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Role of Melatonin in Attenuation of Vascular Angiotensin 1-7 Reactivity *Via* Calcium Channels and Endothelial Derived Relaxing Factors in Induced Diabetic Aortic Rats

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Abstract

Melatonin (MEL) appears to have a regulatory role in vascular tone through enhancing endothelial-derived relaxing factors (EDRFs) and modulation of calcium (Ca^{2+}) influx in both vascular smooth muscle cells (VSMCs) and vascular endothelial cells (VECs). While, such effects on angiotensin 1-7 (Ang 1-7) vasoreactivity in the vascular endothelial dysfunction (VED) remained unclear. Therefore, the current study investigates the vasculoprotective action of MEL on Ang 1-7 dose response curve (DRC) *via* Ca^{2+} channels and EDRFs in streptozotocin (STZ)–induced diabetes mellitus (DM) in male albino rats' isolated aorta. The present study included four experiments. Experiment I, included measurement of tunical intima-media thickness and the histological examination in non-DM, STZ-induced DM and STZ-induced DM treated with MEL. Experiment II included the measurement of the isometric tension using Ang 1-7 DRC with or without N^G-nitro-L-arginine methyl ester (L-NAME), a nitric oxide (NO) inhibitor in the studied groups. Experiment III included the measurement of the isometric tension using Ang 1-7 DRC with or without indomethacin (IND), a non-selective cyclooxygenase (COX) inhibitor in the studied groups. Experiment IV included the measurement of the isometric tension using Ang 1-7 DRC with or without nifedipine (NIF), a voltage-dependent L-type Ca^{2+} channels blocker in the studied groups. The present study found that, MEL demonstrated protective effects against vascular stress through multiple mechanisms. These actions collectively contributed to the maintenance of vascular health, preservation of endothelial function, and prevention of vascular diseases. Also it modulated vascular response to Ang 1-7 by either antioxidant properties or Ca^{2+} vasomotion.

Keywords: melatonin; angiotensin1-7; vascular endothelial dysfunction; endothelial derived relaxing factors; calcium ions channels

دور هرمون الميلاتونين في التقليل من استجابة الأوعية الدموية للأنجيوتنسين 1-7 المتعلقة بقنوات الكالسيوم وعوامل الأستجابة الأوعوية في الشريان الأبهري للجرذان الناجمة عن السكري المستحث

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الخلاصة

يبدو أن الميلاتونين (MEL) يلعب دوراً تنظيمياً في الأوعية الدموية المتوترة من خلال تعزيز عوامل

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الاسترخاء المشتقة من البطانية (EDRFs) وتعديل تدفّق الكالسيوم (Ca^{2+}) في كل من خلايا العضلات الملساء والخلايا البطانية الوعائيتين، بينما ظلت هذه التأثيرات على نشاط الأوعية الدموية للأنجيوتنسين ١-٧ (Ang 1-7) في الخلل الوظيفي البطاني الوعائي (VED) غير واضحة. لذالك تبحث الدراسة الحالية عن تأثير الوقائي للميلاتونين على نشاط أنجيوتنسين ١-٧ المستخدمة بشكل منحنى الاستجابة للجرعة (DRC) في الأوعية الدموية عبر كل من قنوات الكالسيوم وعوامل الاسترخاء المشتقة من البطانية في داء السكري الناجم عن الستربتوزوتوسين (STZ). تضمنت الدراسة الحالية أربع مجموعات تجريبية . التجربة الأولى ، تضمنت قياس سماكة والفحص النسيجي للشريان الأبهري لكل من مجاميع غير المستحث بـ STZ ، المستحث بـ STZ و المستحث بـ الستربتوزوتوسين المعالج بالميلاتونين. تضمنت التجربة الثانية قياس التوتر المتساوي باستخدام منحنى الاستجابة للجرعة للأنجيوتنسين ١-٧ مع أو بدون L-NAME وهو مثبط لأكسيد النيتريك (NO) في المجموعات المدروسة. تضمنت التجربة الثالثة قياس التوتر المتساوي باستخدام منحنى الاستجابة للجرعة للأنجيوتنسين ١-٧ مع أو بدون إندوميثاسين (IND) ، وهو مثبط لانزيمات الأكسدة الحلقية الغير الانتقائية (COX) في المجموعات المدروسة. تضمنت التجربة الرابعة قياس التوتر متساوي القياس باستخدام منحنى الاستجابة للجرعة للأنجيوتنسين ١-٧ مع أو بدون نيفيديبين (NIF) ، وهو مانع قنوات Ca^{2+} المعتمدة على الجهد في المجموعات المدروسة. وجدت الدراسة الحالية أن الميلاتونين أظهر تأثيرات وقائية ضد الإجهاد الوعائي من خلال آليات متعددة. حيث ساهمت هذه الإجراءات بشكل جماعي في الحفاظ على صحة الأوعية الدموية، والحفاظ على وظيفة البطانة، والوقاية من أمراض الأوعية الدموية. كما أنه يعدل استجابة الأوعية الدموية للأنجيوتنسين ١-٧ إما عن طريق خصائص مضادات الأكسدة أومن خلال حركة ايونات الكالسيوم بين طبقات الاوعية الدموية.

