Role of Melatonin in Attenuation of Vascular Ang 1–7 Reactivity *via* Oxidative Stress Enzymes and PI_3K/AKT/eNOS Signalling Pathways in Induced Diabetic Rats

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Abstract. *Diabetes mellitus (DM) is considered as the main complication of the cardiovascular system leading to vascular endothelial dysfunction (VED). Besides, melatonin (MEL) has been known to improve the vascular tone directly or indirectly with MEL receptors (MT_1R and MT_2R) and antioxidant properties, respectively. The rings were extracted from three groups including non-diabetes (non-DM), streptozotocin induced diabetes (STZ-induced DM) and STZ-induced DM treated with MEL (DM+MEL) in male albino rats. The experimental procedure includes thoracic aortic vascular reactivity of angiotensin 1–7 (Ang 1–7) and histological examination. The vascular reactivity was conducted across eight distinct groups, encompassing RO-31-8220 (5 μM), protein kinase C (PKC) inhibitor, Apocyanin [(APO, 10 micromolar (μM)], the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase inhibitor, rotenone (ROT 50 μM), mitochondrial complex I electron transport chain inhibitor, oxypurinol (OXY, 100 μM), xanthine oxidase inhibitor, PI-3065 (1 μM), phosphoinositide 3-kinases (PI_3K) inhibitor, Ipatasertib (1 μM), protein kinase B (AKT) inhibitor, A779 (1 μM), the Mas receptor blocker and N(ω)-nitro-L-arginine methyl ester (L-NAME 200 μM), the nitric oxide (NO) inhibitor pre-incubation. However, it is worth noting that the pre-incubation with OXY resulted in a notably significant rightward shift in this response. Conversely, in the STZ-induced DM group, there was a notable significant rightward shift observed in response to each of APO, ROT, and OXY. MEL appeared to regulate the vascular tone within Ang 1–7 modulation in STZ-induced DM rats. Therefore, MEL could offer many vascular benefits within Ang 1–7 under diabetic condition.*

Introduction

The presence of DM is associated with adverse cardiovascular implications (Aboalgasm et al., 2021). It has been demonstrated that cardiovascular complexities represent a major mortality factor under DM consequences (Beckman and Creager, 2016). Moreover, DM interferes with the protective mechanisms of the cardiovascular system, resulting in a compromised defence system (Chien et al., 2020). Furthermore, the compromised state can lead to dysfunction in the vascular endothelial cells (VECs), marked by a decrease in NO levels and an increase in vasoconstrictors (Sena et al., 2013). Moreover, there is substantial evidence supporting the notion that DM contributes to an elevated production of reactive oxygen species (ROS) in diverse body components, such as VECs, vascular smooth muscle cells (VSMCs), and even within the mitochondria (Tan et al., 2022). Moreover, the elevated glucose levels can serve as a stimulus for the emission of ROS from various origins, including the mitochondrial electron transport chain, NADPH oxidases, xanthine oxidase, and arachidonic acid pathways. Consequently, this may result in the disruption of nitric oxide synthases (eNOS) coupling under such circumstances (Kayama et al., 2015).

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Regarding the build-up of ROS, several studies have indicated that an increased presence of these molecules seems to diminish the availability of NO, potentially leading to intracellular inflammation and apoptosis (Paneni et al., 2013). The renin-angiotensin system (RAS) serves a crucial regulatory function in preserving blood pressure and electrolyte balance within the body (Paz Ocaranza et al., 2020). Specifically, the RAS activity increases with the presence of pathological conditions (Forrester et al., 2018). Initially, renin catalyses the cleavage of angiotensinogen to produce angiotensin 1–10 (Ang 1–10); then, Ang 1–10 is further converted into angiotensin 1–8 (Ang 1–8) through the involvement of angiotensin-converting enzyme (ACE) (Fountain et al., 2023). At the same time, angiotensin-converting enzyme 2 (ACE_2) can cleave both of Ang 1–8 and Ang 1–10 to produce Ang 1–7 and angiotensin 1–9 (Ang 1–9), respectively (Bosso et al., 2020). In recent years, the Ang 1–7 / ACE_2 / MasR axis has been recognised as a critical signalling pathway that neutralizes the vasoconstricting action of the renin / ACE / ANG 1–8 pathway (Tamanna et al., 2021). In addition, the activation of MasR by Ang 1–7 action mostly appears to improve vascular endothelial function *via* reducing ROS production, enhancing endothelial nitric oxide synthase enzyme (eNOS) activity and attenuating NADPH oxidase (Xie et al., 2022). Furthermore, located within cells, PI_3K triggers

signalling cascades that regulate cellular survival, growth, mobility, differentiation, and the modulation of genetic information, thus playing a central role in the progression and development of atherosclerosis (Zhao et al., 2021). Differently, MEL is primarily synthesized by the pineal gland (Karamitri and Jockers, 2019). Many extensive researches have been conducted to explore its significant effects on both the vascular tone and endothelial function (Tobeiha et al., 2022). Particularly, MEL exerts signals directly *via* both of MT_1R and MT_2R in the thoracic aorta (Molcan et al., 2021). The activation of these receptors in VECs is associated with increased NO production as well as vasorelaxation and improved endothelial function (Nikolaev et al., 2021). Correspondingly, MEL could play a role in maintaining proper vascular dilation and regulating blood flow (Ozkalayci et al., 2021). Regarding vasodilation, MT_2R in the VSMCs of the thoracic aorta has been implicated in controlling vascular contractility and inhibiting vasoconstriction, potentially influencing overall vascular tone and blood pressure regulation (Datta et al., 2021). Furthermore, MEL has free radical scavenging, antioxidant, and anti-inflammatory properties, along with a protective effect against cardiovascular disease (Chitimus et al., 2020). Conversely, it has been demonstrated that MEL can increase the presence of the MasR in cardiovascular tissues (Lissoni et al., 2021). Nevertheless, there is an on-going debate regarding the cumulative evidence of MEL's impact on the modulation of Ang 1–7 through oxidative stress enzymes and MasR signalling pathways. The current research seeks to examine how MEL affects the vascular response of the aorta to vasodilation induced by Ang 1–7, both in the presence and absence of RO-31-8220, APO, ROT, OXY, PI-3065, Ipatasertib, A779 and L-NAME pre-incubation in STZ-induced DM rats and non-DM rats.

Materials and Methods

Chemicals

Angiotensin 1–7, RO-31-8220, APO, ROT, OXY, PI-3065, Ipatasertib, A779, L-NAME and STZ were purchased from Sigma Aldrich company (USA).

Animals

The recent research utilized male albino rats (*Rattus norvegicus*) with a body weight (B. wt) ranging from 250 to 300 grams. These rats were bred and housed in the animal house of the Department of Biology at the College of Science, Salahaddin University-Erbil, Iraq. They were allowed to acclimate to standard environmental conditions, which included a temperature of 23 ± 2°C and a 12-hour light / 12-hour dark cycle (with lights on from 06:00 to 18:00). The rats had continuous access to both tap water and food, which was available 24 hours a day. Ethical approval for the study was obtained from the Animal Research Ethics Committee associated with the College of Science at Salahaddin University-Erbil, Erbil, Iraq. This approval, with the reference number 2636, was granted on August 7, 2022.

The present study involved 74 rats randomly and divided into three main groups: non-DM with 34 rats, STZ-induced DM with 18 rats, and STZ-induced DM treated with MEL with 22 rats. The study was commenced on May 6, 2021, and concluded on July 12, 2022. The research was conducted over approximately three distinct phases. The first phase spanned three months and involved the care of rats. Subsequently, diabetes was induced using STZ, and MEL injections were administered daily. The second phase included the assessment of vascular reactivity to Ang 1–7. Finally, the third phase, which lasted about one month, was dedicated to the histological examination.

