets, specifically MELAPLAN 10 mg (PLANTE PHARMA, Poland), were dissolved in sterilized distilled water containing 1% ethanol. This dissolution resulted in an MEL solution with an approximately 150 mg/ml concentration. Following the 14th day after inducing DM in the rats using STZ, the rats were treated. In this treatment, the MEL solution was administered orally through gavage at a daily dosage of 30 mg/kg B.wt for a continuous 14 executive days (29).

RNA isolation and quantitative real-time reverse transcriptase-PCR (qRT-PCR)

To evaluate the aortic AT_1R, ACE_2, and MasR gene expressions with dual replications of non-DM (n=8), STZ-induced DM (n=8), and STZ-induced DM treated with MEL (n=8). Total RNA was purified from isolated aorta using the PureLink™ RNA Mini kit (Invitrogen, Thermo Fisher Scientific Corporation, USA) and quantitated by OneDrop TOUCH Pro/Lite Micro-Volume Spectrophotometer (Biometrics Technologies, USA). The primer sequences (Oligomer biotechnology company, Turkey) used in the present study are shown in table 1. Preparation of cDNA was done using ONE-STEP protocol of 20 µL total volume including Master Mix (green one step low ROX), 5 µL; Forward Primer, 1 µL; Reverse Primer, 1 µL; R.T. mix, 1 µL; dH_2O, 9 µL; Template (RNA), 9 µL with one µg RNA RT-PCR analysis was performed for selected genes using SYBR Green gene Expression Assays (Canvax, Spain) with β-actin as the housekeeping control gene by BIO-RAD (CFX 96) real-time detection system (USA). The One-Step Probe RT-qPCR Kit condition started with reverse transcription for cDNA synthesis at 50°C for 10 minutes, then initial denaturation at 95 °C for 3 minutes, followed by 45 cycles of denaturation at 95°C for 10 s, and annealing/extension at 60°C for 45 s. Delta-CT values were calculated to compare levels of mRNA expression among studied groups. Expression values were normalized to β-actin levels using the formula: average ΔCT = average CT (a target gene) average CT (a reference gene).

Preparation of rat aortic rings

After anesthetization with ketamine-xylazine combination (90 mg/kg and 10 mg/kg; i.p.), the animals' chests were opened by modes of a midline incision in order to isolate the descending thoracic aorta from the aortic arches. Aortae were carefully enucleated and settled in a Petri dish immediately containing cooled Krebs-Henseleit buffer solution (KHBS, in mM): NaCl 122; KCl 4.7; $NaHCO_3$ 15.5; KH_2PO_4 1.2; $CaCl_2$ 2.0; D-glucose 11.5; pH 7.4.

Table 1: Primer sequences for SYBR green qPCR

Gene	Genes sequence (5′ to 3′)	bp
AT_1R-F	CCTACCGCCCTTCAGATAAC	20
AT_1R-R	TCCTCTGGCTTCTGCTGTCA	20
β-actin-F	GCCAACCGTGAAAAGATG	18
β-actin-R	CCAGGATAGAGCCACCAAT	19
ACE_2-F	TCAGAGCTGGGATGCAGAAA	20
ACE_2-R	GGCTCAGTCAGCATGGAGTTT	21
MasR-F	GACCAGCCCACAGTTACCAGTT	22
MasR-R	CCAGGGTTCCCCTTCTGACT	20

F, forward Primer; R, reverse Primer.

Vascular reactivity protocol

The prepared aortic rings were hung horizontally between both L-shaped stainless-steel hooks in a 10-ml organ bath vessel (Automatic organ bath, Panlab Harvard apparatus, (USA) filled with KHBS. The bath solution was maintained at 37 °C and aerated continuously with a mixture of about 95% O_2 and 5% CO_2. The aortic rings were exposed to basal tension of 2.0 g for 60 min. After that, the rings were gradually stretched with a depolarizing modified KHBS with 60 mM KCl and allowed to equilibrate for about 60 minutes, during which rings were repeatedly washed and equilibrated every 15 min. The tension was continuously readjusted until a maximum stable contraction was dominated. The endothelium integrity was assessed by the endothelium-dependent relaxation incubated with one µM acetylcholine (Ach) in 1 µM PE pre-contracted rings. At that point, the preparation was ready to evaluate the changes in the dose-response curve (DRC) of Ang 1-8 ($5x10^{-11}$ - $5x10^{-7}$ µM) induced aortic contraction for 30 mM KCL, Ang 1-7 ($5x10^{-12}$ - 10^{-6} µM) induced aortic vasodilation and MEL ($3x10^{-8}$ - 10^{-3} µM) induced aortic vasodilation.