1. Introduction

Diabetes mellitus (DM) is recognized as a rapidly growing epidemic with far-reaching implications affecting individuals of all age groups worldwide [1, 2]. In addition, DM is considered as risk factor for VED due to enhance vascoactive substances by VECs [3]. This condition leads to various alterations and changes within the VECs. [4]. It has been demonstrated that, VED contributes to complications related to vascular tone and impairs endothelium-dependent vasodilation [5]. In particular, the whole consequences recognized by increased vessel stiffness [6], an elevated oxygen-derived free radical species (ROS) [7] as well as, the alteration of vascular barrier performance [4]. Hence, such factors are contributing in NO bioavailability [8]. Nitric oxide performs several physiological roles [9]. In particular, NO initiates vasodilation through enhancing soluble guanylyl cyclase (sGC) in the VSMCs , followed by production of cyclic guanosine monophosphate (cGMP) [10]. On the other hand, the cyclooxygenases 1 and 2 (COX-1 and COX-2) expression [11] are also targeted by the VED including prostaglandin H_2 (PGH_2), prostaglandin I_2 (PGI_2, prostacyclin), prostaglandin E_2 (PGE_2), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), prostaglandin D_2 (PGD_2), and thromboxane A_2 (TxA_2) were acting as local hormones [12]. More particularly, PGI_2 has been shown to evoke vasodilation [10] through cyclic adenosine monophosphate (cAMP) which activates cytosolic Ca^{2+} outflow and various types of potassium channels [13]. Furthermore, the vascular tone modification under DM also lead to disturbances of the Ca^{+2} channel either on VECs or in VESMCs layers *via* alteration of Ca^{+2} influx through L-type voltage dependent Ca^{+2} channels [14]. Besides, the impaired VSMCs related to such channels have been suggested to be a hallmark dysfunction that contribute in the vascular complications as wel [15].

Likewise, the renin-angiotensin system (RAS) contributes a regulatory role in hemodynamic stability [16] through two physiological axes, that included angiotensin converting enzyme –angiotensin 1-8 – angiotensin type1 receptor (ACE - Ang 1-8 - AT_1) axis and angiotensin converting enzyme$_2$ –angiotensin 1-7 – Mas receptor (ACE_2-Ang 1-7 - Mas) axis [17]. The increment of plasma Ang 1-7 has been well demonstrated through blocking

opposite axis to prevent the decrease of ACE_2 expression [18]. In particular, the vasodilatory signal transduction of the activated Mas improved the endothelial nitric oxide synthase (eNOS) phosphorylation, PGI_2 synthesis augmenting and norepinephrine suppression [19]. The possible systemic mechanism of Ang 1-7 action on the vascular diabetic condition still remained unclear, although previous studies confirming the positive effect of Ang 1-7 on the glucose metabolism through modulation of the blood flow and the inhibition of fibrosis, hence, it stimulates both glucagon and insulin release, respectively [20].

Another important vasodilatory integrated axis has been abundantly pronounced. Melatonin (5-methoxy-N-acetyltryptamine)], the main product of pineal gland which contribute in many physiological aspects directly or indirectly [21]. This product can improve circadian rhythm and the vascular tone regulation [22]. The direct effects of MEL are exerted through the binding to the plasma membrane metabolic receptors known as G protein-coupled receptors (MT_1R and MT_2R), and also acted *via* cytosolic proteins such as calmoduline, calreticulin and tubulin [23]. Additionally, MEL provokes its action through the binding to an orphan nuclear receptor [24]. Earlier attempts have shown a dominance roles of MEL in NO production and/or NO bioavailability by the interaction with MT_2R receptor [25]. Moreover, vasculoprotective effect of MEL has been reported in regressed remodelling tissues along with functional capacity restoration [26].

Despite of accumulated evidences of MEL impact on VED through diabetic induction, as well as MEL receptors alteration at quantitative and qualitative deformities were poorly comprehend and obscure. Therefore, the present study aimed to investigate the vasodilatory effect of MEL on vascular response to Ang 1-7, Ca^{2+} channels and EDRFs in isolated thoracic aorta of male albino rats.

2. Materials and Methods

2.1. Chemicals

Angiotensin 1-7, MEL, L-NAME, IND, NIF and STZ were purchased from Sigma Aldrich (USA).

2.2. Animals

Male albino rats (*Rattus norvegicus*) were weighing about 250–300 gm were used in the recent study. Animals were bred in animal house belonged to the Department of Biology, College of Science, Salahaddin Unuiversity-Erbil, Iraq, and they were acclimatized at standard conditions (23 ± 2 ℃), on a 12 h/12 h light/dark cycle (06:00 - 18:00 light), with free access to tap water and food, 24 h/day *ad libitum*. The current study was confirmed by the Animal Research Ethic Committee belonged to College of Science, Salahaddin University-Erbil, Erbil, Iraq (Reference number; 2636 and Date of Issue; August 7, 2022).

2.3. Diabetes mellitus type-1 induction

The rats were induced with STZ injection intraperitoneally (i.p.) at the dose of 50 mg/kg/body weight of rats that dissolved in sodium citrate buffer (pH 4.5). After STZ injection, the rats were fed with Dextrose 5% for 24 h. The confirmed diabetic rat's condition was tested by a tail blood glucose measurement. The values represent diabetes when they were higher than the 250 mg/dL and 48 h after STZ injection.

2.4. Melatonin dose preparation

Melatonin tablets (MELAPLAN 10 mg, PLANTE PHARMA) were dissolved in sterilized distilled water of 1% ethanol to make a MEL solution about150 mg/ml. After day 14 of STZ-

induced DM, rats are treated with MEL liquid (30 mg/kg BW) orally by gavage for 14 executive days.

2.5. Preparation of rat aortic rings

After anesthetization with ketamine–xylazine combination (90 mg/kg and 10 mg/kg, respectively 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. About 167 aortic rings from 42 rats were prepared. Rings were carefully enucleated and settled in a Petri dish immediately containing cold 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. Then excess surrounding tissues were removed and 4 aortic rings each about 3 mm in length were prepared.

2.6. Vascular reactivity measurements

The prepared aortic rings were hanged on between L-shaped stainless-steel hooks horizontally in 5 ml organ bath vessel (Automatic organ bath, Panlab Harvard apparatus, USA) filled with KHBS. The bath solution was maintained at 37 °C and aerated with a mixture of about 95% O2 and 5% CO2 continuously. The aortic rings were exposed to basal tension 2.0 g for 60 min. Thereafter, the rings were gradually stretched with KHBS and allowed to equilibrate about 60 minutes during which rings were repeatedly washed and equilibrated every 15 min, and the tension was continuously readjusted until a maximum stable contraction was dominated. For the functional integrity of the prepared aortic segments, KCl (60 mM) was used. Thenafter, the endothelium integrity was assessed by the endothelium-dependent relaxation incubated with 1 µM acetylcholine (Ach) in 1 µM PE pre-contracted rings. At that point, the preparation was ready to evaluate the changes in dose-response curve (DRC) of Ang 1-7 induced aortic relaxation.