Diabetes mellitus type 1 induction

To induce diabetes in the rats, about 40 male albino rats received an intraperitoneal (i.p.) injection of 50 mg/kg B.wt of STZ (Mostafavinia et al., 2016). The STZ was dissolved in sodium citrate buffer with a pH of 4.5. After the STZ injection, the rats were given access to a drinking solution containing 5% dextrose for a period of 24 hours. The presence of diabetes was confirmed after 72 hours by assessing the rats' blood glucose levels through the tail blood sample. Diabetes was considered established when the blood glucose levels exceeded 250 mg/dL, and this measurement was made 72 hours after the 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 a MEL solution with a concentration of approximately 150 mg/mL. Following 14 days after inducing DM in the rats using STZ, the rats were subjected to treatment. In this treatment, the administration of the MEL solution was carried out orally through gavage at a daily dosage of 30 mg/kg B.wt for a continuous 14 executive days.

Preparation of rat aortic rings

Following anaesthesia with an i.p. injection of combined 90 mg/kg B.wt ketamine and 10 mg/kg B.wt xylazine, the chests of the animals were opened using a midline incision. This procedure aimed to isolate the thoracic aorta from the aortic arches. A total of 294 aortic rings were prepared from 40 rats of non-DM, SZT-induced DM and STZ-induced DM treated with MEL groups. These rings were carefully removed and immediately placed in a Petri dish filled with cold Krebs-Henseleit buffer solution (KHBS) containing 122 mM NaCl, 4.7 mM KCl, 15.5 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 2.0 mM $CaCl_2$, and 11.5 mM D-glucose., with a pH of 7.4. The excess surrounding tissues were removed, and then, four aortic rings, each approximately 3 mm in length, were obtained for further experimentation.

Vascular reactivity measurements

To evaluate vascular reactivity, the previously

prepared aortic rings were horizontally suspended using L-shaped stainless-steel hooks within a 5 mL organ bath vessel (Automatic organ bath, Panlab Harvard apparatus, USA) filled with KHBS. The bath solution was kept at a constant temperature of 37°C and continuously oxygenated with a gas mixture of approximately 95% oxygen and 5% carbon dioxide. The aortic rings were initially subjected to a basal tension of 2 gm for duration of 60 minutes. Subsequently, the rings were gradually stretched using KHBS and allowed to equilibrate for approximately 60 minutes. During this equilibration period, the rings were periodically washed and adjusted every 15 minutes to maintain a maximum stable constriction. To assess the functional integrity of the prepared aortic segments, a solution containing 60 mM KCl was employed. Following this, the endothelial integrity was evaluated by exposing the aortic rings to a solution of 1 µM acetylcholine in rings that had been pre-contracted with 1 µM phenylephrine (PE). Once these assessments were completed, such prepared rings were ready for the evaluation of the changes in the dose response curve (DRC) of Ang 1–7 induced aortic dilation, allowing for the measurement of how Ang 1–7 affected the dilation of the aortic rings.

Experimental design

The current study included nine experiments (experiments I to VIII), collectively addressing vascular reactivity, involving the assessment of DRC triggered by Ang 1–7 across a concentration range from $5x10^{\wedge-12}$ to $10^{\wedge-6}$ µM. These experiments were conducted in non-DM rats, STZ-induced DM rats, and rats with STZ-induced DM treated with 300 mg/kg body weight of MEL. Each vascular reactivity experiment (I to VIII) included pre-incubation for 20 minutes with specific blockers or inhibitors or without blockers or inhibitors (control group) as following: Experiment I (vascular reactivity): RO-31-8220 (5 µM), a PKC inhibitor; Experiment II (vascular reactivity): APO (10 µM), a NADPH oxidase inhibitor; Experiment III (vascular reactivity): ROT (50 µM), a mitochondrial complex I electron transport chain inhibitor; Experiment IV (vascular reactivity): OXY (100 µM), a xanthine oxidase inhibitor; Experiment V (vascular reactivity): PI-3065 (1 µM), a PI3K inhibitor; Experiment VI (vascular reactivity): Ipatasertib (1 µM), an AKT inhibitor; Experiment VII (vascular reactivity): A779 (1 µM), a MasR blocker; and Experiment VIII (vascular reactivity): L-NAME (200 µM), an NO inhibitor. Meanwhile, experiment IX focused on histological examination of thoracic aortae of non-DM (n = 17), STZ-induced DM (n = 13), and STZ-induced DM treated with 300 mg/kg B. wt of MEL (n = 19). The isolated tissues were fixed in a 10% buffered formo-saline solution and subsequently embedded in paraffin for quantitative histological analysis. Hematoxylin-eosin (H and E) staining was employed for microscopic analysis, which included the counting of smooth muscle cells (SMCs) nuclei and measurement of tunica media thickness (TM). The analysis was performed in a double-blinded manner using ImageJ software version 1.8.0.

Statistical analysis

The study reported various parameters, including maximal effect (Emax), drug potency (pD_2, expressed as $-logIC_{50}$), dAUC% (difference area under the curve percentage), TM thickness, and SMCs nuclei count. These values are present as means and standard errors of the mean (SEM) to distinguish the impact of inhibitors and blockers on vascular responses to Ang 1–7 in aortic segments from non-DM, STZ-induced DM, and STZ-induced DM treated with MEL. The data were analysed using one-way analysis of variance (ANOVA) to compare TM thickness, SMCs nuclei count, Emax, and pD_2 among the studied groups, and the results were displayed as figures and tables. Additionally, two-way ANOVA was employed to compare the groups using DRC, followed by the Dunnett post hoc test. Furthermore, the Student *t* test was employed to compare pD_2 values of the groups against the control group, and this comparison was illustrated in figures. Finally, the level of $P < 0.05$ was employed as the criterion for establishing the statistical significance level in the study.

Results

Effect of melatonin on the vasodilatory response to Ang 1–7 via PKC activity

The isolated aortic rings were pre-incubated by RO-31-8220 (PKC inhibitor) revealing a non-significant difference of a vasodilatory response triggered by Ang 1–7 in the non-DM group as compared with the control group (Figure 1A). On the other hand, the aortic rings of STZ-induced DM rats showed a slight alteration (Figure 1B). Conversely, in diabetic rats treated with MEL, the response was slightly changed, while a significant declination in potency ($P = 0.0067$) occurred as compared with the control rats (Figure 1C). Likewise, the level of dAUC% in STZ-induced DM rats treated with MEL exerted a significant decrease ($P < 0.05$) as compared to the STZ-induced DM rats (Fig. 1D).

Effect of melatonin on the vasodilatory response to Ang 1–7 via NADPH oxidase activity

The aortic rings pre-incubated with APO (NADPH oxidase inhibitor) lead to non-significant changes of the Ang 1–7 effect in non-DM rats. However, the potency was decreased significantly ($P = 0.0003$) as compared with the control (Figure 2A). Conversely, in the STZ-induced DM, the rings showed a significant rightward shift in the vasodilatory response induced by Ang 1–7 at mentioned doses, but the potency remained unchanged as compared with the control group (Figure 2B). On the other hand, in STZ-induced DM treated with MEL, the vasodilatory response induced by Ang 1–7 showed a significant decrease ($P < 0.05$) at the 10^{-6} dose,