Vascular reactivity of melatonin, Ang 1-7 and Ang 1-8

The DRC of MEL was generated in non-DM and STZ-induced DM-isolated aortic rings. The DRC of Ang 1-7 in non-DM, STZ-induced DM, and STZ-induced DM treated with MEL isolated aortic rings with or without MasR blocker (A779, one µM). The DRC of Ang 1-8 in non-DM, STZ-induced DM with or without AT_1R blocker (VAL, one µM) and ACE_2 inhibitor (1 µM) and MEL.

Microscopic examination

Following the last treatment period, the animals were humanely euthanized using ketamine-xylazine combination (90 mg/kg and 10 mg/kg; i.p.) anesthesia. The thoracic aorta was surgically extracted and prepared for further analysis. This preparation involved using a 10% formo-saline solution to fix the tissue. Preserved tissue samples from fixative solution were exposed to serial processes that began with

dehydration, clearing, and impregnation using a series of graded ethanol (Scharlau, Spain) in ascending concentrations and then immersed in xylene (Scharlau, Spain). Finally, tissues were embedded in paraffin wax (Scharlau, Spain) and cooled. The paraffin sections were cut by rotary microtome. After that, samples were stained with hematoxylin and eosin. Finally, photos were taken with a digital microscope, while S.M.C.s nuclei counting and tunica media thickness (TM) were measured double-blinded with image analysis software, explicitly using ImageJ software version 1.8.0.

Statistical analysis

The results of non-DM, STZ-induced DM rats isolated rings presented as the mean ± SEM of the maximum contraction (Emax), the negative logarithm of the molar concentration of MEL, Ang 1-7 and Ang 1-8 produced 50% of the maximum response, $LogIC_{50}$ (pD_2) and $LogEC_{50}$ (pD_2), were analyzed using Tow-way analysis of variance (ANOVA) followed by Sidak post hoc test for pairwise comparisons for all possible pairwise combinations of means, also One-way analysis of variance (ANOVA) followed by Dunnett-test was applied for MEL AUC, Emax and pD_2, genes expression, ACE_2, and histological parameters. Student's T-test (independent type) compared Emax, AUC, Emax, and pD_2 for each of MasR and VAL among the studied groups. P<0.05 was considered a significant level. The statistical package GraphPad Prism, version 8.0.2, was used.

Results

Effects of the melatonin on aortic AT_1R, MasR, and ACE_2 expressions in diabetic rats

The AT_1R mRNA (delta Ct; 3.224± 0.625) had slightly lower expression in the STZ-induced DM rat's aortae as compared to the non-DM group (delta Ct; 4.337± 0.343) (Figure 1A). In contrast, MEL treatment slightly increased AT_1R expression (delta Ct; 3.747± 0.803) compared to the STZ-induced DM group. On the other hand, the outcomes of studies involving the MasR gene expression, while exhibiting subtle variations (delta Ct; 4.773± 0.621) STZ-induced DM rats, have provided valuable insights into the regulatory mechanisms of the RAS as compared to non-DM group (delta Ct; 4.321± 0.399). Within diabetic rats treated with MEL context, subtle changes in MasR expression and activity have been observed (delta Ct; 4.386± 0.842) compared to diabetic rats (Figure 1B). Furthermore, the ACE_2, which mediates the regulatory role between AT_1R and MasR activation, has also demonstrated a slight increase in its function in STZ-induced DM rats (delta Ct; 4.342± 0.590), whereas MEL administration ameliorated diabetic consequences slightly (delta Ct; 3.946± 0.7363) upon ACE_2 activity (Figure 1).

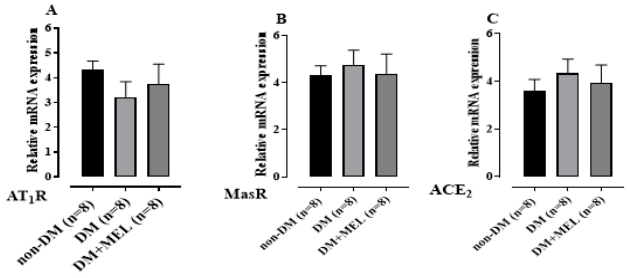

Figure 1: Alteration of aortic AT_1R, MasR, and ACE_2 protein expression in non-DM, STZ-induced DM, and STZ-induced DM treated with MEL male rats. Data were normalized using β-actin expression. The statistical significance of the differences in mean values between the three animal groups was assessed by one-way ANOVA, followed by a post-hoc Tukey test. The higher delta-Ct value represents the lower expression of a gene at the mRNA level.