2.7. Experimental design

Four experiments were carried out, in the experiment I, aorta from 56 rats were removed from non-DM (n=19), STZ-induced DM (n=15) and STZ-induced DM treated with 300 mg/kg BW MEL (n=22) (After day 14^{th} of STZ induction orally by gavage for 14 executive days), then after the isolated tissues were fixed in 10% buffered formo-saline and they paraffinized for the qualitative and semiquantitative histological analysis using hematoxylin-eosin (H and E) staining. The histological examination and tunical intima-media thickness were analysed in a double blinded manner, under image analysis software using CaptaVision software version 2.4.0. The experiment II included the vascular response to Ang 1-7 ($5x10^{-12}$ - 10^{-6} µM) DRC with or without L-NAME (200 µM and 20 min pre-incubation), a NO inhibitor of non-DM, STZ-induced DM and STZ-induced DM treated with 300 mg/kg BW MEL (After day 14th of STZ induction orally by gavage for 14 executive days). The experiment III included the vascular response to Ang 1-7 ($5x10^{-12}$ - 10^{-6} µM) DRC with or without IND (1 mM and 20 min pre-incubation), a non-selective cyclooxygenase (COX) inhibitor of non-DM, STZ-induced DM and STZ-induced DM treated with 300 mg/kg BW MEL (After day 14th of STZ induction orally by gavage for 14 executive days). The experiment IV included the vascular response to Ang 1-7 ($5x10^{-12}$ - 10^{-6} µM) DRC with or without NIF (20 µM and 20 min pre-incubation), a voltage-dependent L-type Ca^{2+} channels blocker of non-DM, STZ-induced DM and STZ-induced DM treated with 300 mg/kg BW MEL (After day 14th of STZ induction orally by gavage for 14 executive days).

2.8. Statistical analysis

The values of maximal effect (Emax), drug potency [pD2 (-logIC50)], AUC and aorta thickness are expressed as the means ± standard error of the mean (SEM) to differentiate the

effect of inhibitors and blocker on vascular responses to Ang 1-7 in aortic segments of both non-DM, STZ-induced DM and STZ-induced DM treated with MEL rats. Results were analysed using one-way ANOVA for comparing aorta thickness, Emax and pD2 between the studied groups presented by figures and table, also two-way analysis of variance (ANOVA) was used for comparison among the studied groups followed by Dunnett post hoc test, while Student T- test was applied to compare pD2 of the studied groups versus control. $P < 0.05$ was considered statistically significant. *p<0.05, **p<0.01 and ***p<0.001.

3. Results

3.1. Vasculoprotective effect of melatonin on impaired aorta with streptozotocin

The thoracic aorta of non-DM group (Figure 1), showed normal histological architecture of the tunical layers measured thickness including wavy of distinct elastic laminae through tunica media, concentrically arrangement, and VSMCs were observed in the interspaces between the concentric lamellae, also the intimal layer exhibited continues endothelial cellular content, additionally, fibrous tissue elements appeared normal recognition of adventitia layer.

In contrast, the DM group was found to have prominent histological damages in which the vascular thickness from STZ-induced DM group was statistically ($p \leq 0.01$) lesser as compared to non-DM group on the expense of media-intima layers associated with disrupted concentric pattern from elastic laminae as compared to non-DM group (Figures 1 A and C). Irregular luminal layers of VECs were observed, and the internal elastic laminae showed discontinuity areas. The vascular structure wall in DM+MEL group showed decreased degeneration of histological interface including increased measured thickness length, at the same time, no obvious disruptions of elastic laminae and VSMCs in the interspaces between the concentric lamellae were shown as compared to DM group as well (Figures 1 A and D).

