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RESEARCH PAPER

The Impact of Melatonin and its Agonist on Endothelin-1 Reactivity in Isolated Aorta in Continuous Light and Pinealectomized Rats

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A B S T R A C T:

The purpose of the present study is to investigate the influence of melatonin and its receptor (MT1, and MT2) agonist (Ramelteon) on vascular endothelin-1 (ET-1) reactivity in isolated aortic rings in continuous light emitting diode (LED) exposure and pinealectomized rats. **Materials and Methods:** Two sets of experiments were performed in this study. In the first experiment, animals were divided into four groups: (control, continuous LED exposure rats, continuous light + Melatonin administration, and continuous light + Ramelteon administration). In the second experiment, animals were categorized into five groups: Control, rats underwent pinealectomy, pinealectomy + Melatonin administration, pinealectomy + Ramelteon administration, and pinealectomy + a continuous LED exposure. **Results:** The obtained results indicated that in constant light exposure of the rats for 10 weeks, the ET-1-induced aortic ring contraction was markedly reduced. Conversely, melatonin and its agonist (Ramelteon) markedly elevated the ET-1-induced aortic ring contraction. Neither, melatonin nor its agonist supplementation significantly increased ET-1 Emax. Similarly, in the second experiment after 6 weeks of pinealectomy and pinealectomy with continuous light exposure to rats significantly decreased aortic ring contraction. Moreover, ET-1 Emax significantly and ET-1 efficiency and area under the curve (AUC) were slightly elevated in pinealectomy with light exposure group compared to the pinealectomized rats. On the other hand, ramelteon shifted the dose-response curve (DRC) of ET-1 to the left side. **Conclusion:** The results suggested that most of the melatonin actions on vascular modulation are due to its binding to MT1and MT2 receptors.

KEY WORDS: Melatonin, Ramelteon, Endothelin-1, Continuous Light, Pinealectomy, Rat aorta
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1. INTRODUCTION:

Melatonin, chemically *N*-acetyl-5-methoxytryptamine, is a tryptophan derivative; the vertebrate pineal gland primarily secretes it as a neurohormone. Nigh-time melatonin secretion is affected by photoperiod. The α1- and β1-adrenoreceptors regulate the manufacture of melatonin by inducing the phosphorylation of Aralkyl amine N-Acetyltransferase by cyclic AMP-dependent protein kinase, a crucial enzyme in the production of melatonin (Acuña-Castroviejo, Escames et al. 2014).

The MT1, MT2, and MT3 receptors are three high-affinity melatonin receptor subtypes that have been discovered. The cardiovascular system has both MT1 and MT2 melatonin receptors which it has complicated impacts on vascular tone; MT1 receptors mediate vasoconstriction(Favero, Rodella et al. 2014), while MT2 mediates vascular dilatation(Zhao, Zhang et al. 2017).

Additionally, various signaling molecules regulate the contraction of vascular smooth muscle cells (VSMC). For instance, endothelial nitric oxide synthase (eNOS) produces NO and reduces the contractility of blood vessels (Zhao, Vanhoutte et al. 2015) . On the other hand, the most effective long-lasting vasoconstrictor is ET-1 (Nichols 2016). ET-1A and ET-1B are two subtypes of

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particular receptors that control the vasoactive effects of ET-1(Agabiti-Rosei, Favero et al. 2017). Whereas the production of NO and prostacyclin by endothelial cells' ET-1B receptors results in vasodilation (Wynne, Chiao et al. 2009)

By eliminating melatonin's circadian production, a pinealectomy and constant light exposure result in a relative melatonin deficiency, and the ensuing hypertension is known as "melatonin-deficient" hypertension (Simko, Reiter et al. 2013). Melatonin induces the relative dominance of the parasympathetic nervous system, which enhances endothelial function and raises the bioavailability of NO, attenuating neurohumoral stimulation (level of renin, norepinephrine, and insulin), and reducing oxidative burden. Furthermore, specific melatonin receptors affect the vasomotor system by decreasing peripheral artery resistance, lowering the circulating fluid volume, and improving the flexibility of big vessels may lower blood pressure (Simko, Reiter et al. 2013, Tousoulis, K Georgakis et al. 2015).(Oxenkrug and Summergrad 2010)

While the exact mechanisms underlying melatonin's antihypertensive effects are still unknown, its central effects, systemic antioxidant, anti-inflammatory, and lipid-lowering effects, as well as its actual effects on the vascular endothelium, will all or part of them have an impact (Paulis, 2007). It is potentiated action on the release of vasodilator chemicals in endothelial cells which is mostly due to factors contributing to vasodilatation under the influence of melatonin. One of these is NO, which relaxes the tension in vascular smooth muscle and prevents hypertension from arising due to the inhibition of the NO-producing eNOS. Furthermore, melatonin triggers the release of the vasodilators bradykinin and prostacyclin from platelet cells into the bloodstream while assuring the accumulation of calcium ions in the vascular endothelium (Pogan, Bissonnette et al. 2002, Paulis, Pechanova et al. 2010, Arushanyan and Shchetinin 2015).