Vascular effects of melatonin on diabetic aortic segments

Melatonin DRC exhibited a significant (P<0.05 and P<0.01) increase of maximal contractility at respect doses $1x10^{-4}$, $3x10^{-4}$, and $1x10^{-3}$ as compared to the non-DM group (Figure 2). As a result, the AUC value was increased significantly (P<0.01) in the STZ-induced DM group as compared to the non-DM group (Figure 2). In light of these developments, Emax and potency decreased slightly compared to the non-DM group (Figure 2).

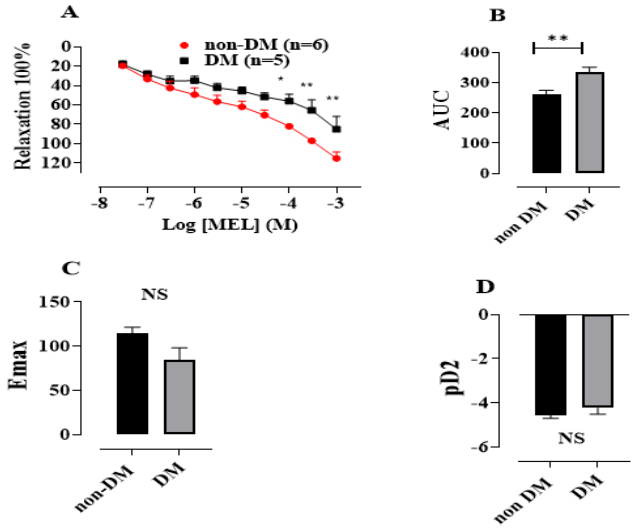

Figure 2: Concentration-response curve of MEL in aortic rings pre-contracted with PE (1 µM). Points represent mean ± SEM of aortic rings from total sample size using two-way ANOVA and T-test. (non-DM, non-diabetes mellitus rats; DM, Streptozotocin induced-diabetes mellitus rats; pD_2, the potency of melatonin; AUC, area under curve; Emax, maximum response of melatonin; NS, non-significant differences, MEL; melatonin). [*, P<0.05; **, P<0.01].

Vascular effects of melatonin on Ang 1-7/MasR signaling pathway in diabetic aortic segments

A diabetic condition caused a significant decrease ($P<0.0001$) in vasodilation induced by Ang1-7 DRC at all doses as compared to a non-DM group, and the MEL reversed the effect of diabetes-treated rats in the same manner (Figure 3). Furthermore, the impact of blocking MasR using A779 was investigated, revealing a moderate enhancement in the impaired Ang 1-7/MasR signaling pathway within the context of non-DM rats compared to the control group (Figure 3). On the other hand, in STZ-induced DM rats, the severity of MasR blockading was also observed slightly (Figure 3). By contrast, the diabetic rats treated with MEL showed significant increase ($P<0.05$) at dosage about $1x10^{-7}$-$1x10^{-6}$ of Ang 1-7 vasodilatory effect pre-treated with A779 (Figure 3).

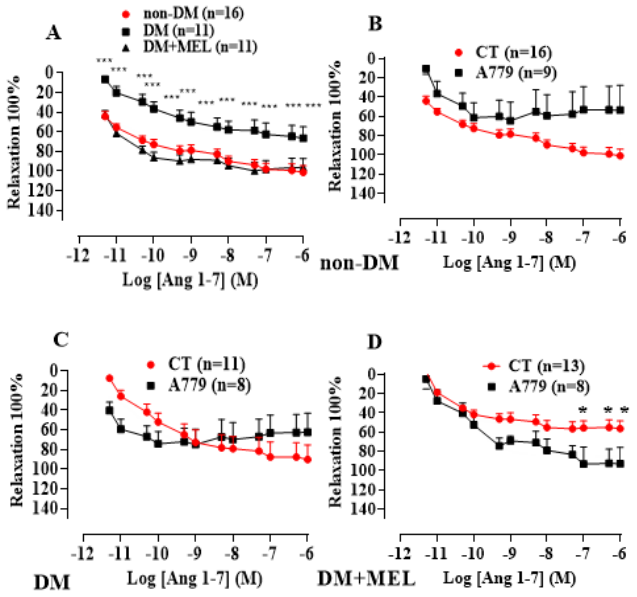

Figure 3: Concentration-response curve of Ang 1-7 in rats' aortic rings pre-contracted with PE (1 µM) in the presence of A779 (1 µM). Points represent mean ± SEM of aortic rings from total sample size using two-way ANOVA. (CT, control; A779, Mas receptor blocker; non-DM, non-diabetes mellitus rats; DM, Streptozotocin induced-diabetes mellitus rats; DM+MEL, Streptozotocin induced-diabetes mellitus in rats treated with melatonin) [*, $P<0.05$; ****, $P<0.0001$].