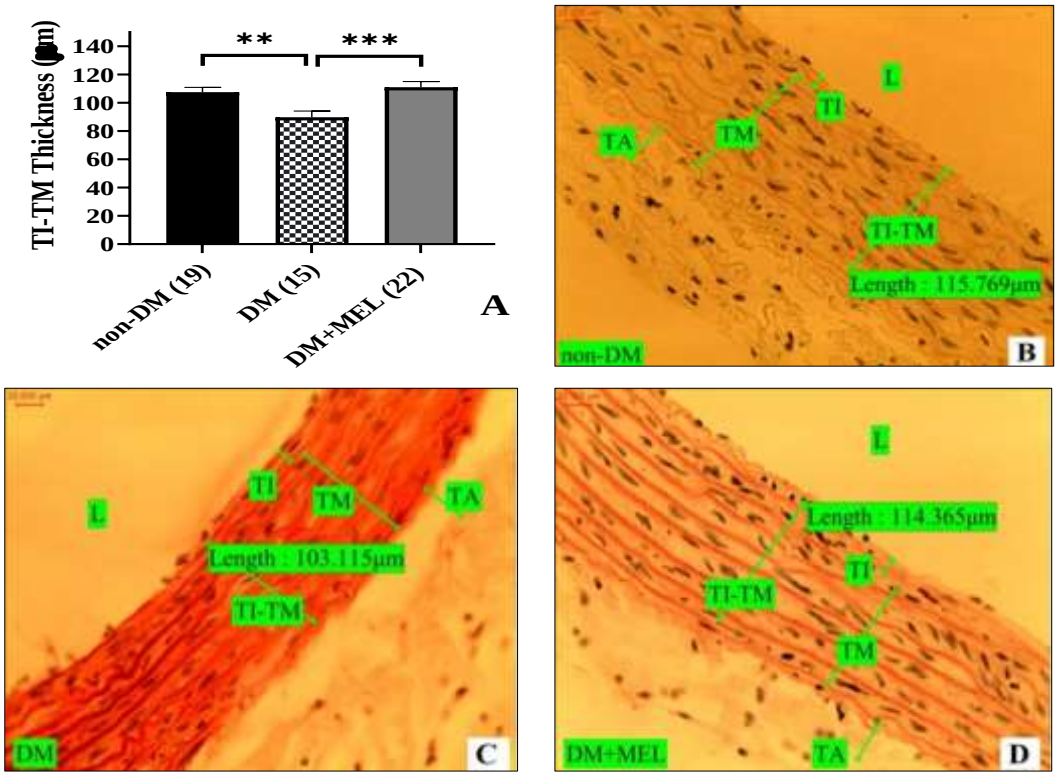

Figure 1: A; showed the cross sections of the aorta shows either decreased or increased thickness layer of smooth muscle cells (intima-media thickness). B, C and D; showed the hematoxylin-eosin staining of the aortic tissues in non-DM, STZ-induced DM and STZ induced DM treated with MEL. Magnification at 400 X. Values are presented as means±

S.E.M. [**; P < 0.001 level, ***; p<0.001]. [non-DM, non-diabetes mellitus rats; DM, STZ induced diabetes mellitus rats; DM+MEL, STZ induced diabetes mellitus rats treated with melatonin; L, lumen; TI, tunica intima; TM tunica media; TA, tunica adventitia; TI-TM, tunica intima-media; S.E.M; standard error of mean].

3.2. Effect of melatonin on the vasodilatory response to Ang 1-7

The vascular response to Ang 1-7 was shifted to left side slightly in STZ-induced DM produced right shift slightly (Figure 2 A). Also the mean maximal relaxation responses were decreased non-significantly (Figure 2 C) with unchanged in potency (Figure 2 B) as compared to non-DM group. In contrast, the AUC was decined non-significantly in DM group as compared to non-DM group as well (Figure 2 D). On the other hand, the orally treated MEL attenuated diabetes consequences non-significantly in STZ-induced DM treated with MEL group through shifting the Ang 1-7 vasodilatory toward right side as compared to DM group (Figure 2 A). Additionally, such vascular impacts of MEL were further confirmed by potrency, maximal effect and AUC, respectively (Figures 2 B, C and D).

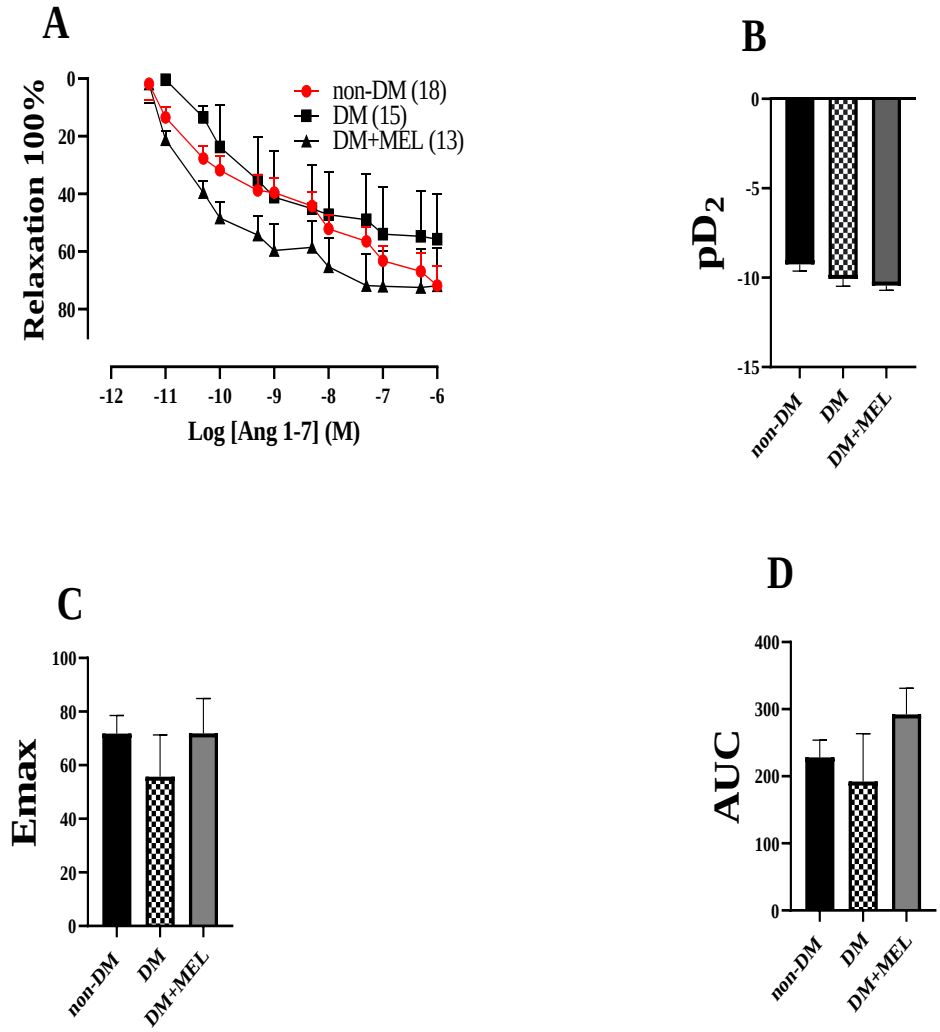

Figure 2: Concentration-response curve of Ang 1-7 in aortic rings pre-contracted with PE (1 µM) in non-DM, DM and DM+MEL. Points represent mean ± S.E.M of aortic rings from a total of animals sample size. (●, non-DM; ■, DM; ▲, DM+MEL; non-DM, non-diabetes mellitus rats; DM, Streptozotocin induced-diabetes mellitus rats; DM+MEL, Streptozotocin induced-diabetes mellitus rats treated with melatonin; AUC, area under curve; pD2, Ang 1-7 potency; Emax, maximum response).