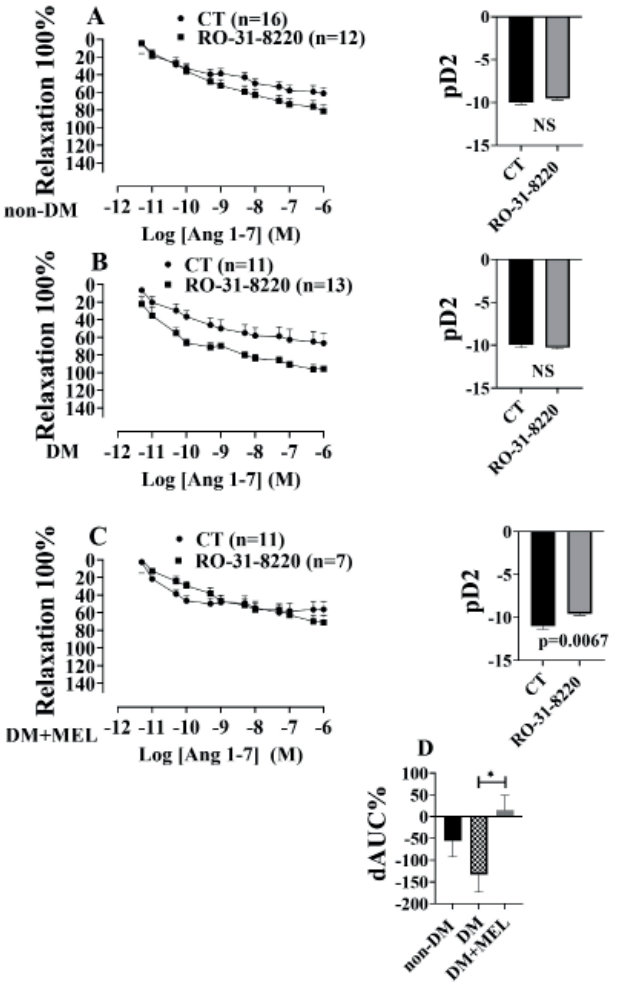

*Fig.1 A*ng 1–7 DRC of aortic rings pre-contracted with PE (1 µM) in the presence of RO-31-8220 (5 µM). The data points in the study are represented as the mean values ± SEM. (CT, control; non-DM, non-diabetes mellitus rats; DM, Streptozotocin induced-diabetes mellitus rats; DM+MEL, Streptozotocin induced-diabetes mellitus in rats treated with melatonin; pD_2, the potency of Ang 1–7; dAUC %, the percentage of difference area under curve; n, sample size). [* $P < 0.05$].

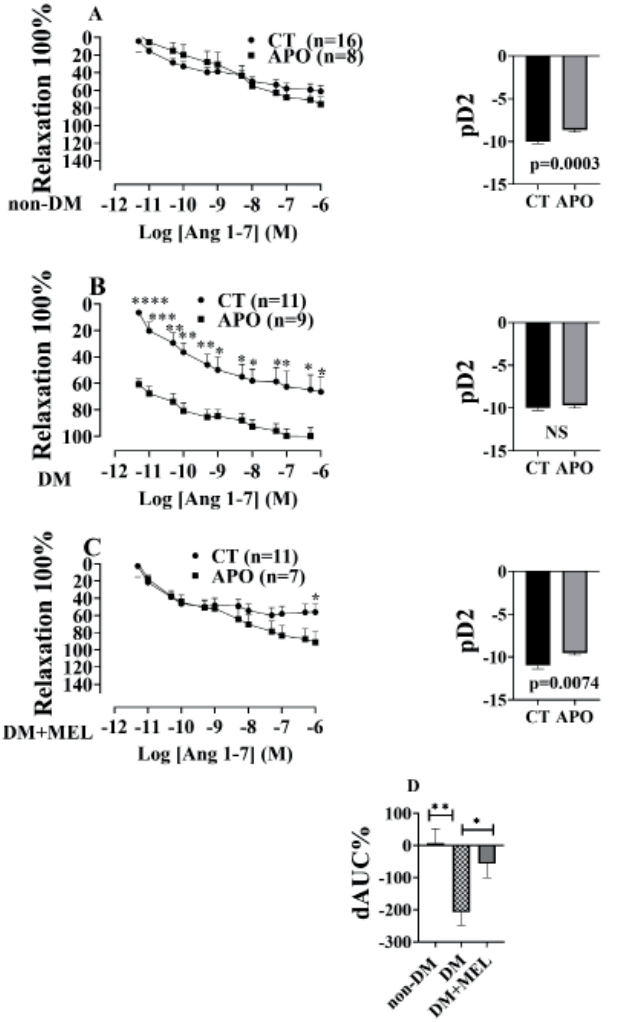

Fig. 2. Ang 1–7 DRC in aortic rings pre-contracted with PE (1 µM) in the presence of APO (10 µM). The data points in the study are represented as the mean values ± SEM. (CT, control; APO, apocyanine; non-DM, non-diabetes mellitus rats; DM, Streptozotocin induced-diabetes mellitus rats; DM+MEL, Streptozotocin induced-diabetes mellitus in rats treated with melatonin; pD2, the potency of Ang 1–7; dAUC%, the percentage of difference area under curve). [*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$].

followed by a significant ($P = 0.0067$) decrease in the potency of Ang1–7 as compared with the control (Figure 2C). Additionally, the dAUC% of such an inhibitor was increased significantly ($P < 0.01$) in the STZ-induced DM group as compared with the non-DM group. However, the dAUC% was declined significantly ($P < 0.05$) in the diabetic group that was administered with MEL as compared with the STZ-induced DM group (Figure 2D).

Effect of melatonin on the vasodilatory response to Ang 1–7 via mitochondrial activity

To investigate whether the complex I electron transport chain of mitochondria has roles in the vascular response to Ang 1–7, ROT was pre-incubated with the aortic rings. In the non-DM group, there were no significant differences observed in the vasodilatory response induced by Ang 1–7, and Ang 1–7 potency remained unchanged (Figure 3A). While diabetes rings exhibited a notable reduction to Ang 1–7 activity, there were no statistically significant alterations in Ang 1–7 potency (Figure 3B). Nonetheless, when isolated rings were subjected to MEL treatment, the Ang 1–7 impact was alleviated through significant ($P = 0.0137$) enhancement in the effectiveness of Ang 1–7 as compared with the control group (Figure 3C). Furthermore, the pre-incubation with ROT caused a significant ($P < 0.05$) increase in dAUC% in the STZ-induced DM group as compared with the control rats. Conversely, under MEL influence, the dAUC% was restored in a significant ($P < 0.01$) fashion as compared with the STZ-induced DM (Figure 3D).

Effect of melatonin on the vasodilatory response to Ang 1–7 via the xanthine oxidase activity

To assess the impact of xanthine oxidase on the vascular response to Ang 1–7, OXY (xanthine oxidase inhibitor) was pre-incubated. In the non-DM group, the vasodilatory response induced by Ang 1–7 exhibited a significant decrease at doses ranging between $5x10^{-7}$ to 10^{-6}, followed by a highly significant ($P = 0.001$) elevation in potency as compared with the control group (Figure 4A). In contrast, the vascular response of STZ-induced DM rings was declined significantly at doses ranging from 10^{-11} to 10^{-6} as compared with the control group along with non-significant changes in potency (Figure 4B). Conversely, in the diabetic condition and MEL administration, the previous effect was restored slightly as compared with the control group, while the potency remained unchanged as well (Figure 4C). Ultimately, the dAUC% value of OXY pre-incubation showed a dramatic ($P < 0.01$) elevation in the STZ-induced DM rats as compared with the non-DM rats. However, the diabetic rings treated with MEL restored diabetic changes significantly ($P < 0.01$) as compared with the diabetic group.

Effect of melatonin on Ang 1–7 Emax via PKC and oxidative stress enzymes activity

Table 1 shows the multiple comparison of the maximal response to the Ang 1–7 effect on isolated aortic rings with or without MEL administration and inhibitors. The control maximal responses to Ang 1–7 was increased significantly ($P = 0.0048$) in STZ-DM groups compared with the control of non-DM aortic rings. On the other hand, the presence of RO-31-8220 produced a significant ($P = 0.0313$) declination in STZ-induced DM treated with MEL against STZ-