Vascular effects of melatonin on Ang 1-8/AT_1R/ACE_2 signaling pathway in diabetic aortic segments

To investigate the role of MEL on the AT_1R/ACE_2 signaling pathway, Ang 1-8 DRC was generated to induce vasoconstriction in diabetic aortic segments. The maximal response of Ang 1-8 was increased significantly ($P<0.05$, $P<0.0001$, and $P<0.01$) in diabetic aortic segments at doses of $5x10^{-9}$- $1x10^{-7}$ as compared to the non-DM group (Figure 4). Likewise, MEL treatment significantly elevated the maximal response of Ang 1-8 level in DM group (Figure 4). On the other hand, the blockade about AT_1R by VAL in non-DM rats showed a dramatic ($P<0.0001$) rightward shift of Ang 1-8 at doses about $5x10^{-9}$- $5x10^{-7}$ compared with control. Similarly, the addition of MEL to the vessel solution appeared to fail VAL abolishment (Figure 4). Besides, the diabetic condition and VAL pre-incubation have also improved the complete rightward shift of Ang 1-8 significantly ($P<0.01$, $P<0.0001$) at doses about $5x10^{-8}$-$5x10^{-7}$ as compared to control (Figure 4). In addition, the pre-incubation of MEL remained unaffected in the same manner. The maximum response to Ang 1-8 after ACE_2 slightly increased in aortic rings from STZ-induced DM compared to control (Figure 4). Additionally, the pre-incubation of ACE_2 and MEL combination caused a significant increment ($P<0.001$, $P<0.0001$) in the Ang 1-7 maximum response value at doses about $5x10^{-8}$- $5x10^{-7}$ compared to the control group (Figure 4).

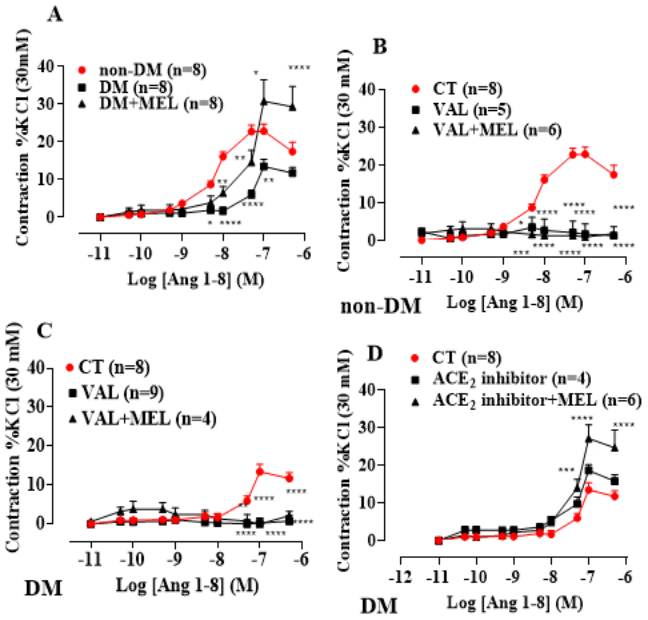

Figure 4: Dose-response curve of Ang 1-8 in rats aortic rings pre-contracted with PE (1 µM) in the presence of VAL (1 µM) and ACE_2 inhibitor (1 µM). Results (mean±SEM) are expressed as a percentage of the response to 30 mmol/l KCl and were analyzed by two-way ANOVA. (CT, control; ACE_2, angiotensin-converting enzyme 2; VAL, AT_1 blocker; non-DM, non-diabetes mellitus rats; DM, Streptozotocin induced-diabetes mellitus rats) [*, $P<0.05$; **, $P<0.01$ ***, $P<0.001$; ****, $P<0.0001$].

Vascular effects of melatonin on Mas receptor AUC, pD_2, and Emax levels in diabetic aortic segments

Under MasR blocking with A779 pre-incubation, both AUC and Emax of Ang 1-7 were slightly lower in non-DM

and STZ-induced DM rat aortic rings than in the control. Besides, in STZ-induced DM treated with MEL, the AUC decreased significantly (P<0.05) as compared to the control, while the Emax level was increased significantly (P<0.05) as compared with the control. In contrast, the potency of Ang 1-7 under MasR blocking increased significantly (P<0.0001) compared to the control. Moreover, this parameter remained unchanged in STZ-induced DM treated with MEL compared to the control (Table 2).

Vascular effects of melatonin on AT1R AUC, pD_2, and Emax levels in diabetic aortic segments

Under AT_1R blocking and VAL pre-incubation, the AUC of Ang 1-8 produced a significant (P<0.001, P<0.05) decrease in all studied groups compared to the control. Similarly, the maximum response (Emax) level of Ang 1-8 was also decreased significantly (P<0.0001) in all studied groups as compared to the control. In contrast, the potency of Ang 1-8 decreased slightly compared to the control (Table 3).