3.3. Effect of melatonin on the vasodilatory response to Ang 1-7 via nitric oxide

The pre-incubation of isolated aortic rings with L-NAME (Figure 3 A) resulted in a non-significant increase in the vascular response to Ang 1-7 in non-DM group, as well as, the potency was decreased significantly ($p < 0.05$) as compared to control (Figure 3 A), while such impact was moderately increased in STZ- induced DM aorta (Figure 3 B) which paralleled with its potency. Differently, rats treated with MEL, restored the vasodilatory response to Ang 1-7 in STZ- induced DM aorta including declined potency ($P < 0.001$) (Figure 3 C). On the other hand, MEL treatment showed modulatory effect on L-NAME pre-incubation tough non-significant restored potency and maximum response mean of Ang 1-7 as compared to STZ-induced DM group (Figure 4).

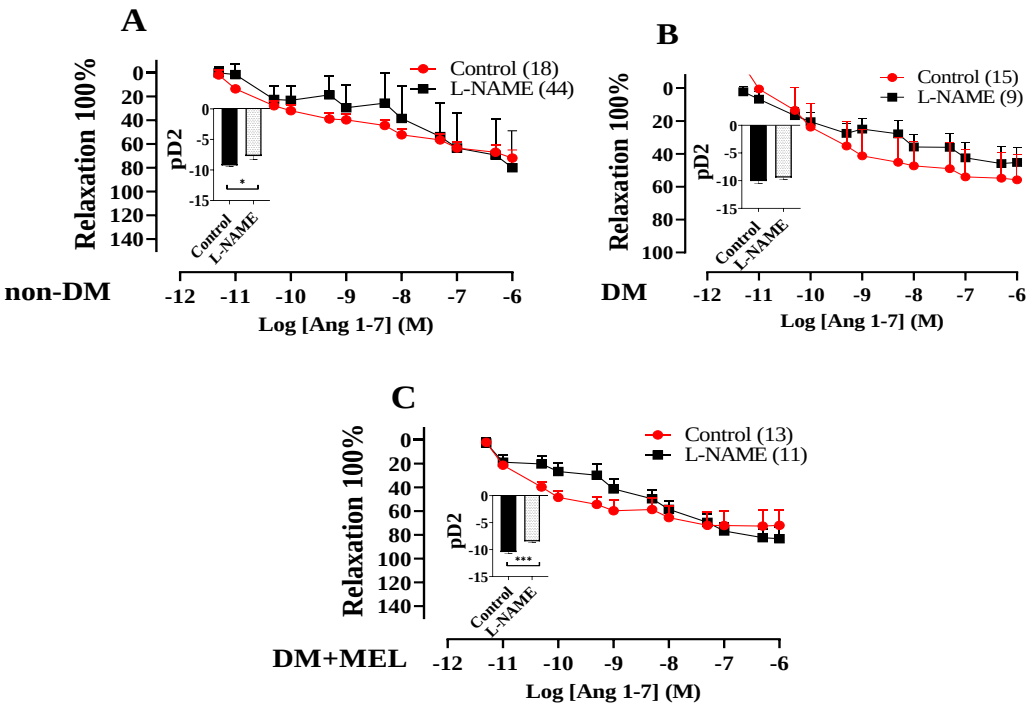

Figure 3: Concentration-response curve of Ang 1-7 in aortic rings pre-contracted with PE (1 µM) in the presence of L-NAME (200 µM). Points represent mean ± S.E.M of aortic rings from a total of animals sample size. (●, control; ■, L-NAME; non-DM, non-diabetes mellitus rats; DM, Streptozotocin induced-diabetes mellitus rats; DM+MEL, Streptozotocin induced-diabetes mellitus rats treated with melatonin; pD2, potency). [***; $P < 0.001$ level, *; $P < 0.05$ level].

Figure 4: Emax and pD2 of vascular response to Ang 1-7 which pre-incubated with L-NAME. [non-DM, non-diabetes mellitus rats; DM, Streptozotocin induced-diabetes mellitus rats; DM+MEL, Streptozotocin induced-diabetes mellitus rats treated with melatonin; pD2, potency; Emax, maximum efficacy; L-NAME, L-N^G-Nitro arginine methyl ester].

3.4. Effect of melatonin on the vasodilatory response to Ang 1-7 via prostaglandins

Angiotensin 1-7 induced the relaxation of the aortic smooth muscle pre-incubated with IND, more intensely in non-DM group at doses 101^{-0} and a10 $^{-9}$ significantly ($p < 0.05$) while such effect was abolished in STZ-induced DM segments (Figures 5 A and B) with slight change in potency from their control groups. In contrast, melatonin potentiated vasodilatory response to Ang 1-7 more obviously; it decreased potency significantly ($p < 0.01$) with slight increase of maximum response mean in STZ-induced DM groups (Figure 5 C). More particularly, under STZ induction the potency of Ang 1-7 in the presence of IND was elevated significantly ($p < 0.001$) and the maximum response mean was decreased slightly (Figure 6) from non-DM group. Whereas, the aortic rings of MEL treated rats was completely restored both potency and maximum response mean values (Figure 6).