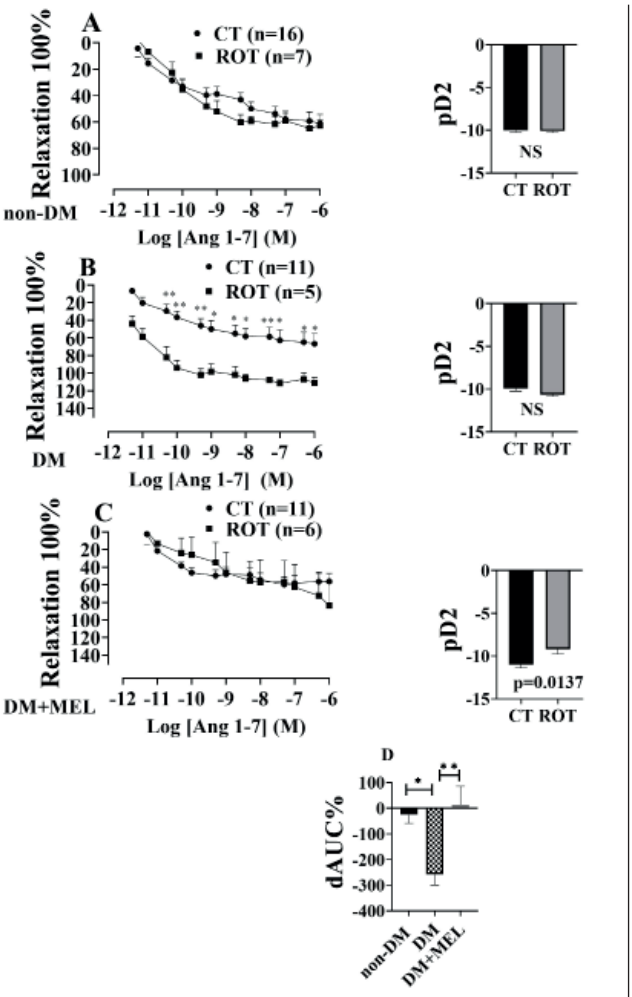

Fig. 3. Ang 1–7 DRC in aortic rings pre-contracted with PE (1 μM) in the presence of ROT (50 μM). The data points in the study are represented as the mean values ± SEM. (CT, control; ROT, rotenone; non-DM, non-diabetes mellitus rats; DM, Streptozotocin induced-diabetes mellitus rats; DM+MEL, Streptozotocin induced-diabetes mellitus in rats treated with melatonin; pD2, the potency of Ang 1–7; dAUC%, the percentage of difference area under curve; n, sample size). [*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$].

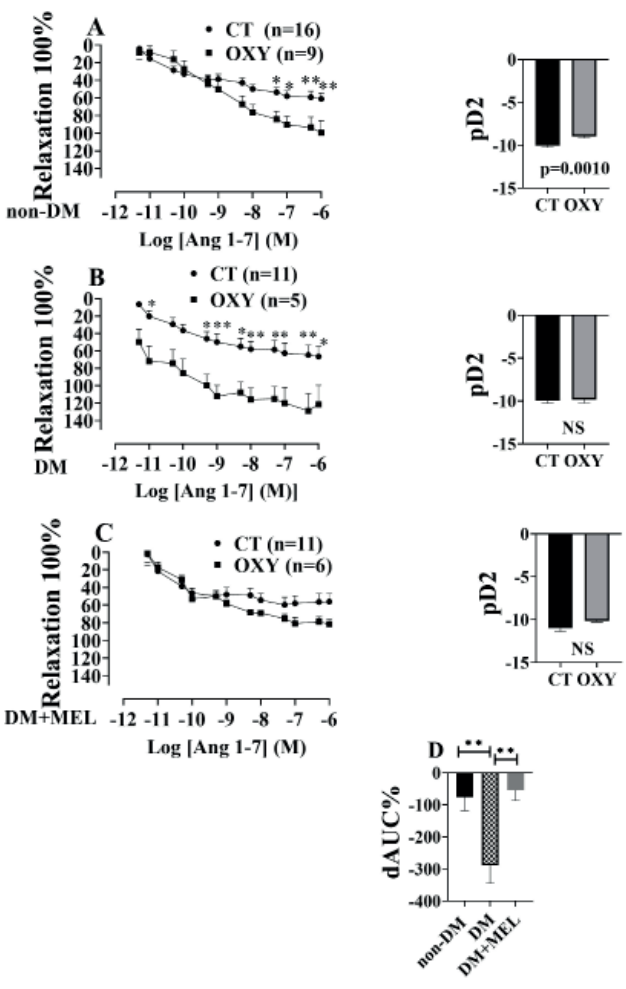

Fig. 4. Ang 1–7 DRC in aortic rings pre-contracted with PE (1 μM) in the presence of OXY (100 μM). The data points in the study are represented as the mean values ± SEM. (CT, control; OXY, oxypurinol; non-DM, non-diabetes mellitus rats; DM, Streptozotocin induced-diabetes mellitus rats; DM+MEL, Streptozotocin induced-diabetes mellitus in rats treated with melatonin; pD2, the potency of Ang 1–7; dAUC%, the percentage of difference area under curve; n, sample size). [*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$].

induced DM rats. Meanwhile, APO mediated slight changes between all compared groups. In contrast, the presence of ROT in that response led to a significant ($P = 0.0437$) increase in diabetic rats treated with MEL as compared with the STZ-induced DM group. Furthermore, the slight difference occurred in the presence of OXY in the studied groups.

Effect of melatonin on Ang 1–7 potency via PKC and oxidative stress enzymes activity

Table 2 shows the multiple comparisons of Ang 1–7 potency with or without MEL administration and applied inhibitors. The Ang 1–7 potency produced slight changes of control groups in all comparison groups. In contrast, the Ang 1–7 potency in the presence of RO-31-8220 was increased dramatically ($P = 0.0133$) in STZ-induced DM as compared with the non-DM group, while the selected parameter was decreased significantly ($P = 0.0300$) in STZ-induced DM treated with MEL as compared with the STZ-induced DM group. Similarly, the pre-incubation aortic ring with APO exhibited a significant ($P = 0.0211$) declination of potency in STZ-induced DM as compared with the DM group. On the other hand, the multiple comparison of ROT pre-incubation revealed a significant ($P = 0.0220$) decrease of Ang 1–7 potency in diabetic rats treated with MEL from the diabetic group. Additionally, in presence of OXY, the Ang 1–7 potency of isolated rings was also increased significantly ($P = 0.0082$) in STZ-induced DM treated with MEL as compared with the non-DM group.

Effect of melatonin on the vasodilatory response to Ang 1–7 via the PI_3K signalling pathway

In the presence of PI-3065 (PI_3K inhibitor), the maximal vasodilatory response induced by Ang 1–7 and Ang 1–7 potency showed non-significant changes in the non-DM group as compared with the control group (Figure 5A). On the other hand, the presence of PI-3065 produced a slight increase in the response with unchanged potency as compared with the control group (Figure 5B). Correspondingly, the STZ-induced DM treated with MEL also produced slight changes of the vasodilatory response induced by Ang 1–7 with unchanged potency as compared with the control groups (Figure 5C). Besides, the dAUC% exhibited no changes among the studied groups (Figure 5D).

Table 1. The Emax value for Ang 1–7 reactivity *via* PKC enzyme and NADPH oxidase activity

Groups	Emax (PE)			Multiple Comparison
	non-DM (A)	DM (B)	DM+MEL ©	
CT	50.55 ± 8.922	66.46 ± 11.53	96.00 ± 8.000	(A vs. C) $P < 0.0048$
RO-31-8220	81.20 ± 6.697	95.54 ± 2.909	70.79 ± 7.761	(B vs. C) $P < 0.0313$
APO	75.60 ± 8.018	102.9 ± 6.283	91.02 ± 11.93	NS
ROT	62.77 ± 4.304	111.2 ± 6.174	83.49 ± 24.84	(A vs. B) $P < 0.0437$
OXY	99.45 ± 13.59	121.4 ± 21.78	81.6 ± 5.472	NS

Data are presented as mean ± SEM. One-way ANOVA was employed to analyse the studied groups followed by the Tukey test as post hoc. [Emax, maximum response; n, sample size, CT; control; MEL, melatonin; APO, apocyanin; OXY, oxypurinol; ROT, rotenone; non-DM, non-diabetes mellitus; DM, STZ-induced diabetes mellitus; *P*, probability value; PE, phenylephrine; NS, non-significant difference; vs, versus].

Table 2. The potency value for Angiotensin 1–7 reactivity *via* PKC enzyme and NADPH oxidase activity

Groups	pD_2			Multiple Comparison
	Non-DM (A)	DM (B)	DM+MEL (C)	
CT	–10.03 ± 0.207	–9.947 ± 0.305	–11.00 ± 0.399	NS
RO-31-8220	–9.516 ±0.180	–10.27 ± 0.159	–9.561 ± 0.190	$P < 0.0133$ (A vs. B)
				$P < 0.0300$ (B vs. C)
APO	–8.648 ± 0.249	–9.688 ± 0.292	–9.520 ± 0.249	$P < 0.0211$ (A vs. B)
ROT	–10.12 ±0.172	–10.64 ± 0.250	–9.202 ± 0.566	$P < 0.0220$ (B vs. C)
OXY	–8.965 ± 0.179	–9.831 ± 0.416	–10.14 ± 0.145	$P < 0.0082$ (A vs. C)

Data are presented as mean ± SEM. One-way ANOVA was employed to analyse the studied groups followed by the Tukey test as post hoc. [pD_2, Ang1–7 potency; n, sample size, CT; control; MEL, melatonin; APO, apocyanin; OXY, oxypurinol; ROT, rotenone; non-DM, non-diabetes mellitus; DM, STZ-induced diabetes mellitus; *P*, probability value; NS, non-significant difference; vs, versus].