Vascular effects of melatonin on ACE2, AUC, pD_2, and Emax levels in diabetic aortic segments

Table 4 shows the effect of MEL on ACE_2 activity in diabetic isolated aortae. The pre-incubation of ACE_2 inhibitor, the AUC of Ang 1-8 DRC, showed no significant changes in STZ-induced DM rats' aortic rings compared to the non-DM group. Likewise, a non-significant difference was observed in STZ-induced treated with MEL compared to non-DM and DM, respectively. Surprisingly, under the diabetic condition, the Emax value increased significantly (P<0.05) compared to the non-DM group. Furthermore, the combined diabetes induction and MEL administration produced a significant elevation of Emax significantly (P<0.01, P<0.001) as compared to DM and non-DM, respectively (Figure 4).

Table 2: Effects of melatonin on Mas receptor *via* Ang 1-7 activity in diabetic rat's aortae

Parameters	Groups	non-DM	DM	DM+MEL
AUC	CT	287.4±29.36	416.5±50.3	291.4±26.97
	A779	244.4±72.61	388.4±53.25	173.5±33.6*
Emax	CT	101.1±6.609	90.08±14.78	43.47±7.957
	A779	87.71±15.69	77.65±19.22	93.01±16.89*
pD_2	CT	-10.02±0.1689	-10.09±0.274	-10.87±0.2957
	A779	-15.69±0.2689****	-16.25±0.374****	-10.14±0.244

Values in the table are means±SEM Data were statistically analyzed using an independent T-test to compare the A779 value in each row versus control in the same column. [non-DM, non-diabetic rats; DM, streptozotocin induced diabetes; DM+MEL, diabetic rats treated with melatonin; CT, control; A779, Mas receptor blocker; AUC, area under curve of Ang 1-7; Emax, maximum response of Ang 1-7; pD_2, the potency of Ang 1-7; *P<0.05; ****P<0.0001].

Table 3: Effects of melatonin on AT_1 receptor *via* Ang 1-8 activity in diabetic rats aortae

Parameters	Groups	non-DM	DM	DM+MEL
AUC	CT	44.69±3.845	17.58±3.653	41.7±9.606
	VAL	8.613±5.637***	2.217±3.215*	9.851±3.36*
Emax	CT	21.88±0.9576	14.17±1.497	34.2±3.733
	VAL	2.144±0.9913****	0.334±0.6295****	0.8687±0.998****
pD_2	CT	-8.305±0.1047	-7.35±0.173	-7.368±0.1791
	VAL	-9.268±3.959	-8.179±0.452	-8.162±1.151

Values in the table are means±SEM Data were statistically analyzed using an independent T-test to compare the VAL value in each row versus control in the same column. [non-DM, non-diabetic rats; DM, streptozotocin induced diabetes; DM+MEL, diabetic rats treated with melatonin; CT, control; VAL, AT_1R blocker; AUC, area under curve of Ang 1-8; Emax, maximum response of Ang 1-8; pD_2, the potency of Ang 1-8; *P<0.05; ***P<0.001; ****P<0.0001].

Histological and morphometric effect of melatonin on diabetic rats' aortae

The obtained qualitative analyses of the non-DM group showed the normal histological texture, including flattened nuclei (blue arrowhead), tunica media with smooth muscles having single oval nuclei (green arrowhead), and parallel distribution of elastic fibers lamellae (yellow arrowhead), and packed, wavy connective tissue in tunica adventitia fiber (Figure 5). In contrast, the diabetic rat's aortae texture showed detrimental degeneration, including irregular tunica intima with protruding nuclei toward the lumen (blue arrowhead), irregular nuclei position smooth muscle muscles of tunica media (green arrowhead), and dysregulation area with thin elastic fibers lamellae (yellow arrowhead), and

separated connective tissue in tunica adventitia with interrupted nuclei (black arrowhead) as shown in figure 5. Similarly, the morphometric analyses also demonstrated a significant (P<0.05) decline in both aortic tunica media wall thickness and smooth muscle cells nuclei count (P<0.01) compared to the non-DM group (Table 5). On the other hand, the diabetic rats were treated with MEL demonstrated a dramatic decrease in diabetes consequences, which includes restored nuclei position (blue arrowhead), increased nuclei of tunica media (green arrowhead), and decreased dysregulation area with elastic fibers lamellae (yellow arrowhead), and less interrupted nuclei (black arrowhead) of connective tissue in tunica adventitia (Figure 5). Relatively, the tunica media wall thickness and smooth muscle cells nuclei count were recovered dramatically (P<0.05) compared to those in non-DM and STZ-induced DM groups, respectively (Table 5).