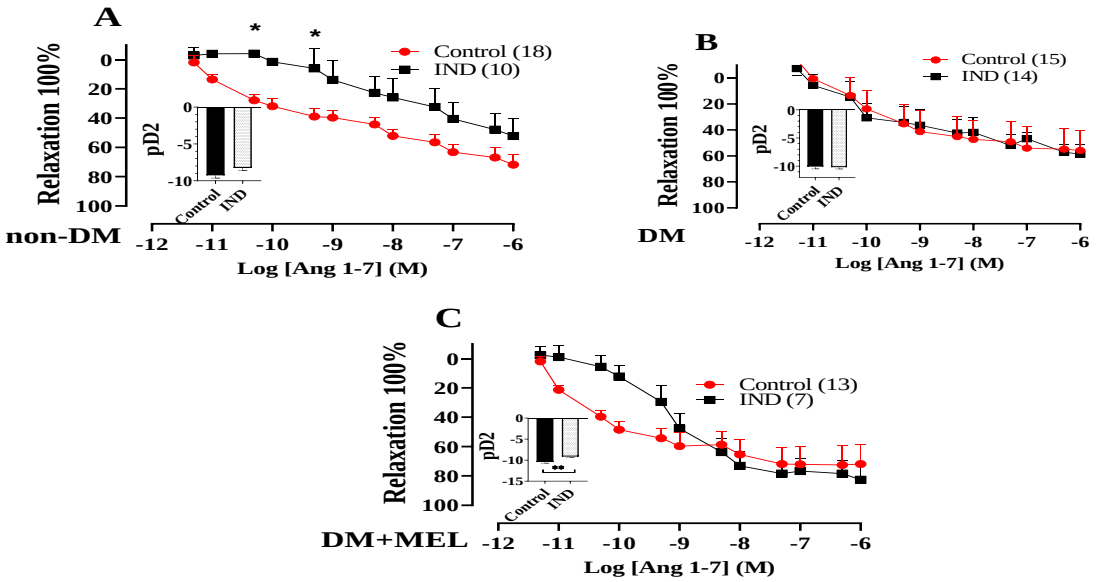

Figure 5: Concentration-response curve of Ang 1-7 in aortic rings pre-contracted with PE (1 µM) in the presence of IND (1 mM). Points represent mean ± S.E.M of aortic rings from a total of animals sample size. (●, control; ■, IND; non-DM, non-diabetes mellitus rats; DM, Streptozotocin induced-diabetes mellitus rats; DM+MEL, Streptozotocin induced-diabetes mellitus rats treated with melatonin; pD2, potency). [*; $P < 0.05$ level, **; $P < 0.01$ level].

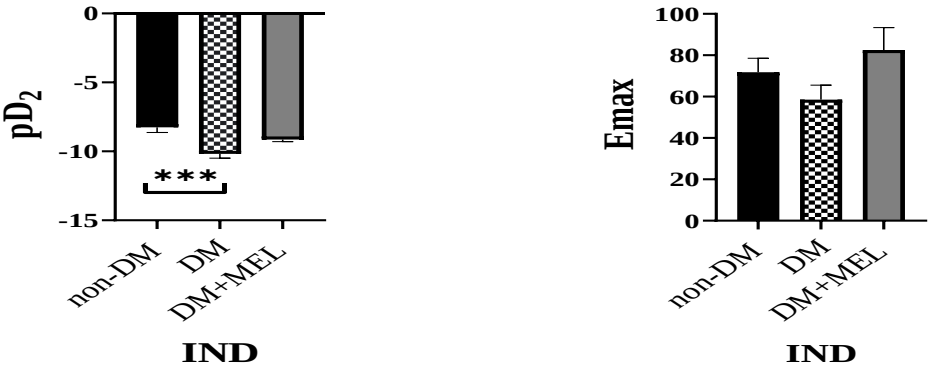

Figure 6: Emax and pD2 of vascular response to Ang 1-7 which pre-incubated with IND. [non-DM, non-diabetes mellitus rats; DM, Streptozotocin induced-diabetes mellitus rats; DM+MEL, Streptozotocin induced-diabetes mellitus rats treated with melatonin; pD2, potency; Emax, maximum efficacy; IND, Indomethacin]. [***; $P < 0.001$ level].

3.5. Effect of melatonin on the vasodilatory response to Ang 1-7 via calcium ion channels

The DRC to Ang 1-7 of pre-incubated aorta with NIF showed left warded-shift without any significant change in potency and maximum response mean in non-DM and STZ-induced DM aorta from control groups (Figures 7 A). Interestingly, while the aortic rings of MEL treated rats induced right warded-shift relaxation to Ang 1-7 in SZT-induced DM from control, and such effect was confirmed with maximum response mean value (Figure 7 C and Table 1). Besides, the values of Ang 1-7 including potency and maximum response mean were increased non-significantly in STZ-induced DM from non-DM followed with the same observation in rats treated with MEL in between (Figure 8).