Effect of melatonin on the vasodilatory response to Ang 1–7 via the AKT signalling pathway

The aortic rings were pre-incubated by Ipatasertib (AKT inhibitor), showed slight changes in the maximal vasodilatory response induced by Ang 1–7 and potency in the non-DM group as compared with the control group (Figure 6A). Likewise, that response was changed slightly in the STZ-induced DM group with no changes in potency as compared with the control group (Figure 6B). Similarly, the STZ-induced DM treated with MEL also produced slight changes in the vasodilatory response induced by Ang 1–7 with no changes in its potency against the control group (Figure 6C). Additionally, the dAUC% showed slight changes in all studied groups (Figure 6 D).

Effect of melatonin on the vasodilatory response to Ang 1-7 via Mas receptor

The maximum vasodilatory response caused by Ang 1–7 revealed slight changes of isolated aortic rings pre-incubated with A779 in the non-DM group as compared with the control. However, the Ang 1–7 effectiveness produced a significant decrease ($P = 0.0001$) in the diabetic group as compared with the control group (Figure 7A). Similarly, under the STZ effect, the vasodilatory response triggered by Ang 1–7 showed a significant decrease ($P = 0.002$) in the potency as compared with the control groups (Figure 7B). In contrast, under the diabetic condition with MEL treatment, the significant decrease ($P < 0.05$) of the vasodilatory response occurred at 10^{-7}, $5x10^{-7}$, and 10^{-6} concentration, but the potency of Ang 1–7 remained unchanged as compared with the control group (Figure 7C). Conversely, the dAUC% showed slight changes in all the studied groups (Figure 7D) as well.

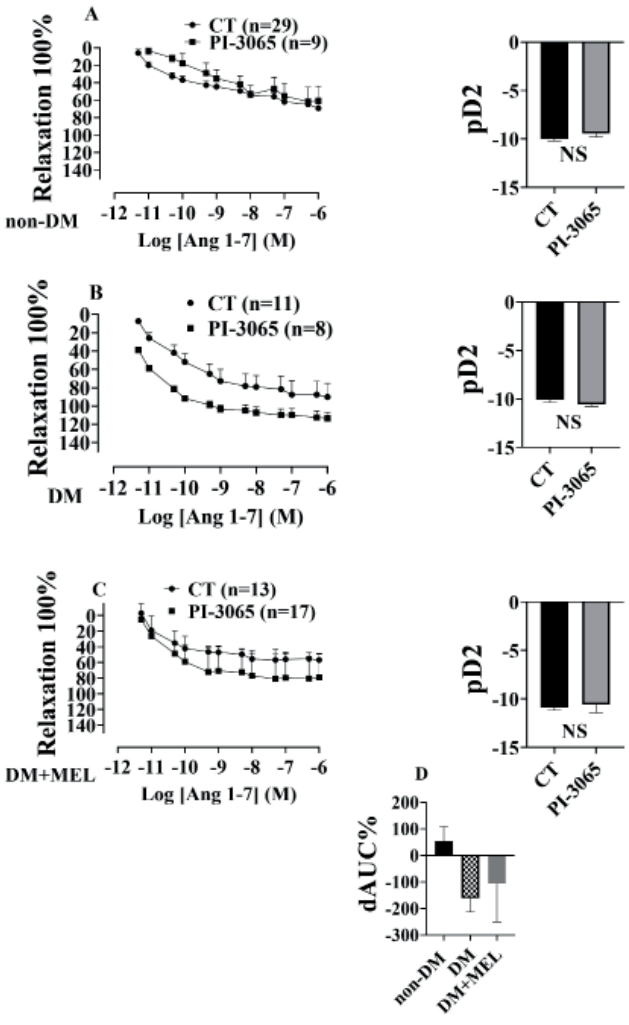

Fig. 5. Ang 1–7 DRC in aortic rings pre-contracted with PE (1 µM) in the presence of PI-3065 (1 µM). The data points in the study are represented as the mean values ± SEM. (CT, control; PI-3065, PI_3K inhibitor; non-DM, non-diabetes mellitus rats; DM, Streptozotocin induced-diabetes mellitus rats; DM+MEL).

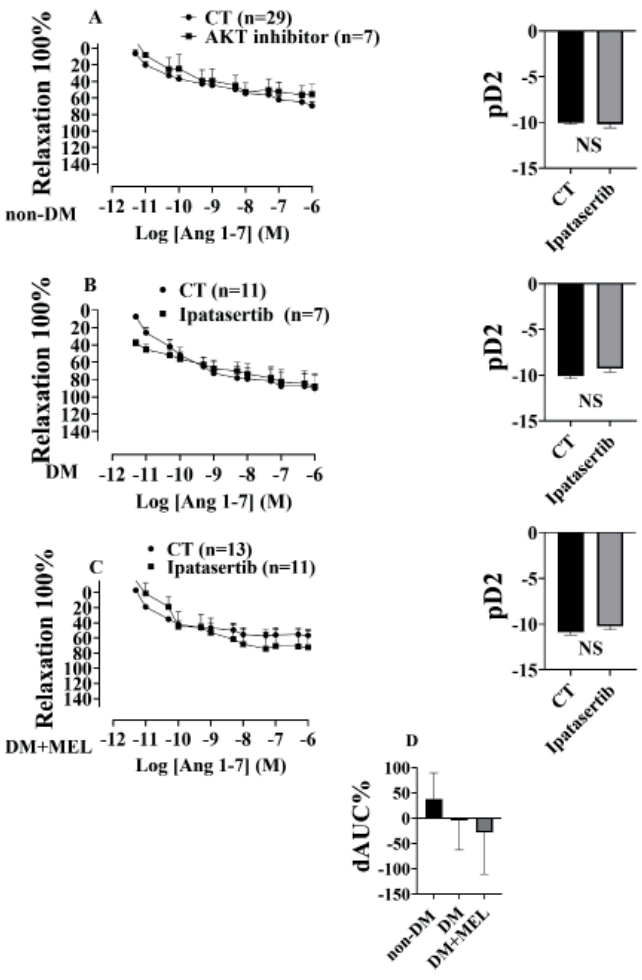

Fig. 6. Ang 1–7 DRC in aortic rings pre-contracted with PE (1 µM) in the presence of Ipatasertib (1 µM). The data points in the study are represented as the mean values ± SEM. (CT, control; Ipatasertib, AKT inhibitor; non-DM, non-diabetes mellitus rats; DM, Streptozotocin induced-diabetes mellitus rats; DM+MEL, Streptozotocin induced-diabetes mellitus in rats treated with melatonin; pD2, the potency of Ang 1–7; dAUC%, the percentage of difference area under curve).

Effect of melatonin on the vasodilatory response to Ang 1–7 via the NO activity

The Ang 1–7 effect of aortic rings pre-incubated with L-NAME showed non-significant changes in the non-DM group as compared with the control. However, in diabetic rats, the potency was elevated dramatically ($P = 0.0001$) from to the control rats (Figure 8A). Similarly, in STZ-induced DM, the vasodilatory response triggered by Ang 1–7 showed non-significant changes, but the dramatic increase ($P = 0.001$) in the potency occurred (Figure 8B). In contrast, in diabetic rats treated with MEL, a significant decrease ($P \leqslant 0.05$) in the vasodilatory response of Ang 1–7 occurred at concentrations of $5x10^{-7}$ and 10^{-6}, while the potency of Ang 1–7 remained unchanged as against control rats (Figure 8C). Additionally, the dAUC% showed slight differences in all the studied groups (Figure 8D).