Table 4: Effects of melatonin on ACE_2 activity *via* Ang 1-8 in diabetic rats aortae

Parameters	Multiple comparisons	non-DM (A)	DM (B)	DM+MEL (C)
AUC	N.S.	21.50±5.768	29.59±2.750	35.85±6.102
Emax	A vs B * A vs C *** B vs C **	9.974±1.650	18.46±1.593	29.12±2.749
pD_2	N.S.	-8.022±0.404	-7.497±0.166	-7.414±0.158

Values in the table are means±SEM Data were statistically analyzed using one-way ANOVA to compare mean values in each row. [non-DM, non-diabetic rats; DM, streptozotocin induced diabetes; DM+MEL, diabetic rats treated with melatonin; CT, control; NS, non-significant; AUC, area under curve of Ang 1-8; Emax, maximum response of Ang 1-8; pD_2, the potency of Ang 1-8; *P<0.05; **P<0.01; ***P<0.001].

Table 5: Effects of melatonin on tunica media layer thickness and smooth muscle cells nuclei

	Non-DM (n=17) (A)	DM (n=13) (B)	DM+MEL (n=19) (C)	Multiple
Tunica media layer thickness (µM)	68.08±2.214	57.89±1.834	66.90±2.834	A vs. B * B vs C *
Smooth muscle cells' nuclei count	133.7±16.23	77.38 ±10.82	89.25±7.036*	A vs. B ** A vs. C *

Values in the table are means±SEM Data were statistically analyzed using one-way ANOVA to compare mean values in each row. [non-DM, non-diabetic rats; DM, streptozotocin induced diabetes; DM+MEL, diabetic rats treated with melatonin. [*P<0.05; **P<0.01].

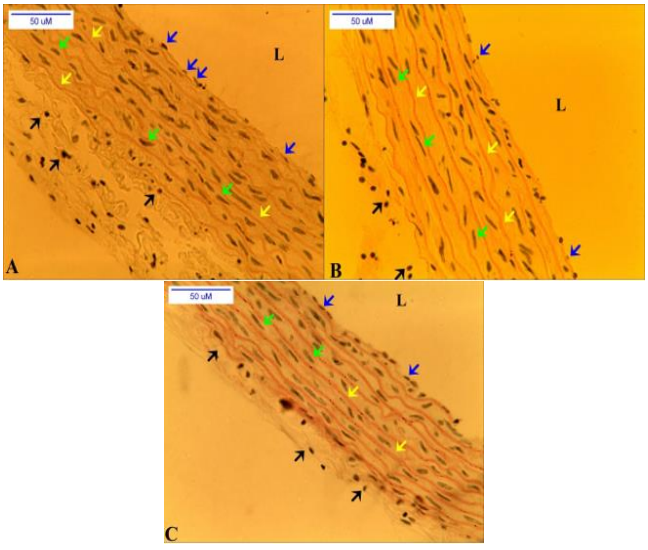

Figure 5: (A) the histological section of the thoracic aorta of the non-DM group showing the typical texture of tunica intima cells having flattened nuclei (blue arrowhead), tunica media with smooth muscles having single oval nuclei (green arrowhead) and parallel distribution of elastic fibers lamellae (yellow arrowhead), and packed, wavy connective tissue in the tunica adventitia. (B) the histological section of the thoracic aorta of the DM group shows irregular tunica intima cells having protruding nuclei toward the lumen (blue arrowhead), tunica media with smooth muscles having fewer nuclei and irregular nuclei position (green arrowhead), and dysregulation area with thin elastic fibers lamellae (yellow arrowhead), and separated connective tissue in tunica adventitia with interrupted nuclei (black arrowhead). (C) the histological section of the thoracic aorta of DM treated with melatonin group showed the regular tunica intima cells having normal nuclei position (blue arrowhead), tunica media with smooth muscles having increased nuclei and regular nuclei position (green arrowhead) and decreased dysregulation area with standard elastic fibers lamellae (yellow arrowhead), and packed connective tissue in tunica adventitia with less interrupted nuclei (black arrowhead). [non-DM, non-diabetes mellitus; DM, STZ-induced diabetes mellitus; L, lumen (H. and E., x400)].