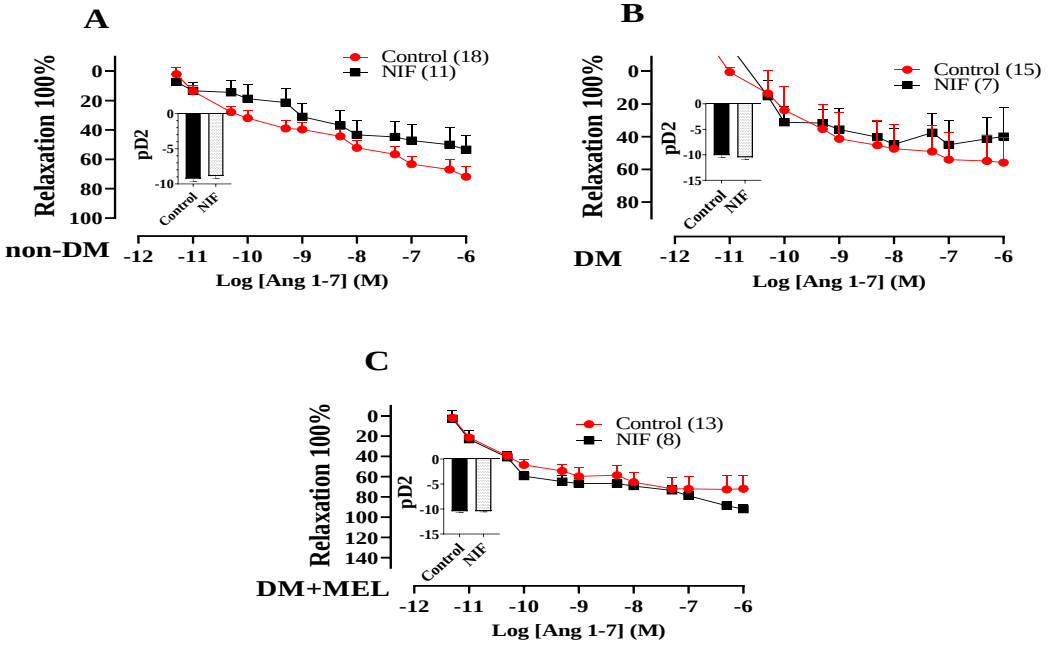

Figure 7: Concentration-response curve of Ang 1-7 in aortic rings pre-contracted with PE (1 µM) in the presence of NIF (20 µM). Points represent mean ± S.E.M of aortic rings from a total of animals sample size. (●, control; ■, NIF; non-DM, non-diabetes mellitus rats; DM, Streptozotocin induced-diabetes mellitus rats; DM+MEL, Streptozotocin induced-diabetes mellitus rats treated with melatonin; pD2, potency).

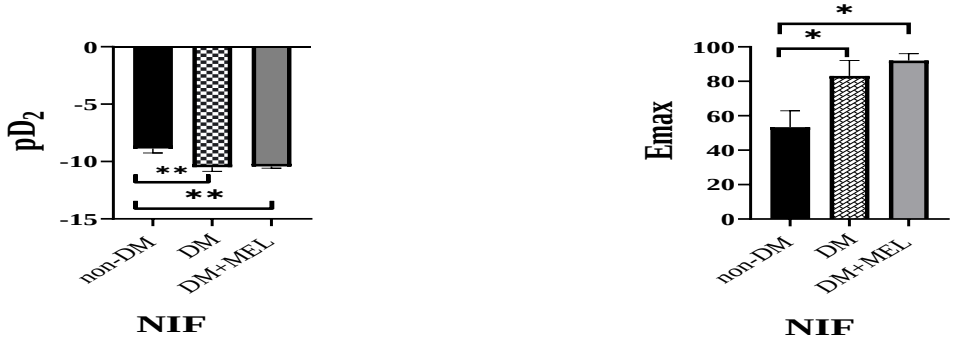

Figure 8: Emax and pD2 of vascular response to Ang 1-7 which pre-incubated with IND. pre-incubated with NIF. [non-DM, non-diabetes mellitus rats; DM, Streptozotocin induced-diabetes mellitus rats; DM+MEL, Streptozotocin induced-diabetes mellitus rats treated with melatonin; pD2, potency; Emax, maximum efficacy; NIF, Nifedipine]. [*; $P < 0.05$ level, **; $P < 0.01$ level].

3.6. Effect of melatonin on Ang 1-7 potency and maximum response via nitric oxide, cyclooxygenases and calcium ion channels

The pre-incubation of isolated aortic rings with IND showed non-significant change in Ang 1-7 potency, except that to STZ-induced DM treated with MEL which decreased significantly ($P < 0.01$) as compared to control (Table 1). In contrast, the maximum response was increased slightly in both non-DM and STZ-induced DM treated with MEL with unchanged such value in STZ-induced DM groups as compared to control. However, in pre-incubated vessels with L-NAME, the potency of Ang1-7 produced significant decrease ($p < 0.05$) in non-DM group and significant increase ($p < 0.001$) in STZ-induced DM treated with MEL as compared to control, meanwhile, non-significant change was observed in STZ-induced DM group as compared to control. On the other hand, the maximum response of Ang 1-7 was slightly increased in both non-DM and STZ-induced DM treated with MEL, beside, such effect was slightly decreased in STZ-induced DM group as compared to control. In the latter pre-incubation, the NIF produced in significant changes in Ang 1-7 potency in all studied groups as compared to control, surprisingly, the maximum response of Ang 1-7 was slightly decreased in both non-DM and STZ-induced DM as compared to control. Differently, NIF pre-incubation produced non-significant increase of Ang 1-7 in STZ-induced treated with MEL as compared to control.

Table 1: Effects of melatonin on the vascular Ang 1-7 reactivity via calcium channels and the EDRF in induced diabetic aortic rats

	pD2			***Emax***			**n**		
	non-DM	**STZ-induced DM**	**STZ-induced DM treated with MEL**	**non-DM**	**STZ-induced DM**	**STZ-induced DM treated with MEL**	**non-DM**	**STZ-induced DM**	**STZ-induced DM treated with MEL**
Control	-9.259± 0.369	-10.07± 0.413	-10.44± 0.269	71.76 ± 6.754	55.74± 15.54	71.85± 13.03	18	15	13
L-NAME	**-7.313 ± 0.467***	-9.435± 0.352	**-8.462±*** 0.185**	100.0 ± 31.11	45.00± 9.000	83.13± 9.000	21	9	11
Indomethacin	-8.262± 0.369	-10.18± 0.311	**-9.142±** 0.152**	58.51 ± 11.00	58.51± 7.067	82.54± 10.86	10	9	7
Nifedipine	-8.875± 0.378	-10.50± 0.360	-10.43± 0.155	53.35 ± 9.562	39.86± 17.59	92.04± 4.000	11	7	8

Data are presented as mean± standard error of mean. All studied groups were compared with their control using One way ANOVA. [*; $P < 0.05$ level, **; $P < 0.01$ level, ***; $P < 0.001$ level]. [non-DM, non-diabetes mellitus rats; DM, Streptozotocin induced-diabetes mellitus rats; DM+MEL, Streptozotocin induced-diabetes mellitus rats treated with melatonin; pD_2, potency; Emax, maximum response; n, sample size].