Effect of melatonin on the maximal response to Ang 1–7 reactivity via $PI_3K/AKT/eNOS$ signalling pathway

Table 3 shows the multiple comparison of the maximal response to Ang 1–7 with or without MEL and applied inhibitors as well as blockers. The control maximal response in diabetic rats administered with MEL was decreased significantly ($P = 0.0492$) as compared with the control in STZ-induced DM rats. In contrast, the maximal response to Ang 1–7 with each of PI-3065, Ipatasertib and L-NAME pre-incubation was declined slightly in diabetic rats treated with MEL as compared with the diabetic rats, except in the presence of A779 that increased non-significantly.

Effect of melatonin on to Ang 1–7 potency via the $PI_3K/AKT/eNOS$ signalling pathway

Table 4 shows multiple comparisons of Ang

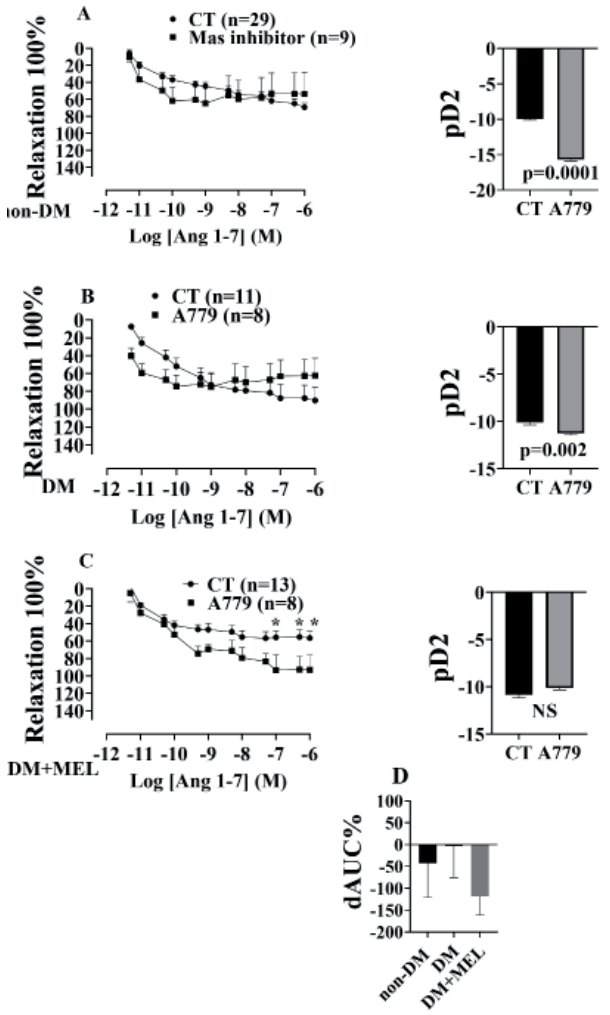

Fig. 7. Ang 1–7 DRC in aortic rings pre-contracted with PE (1 μM) in the presence of A779 (1 μM). The data points in the study are represented as the mean values ± SEM. (CT, control; A779, Mas receptor blocker; non-DM, non-diabetes mellitus rats; DM, Streptozotocin induced-diabetes mellitus rats; DM+MEL, Streptozotocin induced-diabetes mellitus in rats treated with melatonin; pD2, the potency of Ang 1–7; dAUC%, the percentage of the difference area under curve). [*, $P < 0.05$; **, $P < 0.01$].

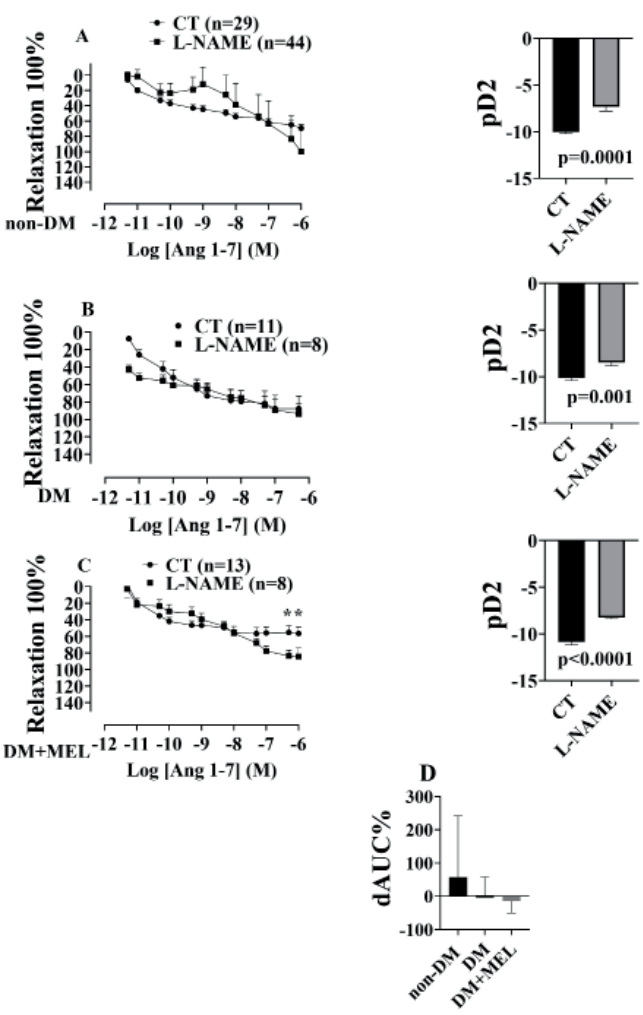

Fig. 8. Ang 1–7 DRC in aortic rings pre-contracted with PE (1 μM) in the presence of L-NAME (200 μM). The data points in the study are represented as the mean values ± SEM. (CT, control; L-NAME, NO inhibitor; non-DM, non-diabetes mellitus rats; DM, Streptozotocin induced-diabetes mellitus rats; DM+MEL, Streptozotocin induced-diabetes mellitus in rats treated with melatonin; pD2, the potency of Ang 1–7; dAUC%, the percentage of difference area under curve). [**, $P < 0.01$].

Table 3. The Emax value to Ang1–7 reactivity *via* the PI3K/AKT/eNOS signalling pathway

Groups	Emax (PE)			Multiple Comparison
	Non-DM (A)	DM (B)	DM+MEL ©	
CT	69.29 ± 5.109	90.08 ± 14.78	56.53 ± 7.957	(B vs. C) $P < 0.0492$
PI-3065	60.76 ± 16.60	113.5 ± 6.611	79.11 ± 29.45	NS
Ipatasertib	55.41 ± 11.85	88.08 ± 14.41	72.00 ± 21.63	NS
A779	53.74 ± 25.71	62.35 ± 19.22	93.01 ± 16.89	NS
L-NAME	100.0 ± 31.11	93.32 ± 11.41	84.21 ± 10.11	NS

Data are presented as mean ± SEM. One-way ANOVA was employed to analyse the studied groups followed by the Tukey test as post hoc. [Emax, maximum response; CT, control; MEL, melatonin; Ipatasertib, AKT inhibitor; L-NAME, NO inhibitor; A779, Mas receptor blocker; PI-3065, PI_3K inhibtor; non-DM, non-diabetes mellitus; DM, STZ-induced diabetes mellitus; *P*, probability value; PE, phenylephrine; NS, non-significant difference; vs, versus].

Table 4. The potency value to Angiotensin 1–7 reactivity *via* the PI_3K/AKT/eNOS signalling pathway

Groups	pD_2			Multiple Comparison
	Non-DM (A)	DM (B)	DM+MEL (C)	
CT	–10.02 ± 0.1689	–10.09 ± 0.274	–10.87 ± 0.2957	$P = 0.0296$ (A vs. C)
PI-3065	–9.446 ± 0.3513	–10.6 ± 0.1584	–10.6 ± 0.8261	NS
Ipatasertib	–10.18 ± 0.4124	–9.276 ± 0.4164	–10.23 ± 0.3993	NS
A779	–15.69 ± 0.2132	–11.27 ± 0.1593	–10.14 ± 0.1907	$P < 0.0001$ (A vs. B)
				$P < 0.0001$ (A vs. C)
				$P = 0.001$ (B vs. C)
L-NAME	–7.313 ± 0.4674	–8.482 ± 0.3555	–8.239 ± 0.1455	NS

Data are presented as mean ± SEM. One-way ANOVA was employed to analyse the studied groups followed by the Tukey test as post hoc. [pD_2, Ang1–7 potency; CT, control; MEL, melatonin; Ipatasertib, AKT inhibitor; L-NAME, NO inhibitor; A779, Mas receptor blocker; PI-3065, PI_3K inhibitor; non-DM, non-diabetes mellitus; DM, STZ-induced diabetes mellitus; *P*, probability value; PE, phenylephrine; NS, non-significant difference; vs, versus].