Discussion

The outcomes of the present experimental investigation reveal that diabetes induced by STZ. Results in harmful complications, which include upregulation of AT_1R, a potent receptor for Ang 1-8, subsequently leading to vasoconstriction (Figure 1 A). It has been indicated that an elevated glucose concentration enhances Ang 1-8 mediated contractions through the involvement of AT_1R, with ROS partially contributing to the amplified contraction (30). In particular, it was evident that rats with diabetes displayed increased mRNA and protein expression of AT_1R, with no impact on the expression of the AT_2R receptor, which remained consistent (31). Under MEL treatment, the AT_1R was slightly elevated. Melatonin has been shown to have vasodilatory effects, which means it can relax and widen blood vessels (32). This effect could potentially counteract the vasoconstrictive effects of Ang 1-8 on AT_1R (33). Melatonin's ability to promote vasodilation might help lower blood pressure and improve vascular function (34). The critical discrepancy about the MEL effect arises because MEL DRC provided valuable outcomes in DM rats (35). Nonetheless, when MEL pre-incubated, it did not eliminate the Ang 1-8 DRC in the diabetes model, as shown in Figure 4 A. This lack of effect might be linked to the dysregulation of MEL receptors in both VECs and VSMCs within the aorta (36). These findings highlight the possibility that multiple mechanisms or factors are at play in developing diabetic endothelial dysfunction. This complexity may explain why our results, which involve the long-term administration of MEL, are a novel addition to this field of study.

Our current research has revealed improved gene expressions of MasR and ACE_2 in diabetic rats. This prompts us to re-evaluate the functional importance of these target genes. It is well known that ACE_2 and MasR are interconnected in the RAS (37), with ACE_2 playing a pivotal role in generating Ang 1-7 endogenously, activating MasR (14). This interaction contributes to regulating blood pressure and vascular tone (38). Regarding the ACE_2/Ang-7/MasR axis, ACE_2 maintains the RAS inversely mostly by reducing the Ang 1-8 to the Ang 1-7 (39). In particular, several factors contribute to diabetes complications upon gene expression, including damaging D.N.A., altering transcription factors through direct ROS action (40), and both inflammatory mediators and inflammatory cytokines, such as tumor necrosis factor-alpha (TNF-alpha) and interleukin-6 (IL-6), can impact the expression of these genes (41). On the other hand, our *in vitro* observation of our results indicated that a pattern of reciprocal changes in ACE_2 within diabetic and Ang 1-8 DRC suggests the possibility of reciprocal inhibition of protein expression between such DRC with or without ACE_2 inhibitor. Regarding the gene MasR expression, the *in vitro* results of Ang 1-7 DRC under A779 also support that expression level in DM rats. Previous studies have revealed that rats with DM prefer the harmful Ang 1-8 metabolite while exhibiting a reduced inclination for the beneficial Ang1-7 metabolite (42). The extant results of MEL administration and diabetes condition demonstrate a restored vascular tone upon ACE_2 and MasR activity. It is widely recognized that MEL employs various mechanisms to combat diabetic complications at different levels (43). Previous experiments have validated that MEL enhances the functioning of glucose-6-phosphate dehydrogenase (G6PD) in red blood cells, followed by NADPH augmentation and enhancing glutathione synthesis (43). Our findings also verified that MEL interventions contributed to restoring these genes within the vascular system. This was further confirmed by the vascular effects of Ang 1-7, as depicted in Figure 3 D, and Ang 1-8, as shown in Figure 4 D. Several research investigations have proposed that the impairment of VED resulting from a combination of ACE_2 deficiency is closely linked to oxidative stress (44). Furthermore, multiple research studies have demonstrated that the interaction between Ang 1-7 and the MasR triggers a range of beneficial cardiovascular responses, including NO production (45). On the contrary, additional research suggests that Ang 1-7 might operate *via* the AT_2R, and the MasR can counteract the effects of the AT_1R (46). Correspondingly, MEL functions to counteract oxidative free radicals, producing metabolites (47).

Recent results have also unveiled the underlying mechanisms responsible for the diabetes-related effects on aortic components. These effects manifest as a reduction in the thickness of the tunica media layer and a decrease in the number of nuclei in smooth muscle cells. The altered metabolic condition linked to diabetes leads to various changes in the arterial system, and the resulting vascular dysfunction could serve as a pathological connection between diabetes and cardiovascular risk (48). From a pathological perspective, one such change may involve a decrease in the thickness of the tunica media in specific arteries (49). The chronic hyperglycemia associated with type 1 diabetes can led to structural alterations and may include advanced glycation end products (AGEs) production and generation of oxidative stress (50).

Conversely, the administration of MEL in parallel with diabetes has demonstrated the ability to ameliorate these effects through various mechanisms. Studies have indicated that MEL plays a substantial role in repairing and rejuvenating the vascular tunica media (32). More specifically, MEL possesses potent antioxidant properties that play a crucial role in safeguarding the thoracic aorta tissue against the detrimental effects of oxidative stress and the damage induced by free radicals (51). Additionally, MEL has a unique combination of hydrophilic and lipophilic characteristics that allows it to traverse various morphophysiological barriers quickly, facilitating its rapid distribution throughout organs and bodily fluids (52). Recent research suggested that exposing cells to MEL could enhance its antioxidant properties (53).