4. Discussions

The dramatic vasculoprotective roles of MEL in STZ-induced thoracic aorta was predominantly achieved by attenuated diabetic consequences. One of the primary consequences of diabetes on the vascular system is the development of VED followed by impairment of vascular tone [27]. Hence, the production of NO is decreased, as well as increased production of vasoconstrictor substances such as endothelin-1 [28]. Furthermore,

the chronic hyperglycemic state in diabetes promotes the formation of advanced glycation end products (AGEs) that are formed by the non-enzymatic reaction between glucose and proteins, and their accumulation in blood vessels leads to increased oxidative stress and inflammation [29].

It has been demonstrated that MEL affect vascular cells through activating MT_1R and MT_2R or by *via* scavenging free radicals [30, 31]. Additionally, MEL enhances the activity of endogenous antioxidant enzymes, such as superoxide dismutase and glutathione peroxidase, further reducing oxidative stress [32]. Likewise, it modulates inflammatory processes by inhibiting the activation of pro-inflammatory signaling pathways, such as nuclear factor-kappa B (NF-κB) [33]. On the other hand, It also suppresses the production of pro-inflammatory cytokines, including interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) [34]. Such actions produce decreased degeneration of histological texture [35].

Similarly, MEL provoked vasodilatory effect on Ang 1-7 in STZ-induced DM aorta through the elimination of dysfunction consequences. As a result, the rightward shift was observed most. Our results are supported by the finding demonstrated that MEL could attenuate DM exerts ,thereby, [36, 37]. Moreover, this modulation was also confirmed by maximum response and potency of Ang 1-7 (Figure 2 B and C). It has been shown that MEL improves endothelial function in terms of enhancement of NO synthesis and reduction of NO degradation [38].

The impacts of MEL on Ang 1-7 relaxation pre-incubated with L-NAME in DM condition generated the slight rightward shift curve. Interestingly, MEL receptors expression are attributed upon their localization in different parts of the cardiovascular system [39]. Therefore, the MT_2R activation in VED condition was confirmed in earlier studies observed [40]. In particular, the MT_1 found dominantly to be exist in both adventitia and in the endothelial cell layer [41]. Therefore, in this study the impact of administered MEL on Ang 1-7 relaxation was appeared most probably in DM condition under L-NAME pre-incubation. On the other hand, the significant decrease of Ang 1-7 potency was shown in both non-DM and STZ-induced DM followed by MEL administration. However, the lower production of NO, results in cGMP and protein kinase G (PKG) decrement [42]. Additionally, the majority of VED mostly contributable to imbalance release of vasoconstrictor and vasodilator factors, thereby, enhancement of vascular contraction function [43]. Eventually, the present results showed relaxatory response under MEL administration that confirmed by non-significant increase in maximum response against STZ-induced DM. It has been suggested that, MEL could abolish oxidative damage and VSMC loss *via* receptor-dependent mechanism [44].

In the present result, the pre-incubated aorta with IND offered the rightward shift curve of Ang 1-7 in non-DM condition that confirmed by the slight maximum response (Table 1). It has been demonstrated that VECs release EDRFs, thereby enhancing endothelium-dependent vasorelaxant action [45]. Herein, the PGI_2, is well-characterized as EDRFs that derived from endothelium arachidonic acid [46]. In contrast, such changes in STZ-induced DM, didn't generate such attenuation. This result is mostly states the over expressed COXs induced diabetes, however, there is no an obvious linkage between COX activity, NO and oxidative stress under VED condition [47]. The present results seem to indicate that, IND is more selective for COX-1 inhibition over COX-2 inhibition. Despite that, the inhibited COX had no impact on vasodilation to Ang 1-7 induced by PE in DM aortic rings. Similarly, additive MEL had abolished the degenerated consequences induced by STZ slightly; besides the Ang 1-7 potency was elevated significantly, but not maximum response. Such results may attribute to

release of vasoconstrictors COX related with pathway to enhance receptor–dependent counteracts EDRFs under DM condition [18]. Therefore, it is still unclear if IND-induced endothelium-dependent vasodilation could damage or protect the cardiovascular apparatus.

Our results also show that, MEL administration reduced DM consequences on Ang 1-7 relaxation of rings pre-incubated with NIF as non-selective L-type Ca^{2+} channel blocker slightly compared with DM condition (Figure 7 A, B and C). Recent study has indicated that, the free form of intracellular Ca^{2+} is remarkably contributes as an vital second messenger in smooth muscles contractility mediation [48]. Besides, the VSMCs' L-type voltage-gated Ca^{2+} channels function to uptake such vasoelement dominantly [49]. Hence, the generated ROS under DM condition provokes endothelium dependent contraction factors [50]. In contrast, the previous study showed that NIF contributes a dose-dependent vasorelaxation in endothelium-denuded aortic rings [51]. Meanwhile, another study has demonstrated that NO was included in the blocking of L-type Ca^{2+} influx *via* the NO-cGMP signaling [52]. In particular, the VSMCs dilation is dominantly attributed through either influx Ca^{2+} to VEDCs to promote NO production and its efflux to VSMCs by protein kinase G (PKG) activation [53]. Our study is the first indication that MEL most probably showed vasodilatory modulation in Ang1-7 by enhancing calcium vasomotion. Also such confirmed mechanism is agreed with finding that VED changes contributed in downregulation of such involved channels indirectly [54].

Conclusions

The present result demonstrated that MEL reduced the degenerative consequences of induced rats with STZ. In addition, MEL improves the vasodilatory attenuation of Ang 1-7 reactivity. The enhancement of MEL on endothelium-dependent vasodilation through Ang 1-7 reactivity shows a modulatory effect through providing antioxidant properties against VED, however, MEL did not change the level of its target action in NO-deficient induced by L-NAME. Additively, MEL in somehow abolished the degenerated consequences induced by STZ slightly under IND pre-incubation. Hence, the slight increment was observed in Ang 1-7 with additive MEL. In contrast, MEL interfereed with L-type Ca^{2+} channels through and enhanced Ca^{2+} vasomotion.

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Conflict of Interest

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

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