1–7 potency with or without MEL and applied inhibitors as well as blockers. The potency of Ang 1–7 of diabetic rats treated with MEL was decreased dramatically ($P = 0.0296$) as compared with the control diabetic rats. On the other hand, the Ang 1–7 potency with A779 pre-incubation showed a significant ($P < 0.0001$) decrease in the diabetic group as compared with the non-DM group. Meanwhile, the potency of Ang 1–7 in diabetic rats treated with MEL under A779 produced a significant ($P < 0.0001$) decrease as compared with both STZ-induced DM and non-DM groups, respectively. In contrast, this potency showed slight changes in the presence of PI-3065, Ipatasertib and L-NAME between the studied groups.

Effect MEL tunica medial layer thickness and smooth muscles nuclei count

To evaluate whether the histological examination of MEL on diabetic rats' aortae, the TM layer thickness (μm) and SMCs nuclei count were measured. Tunica media layer thickness was decreased significantly ($P < 0.05$) in diabetic rats from the non-DM group (Figure 9A). In contrast, MEL administration ameliorated the diabetic effect through a compensation of such degeneration significantly ($P < 0.05$) as compared with the STZ-induced DM group. On the other hand, the SMCs nuclei count was decreased dramatically ($P < 0.01$) in STZ-induced DM groups as compared to the non-DM group (Figure 9B), whereas the value in SZT-induced DM treated with MEL was increased non-significantly as compared with STZ-induced DM, and it was decreased significantly ($P < 0.05$) compared with non-DM rats.

Discussion

The obtained results revealed a minor rightward shift of the Ang 1–7 response when a PKC inhibitor was used in aortic rings of non-DM subjects. It has been suggested that PKC could offer a regulatory role of the vascular tone, which influences the diameter of blood vessels as well (Khalil, 2013). It is important to note that various subtypes of PKC can have different effects, with some promoting vasoconstriction and others promoting vasodilation (Thengchaisri et al., 2021a). Regarding PKC roles, the complexity of subtypes could highlight their significance roles

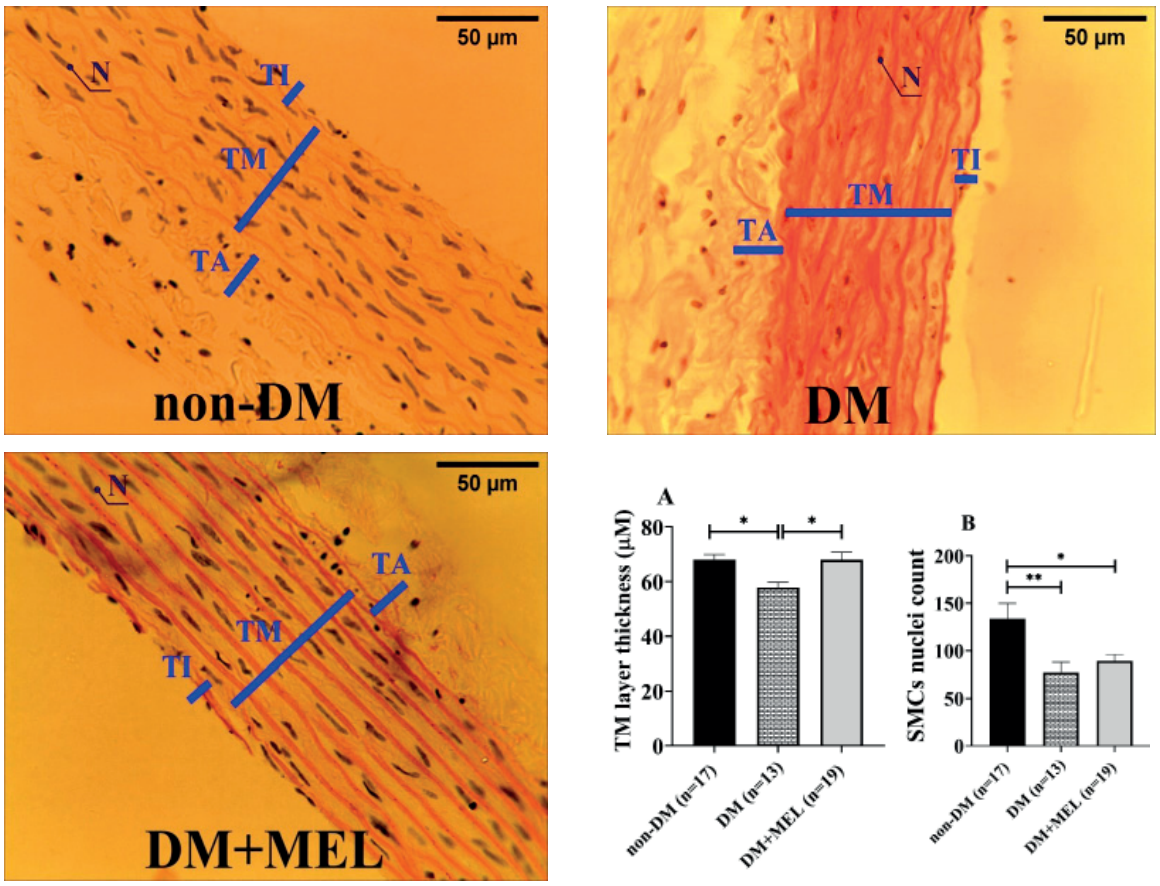

Fig. 9. Quantitative effect of MEL on aortic histology, A; tunica media thickness (µm), B; smooth muscle nuclei count. The bar graph represents the mean ± SEM from the total sample size. (non-DM, non-diabetes mellitus rats; DM, Streptozotocin induced-diabetes mellitus rats; DM+MEL, Streptozotocin induced-diabetes mellitus in rats treated with melatonin; SMCs, smooth muscle cells; n, sample size; N, smooth muscle cell nucleus; TI, tunica intima; TM, tunica media; TA, tunica adventitia). [*, $P < 0.05$; **, $P < 0.01$].

in modulating the blood vessel responsiveness and ultimately affecting the vascular function (Ringvold and Khalil, 2017). In contrast, in the STZ-induced DM group, the vasodilatory response induced by Ang 1–7 was more pronounced. This suggests that in the presence of diabetes and VED, PKC provokes a significant role in the functioning of VECs (Knapp et al., 2019). This heightened response of Ang 1–7 in the presence of diabetes and elevated PKC activity underscores the complex regulatory mechanisms involved in the vascular function under diabetic conditions (Wang et al., 2015). Additionally, one of the main outcomes of diabetes on the vascular system is the emergence of VED, which is subsequently followed by a decline in the vascular tone (Nie et al., 2019). Furthermore, under the diabetes condition, PKC mediates some vasoconstrictors to induce vasoconstriction (Jackson et al., 2016). Based on our findings, the isolated aorta exhibited strong vascular responses toward Ang 1–7 under diabetic conditions; hence, the dAUC% provided such an effect. Existing data suggest that the inhibition of PKC may be involved in vascular tone regulation through MEL antioxidant activity (Huang et al., 2022). Regarding PKC, MEL could exert its modulatory effects *via* specific receptor binding including MT_1R and MT_2R through various signalling pathways (Liu et al., 2016).

The obtained results of NADPH oxidase inhibitor demonstrated a dramatic increase in the potency of Ang 1–7 in both non-DM and STZ-induced DM rats treated with MEL. Recent studies have suggested that the elevated NADPH oxidase activity can lead to pathological changes and a reliance on hydrogen peroxide (H_2O_2) to regulate the vascular tone due to excessive production of superoxide (O^{2-}) and H_2O_2 (Sylvester et al., 2022). Additionally, the excessive production of O^{2-} is associated with various pathological obstacles, including vascular complications of diabetes (Thompson et al., 2017). However, in diabetic rats, the Ang 1–7 by itself improved the vascular tone by inhibiting oxidative enzymes (Raffai et al., 2011a). Furthermore, the significant effects were observed with dAUC% in the diabetic group treated with MEL. Our findings align with previous research and reinforce the evidence from other studies that MEL has beneficial effects in mitigating diabetic complications through various mechanisms (Pourhanifeh et al., 2020).