Conclusions

The critical role of MEL in the cardiovascular system is mainly associated with its positive influence on glucose metabolism and its effect on vascular tone. Most research findings support the notion that MEL has advantageous effects on diabetic outcomes, either at the genetic level or in terms of physiological consequences. The administration of MEL during diabetic conditions modulates the RAS elements in distinct ways. The vascular improvement of MEL serves as a preventive measure of AT_1R, MasR, and ACE_2 gene expression against those complications of diabetic exposure. The findings not only shed light on potential pathways involved in vascular tone regulation but also emphasize the modifiable effects of MEL. Furthermore, in an *in vitro* diabetic aorta model, MEL also demonstrated a reduction in its effects. Melatonin may modulate vascular responses and enhance vasorelaxation in diabetic conditions, mainly when the MasR is blocked. Besides, our findings suggest that MEL significantly impacts Ang 1-8 vasoconstriction in diabetic rats, particularly in cases where ACE_2 is blocked. Notably, this effect occurs without any discernible influence on blocked AT_1R activity. The administration of MEL further attenuated the degeneration of pathohistological manifestations. This emphasizes MEL potential protective and therapeutic effects of MEL, suggesting that it may have a role in mitigating pathological changes and improving tissue health.

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Conflicts of interest

All the authors declare that there are no conflicts of interest regarding the publication of this manuscript.

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تأثيرات الوقائية للميلاتونين على التعبير الجيني لمستقبلات الأنجيوتينسين ١-٨ من النوع الأول، ماس والأنزيم المحول من النوع الثاني للأنجيوتينسين في الأوعية الدموية للفئران المجهزّة بالسكري المستحث

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الخلاصة

يعتبر داء السكري تهديدا لنغمة الأوعية الدموية، بينما يظهر الميلاتونين تأثيرات واسعة الطيف عبر أعضاء متعددة ويعرض خصائص متعددة الاتجاهات. تهدف الدراسة الحالية إلى الكشف في التأثير الوعائي للميلاتونين إما في الجسم الحي أو في المختبر على التعبير الجيني المتضمن لمستقبلات الأنجيوتينسين ١-٨ من النوع الأول، ماس والأنزيم المحوّل من النوع الثاني للأنجيوتينسين في الشريان الأورطي الصدري المعزول للفئران المصابة بالسكري المستحدثة عن طريق الستربتوزوتوسين. تم عزل الشريان الأورطي الصدري من أجل التحقيق في تأثير الميلاتونين على مستقبلات الأنجيوتينسين ١-٨ من النوع الأول، ماس و الأنزيم المحوّل من النوع الثاني للأنجيوتينسين بواسطة تفاعل البوليميرات المتسلسل بالزمن الحقيقي، كما تم قياس منحنى الاستجابة للجرعة للميلاتونين، والأنجيوتنسين ١-٧ مع أو بدون حاصر مستقبل ماس والأنجيوتنسين ١-٨ مع أو بدون حاصر مستقبل الأنجيوتينسين ١-٨ من النوع الأول (فالسرتان) ومثبط الأنزيم المحوّل من النوع الثاني للأنجيوتينسين بواسطة جهاز حمام الأعضاء، كما تشمل الدراسة الحالية الفحص المرضي لأنسجة الشريان الأورطي الصدري. تم تقسيم ذكور الفئران البيضاء بشكل عشوائي الى ثلاث مجموعات، المجموعة الأولى شملت فئران غير مصابة بالسكري، والمجموعة الثانية شملت فئران مصابة بالسكري الناجم عن الستربتوزوتوسين، والمجموعة الثالثة شملت فئران مصابة بالسكري الناجم عن الستربتوزوتوسين م المعاملة بالميلاتونين. أظهر الشريان الأورطي للمجموعة المصابة بالسكري انخفاضا طفيفا في التعبير الجيني في مستقبل الأنجيوتينسين ١-٨ من النوع الأول وزيادة طفيفة في كل من ماس والأنزيم المحوّل من النوع الثاني للأنجيوتينسين، بينما أظهرت مجموعة السكري المعاملة بالميلاتونين تمكين استعادة هذه الجينات المستهدفة بشكل قليل. أظهر الميلاتونين آثارا إيجابية على التعبير الجيني وتفاعل الأنجيوتينسين ١-٧ والأنجيوتنسين ١-٨ في الشريان الأورطي الصدري للفئران المصابة بالسكري من خلال آليات متميزة.