In our study, the inhibited mitochondrial electron transport chain showed a reduction in the vascular response triggered by Ang 1–7 in diabetic subjects. However, such a response was reversed significantly

in the STZ-induced DM treated with MEL group. It is broadly recognized that mitochondria provide a central role in cellular ROS generation through their catabolic processes (Supinski et al., 2020). Regarding ROS activity, it may be attributed to the positive outcomes of MEL which could be linked to its capacity in mitochondrial ROS reduction.

The observations of xanthine oxidase inhibition were also involved in our results; the Ang 1–7 vasodilatory response induced in diabetic rats increased dramatically. However, when diabetic rats were treated with MEL, this effect was reduced. Interestingly, in the group of diabetic rats treated with MEL, such responsiveness may be attributed to MEL's counteraction against VED and the declined vasodilatory response to Ang 1–7. Our explanation is supported by a previous attempt which proposed that the inhibition of xanthine oxidase-mediated O^{2-} generation with a substance like OXY can improve endothelium-dependent vasodilator responses (Thengchaisri et al., 2021b). In particular, xanthine oxidase is well-known to be a potent source of oxidative stress in the vascular system through the production of uric acid (Liu et al., 2021). Furthermore, studies have depicted that the protective effects of MEL are in part mediated through its binding to MT_2R (González-Flores et al., 2023). Additionally, MEL has been shown to decrease certain pro-inflammatory factors in STZ-induced diabetic rats (Maher et al., 2020).

Data obtained from PI_3K inhibitor reveal the dysregulation of vasodilation by Ang 1–7. Numerous attempts have shown that the PI_3K regulatory roles through different physiological mechanisms were linked to a variety of intracellular growth factors (Zhao et al., 2021). However, diabetes could generate both the advanced glycation end products (AGEs) and the receptor of advanced glycation end product (RAGE) contributing to the improvement of downstream PI_3K/AKT signals (Figure 5) (Yuan et al., 2020). Surprisingly, in diabetic rats treated with MEL, the Ang 1–7 vasodilation under PI3K inhibitor effect was increased. Experimental research has supplied findings that endorse the notion that MEL has the potential to enhance insulin signalling, potentially improving insulin sensitivity. This is achieved through the production of insulin-like growth factor and the augmentation of phosphorylation on the tyrosine residues of insulin receptors (Picinato et al., 2008). Hence, the increased vasodilation under MEL action could be involved through insulin improvement.

In our results, the Akt molecule inhibition by Ipatasertib revealed non-significant changes of vasodilation triggered by Ang 1–7 in all studied groups. It confirmed that hyperglycaemia could cause glycosylation to occur at the AKT phosphorylation site in eNOS, ultimately leading to the suppression of the eNOS function (Figure 6A, B, C) (Alghanem et al., 2021). On the other hand, dysregulation of AKT signalling pathways can lead to abnormal vascular remodelling (Jia and Sowers, 2021). In contrast, MEL could offer antioxidant properties of VECs activity through direct free radical scavenging, declined NADPH oxidase, and elevating SOD activity, hence, promoting NO and/or vasodilation *via* MT_2R as well (Song et al., 2022).

Our findings under MasR blocker indicate that Ang 1–7 effectively promotes the dilation of the thoracic aorta, and its potency is notably enhanced in the group with experimentally induced diabetes using STZ. Furthermore, the administration of MEL also significantly induced vasodilation. These results demonstrate that the external Ang 1–7 successfully restored the dilatory capacity of diabetic rats' aortae (Zhang et al., 2015). Additionally, it has been suggested that the signalling pathway of Ang 1–7 primarily operates through the MasR (Chen et al., 2023). Indeed, Ang1–7 *via* its interaction through MasR triggers specific intracellular signalling pathways, resulting in the generation of NO and the stimulation of cAMP release (Tetzner et al., 2016). Meanwhile, several studies have reported that Ang 1–7 can also communicate through the AT_2R (Raffai et al., 2011b). Thus, our finding has uncovered a novel aspect suggesting that both MEL and Ang 1–7 could synergistically potentiate the vascular tone through eNOS activity.

The results obtained from using the NO inhibitor by L-NAME revealed a reduction in the potency of Ang 1–7 without a change in vasodilation, as well as in dAUC% in both diabetic rats and diabetic rats treated with MEL. This suggests that there may be impaired endothelial eNOS signalling pathways, leading to a subsequent decrease in NO production (Xie et al., 2021). Meanwhile, in the STZ-induced DM treated with MEL group, the elevated maximum response was observed (Figure 8C). Studies have indicated that MEL has the potential activity to restore or compensate the decreased NO levels (Simko et al., 2018).

The quantitative histopathological findings demonstrate dysfunction in the development of aortic layers, characterized by a reduction in the number of VSMCs nuclei and a decrease in TM thickness, as well as the disrupted structural organization. These changes are attributed to the significant impact of diabetes on VSMCs, primarily resulting from prolonged exposure to hyperglycaemia followed by vascular complications (Ullah Wazir et al., 2023). On the other hand, the precise cellular-level mechanism responsible for these changes remains unclear. Furthermore, the contraction or shrinking of VSMCs and an increase in collagen bundles within the tissue can lead to alterations in the size, shape, and number of cell nuclei (Silva-Velasco et al., 2023). These combined factors are attributed to be responsible for the observed changes in the aortic diameter. However, the results of STZ-induced DM treated with MEL

showed a notable reduction in the adverse effects of diabetes, and significant improvement was observed, indicating a substantial amelioration of diabetic-related consequences. Indeed, accumulated evidence supports the idea that MEL can improve metabolic irregularities and dysfunction in the adipose tissue due to its antioxidant properties (Cui et al., 2023). Additionally, MEL's effects may be mediated through their membrane receptors (Xia et al., 2020). Moreover, MEL has been found to enhance the activity of the mitochondrial activity, especially those mechanisms linked to oxidative enzymes (Reiter et al., 2016).

Conclusions

The study findings indicate that STZ negatively affects the vascular response to Ang 1–7 in isolated thoracic aortae. However, the administration of MEL effectively reversed these diabetes-induced alterations, leading to improved vasodilation in response to Ang 1–7. The involvement of PKC and NADPH oxidase had minimal effects on Ang 1–7-induced vasodilation in non-diabetic aortic rings. Conversely, MEL induced slight changes in the diabetic group induced by STZ, suggesting that MEL plays a regulatory role in vascular responsiveness to Ang 1–7 in diabetic aortic rings. Particularly, MEL mitigates the adverse effects of mitochondrial complex I inhibition on the vascular function in diabetes and partially counteracts the effects of xanthine oxidase by modulating oxidative stress pathways.

The study also suggests that PI3K may not be a primary regulator of Ang 1–7-induced vasodilation in these experimental conditions. The impact of AKT inhibition on the vascular response to Ang 1–7, with or without MEL treatment, was relatively modest across the experimental groups. The study underscores the crucial roles of MasR in maintaining the efficacy of Ang 1–7-induced vasodilation in both non-diabetic and diabetic conditions. However, under MEL treatment in diabetic rats, there was a reduction in the maximal vasodilatory response to Ang 1–7 when MasR was blocked, although these interventions on vascular responses were subtle.

Furthermore, the study concludes that NO has a counterregulatory role in the efficacy of Ang 1–7-induced vasodilation, as evidenced by a significant increase in potency when NO is inhibited. The complex interactions among NO, MEL, and Ang 1–7 in regulating vascular responses provide valuable insights into potential therapeutic strategies for addressing vascular dysfunction in diabetes. Finally, MEL may have potential protective and restorative effects on the structural integrity of the tunica media layer thickness and vascular smooth muscle cell (VSMC) density in diabetic conditions.

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Conflict of Interest

There were no conflicts of interest as declared by the authors.

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