As a melatonin agonist, Ramelteon was given FDA approval in 2005. It has an affinity for the SCN's melatonin MT1 (which blocks the arousal impulses that keep people awake) and MT2 (which synchronizes the biological clock to the day-night cycle) receptors. Ramelteon as a melatonergic receptor agonist reduces sleep latency and might also boost sleep quality, efficiency, and duration (Nishikimi, Numaguchi et al. 2018, Cox and Takahashi 2019). Moreover, ramelteon significantly impacts the cardiovascular like maintaining heart rate variability, which has a therapeutic effect on cardiac activity and may prevent cardiac dysfunction while you sleep (Yoshimoto, Yamashiro et al. 2021), and chronic ramelteon medication improves the age-associated increase of systolic blood pressure (SBP) (Oxenkrug and Summergrad 2010).

Melatonin has been known to regulate vascular tone because continuous light exposure and pinealectomy reduce melatonin production, the vascular tonicity tends to increase and as a concomitant, the contractility of the arteries will rise. Furthermore, the exact mechanism of such change has not been understood yet, therefore the current study aims to investigate the influence of melatonin and its receptor (MT1, and MT2) agonist (Ramelteon) on vascular ET-1 reactivity in isolated aortic rings in continuous LED exposure and pinealectomized rats.

2. Material and method

2.1. Laboratory animals

Adult male albino rats (*Rattus norvegicus*) with 150-250 g body weights were used in this study. Animals were placed in plastic cages and kept in the Animal House of the Department of Biology, College of Science, Salahaddin -University –Erbil under standard laboratory conditions. (wheat 66.6% ,soya 25.6%, corn oil 4.4%, limestone 1.5%, salt 0.63%, methionine 0.158%, Lysine 0.24%choline chloride 0.062%, multivitamins 0.5% and trace elements 0.05%) and tap water *ad libitum*. The room temperature was controlled in the 22±4 ºC and the photoperiod at about 12/12 dark/light cycle.

2.2. Experimental design

In this study, two sets of experiments were carried out to ascertain the influence of melatonin and its agonist (Ramelteon) on a vascular ET-1 dose-response curve in isolated thoracic aorta in continuous light exposure rats for 10 weeks and pinealectomized rats for 6 weeks. In the first experiment, 35 rats were used**.** Animals were randomly separated into 4 sets of 6-8 rats placed in special cages, and it was designed as the

following: the first group represents a control (n=8): they received a standard diet and about 12/12 dark/light cycle photoperiod. The second group (n=8) was exposed to continuous light (LED) (24 h/day, light intensity of> 600 lux) and received a standard diet and water *ad libitum*. The third group (n=9) exposed to continuous light (LED) (24 h/day, light intensity of> 600 lux) + Melatonin was administrated daily orally by gavage (10 mg/kg body weight of rats) every morning (09:00-11:00). Fourth group (n=10) exposed continuous light (LED) (24 h/day, light intensity of> 600 lux) +Ramelteon was administrated daily orally by gavage (10 mg/kg body weight of rat) every morning (09:00-11:00).In the second experiment, 40 rats were used. Animals were categorized into five groups, and it was designed as the following: The first group represents a Control (n=7): they received a standard diet, water, and about 12/12 dark/light cycle photoperiod. The second group (n=9) of rats underwent pinealectomy and received a standard diet and water *ad libitum*. The third group (n=7) of rats underwent pinealectomy and administrated Melatonin (10 mg/kg body weight of rat) in drinking water. The fourth group (n=9) of rats underwent pinealectomy and administrated ramelteon (10 mg/kg body weight of rat) in drinking water. Finally, the fifth group underwent pinealectomy and was exposed to continuous light.

2.3. Pinealectomy

Pinealectomies were carried out following the typical protocol outlined by Hoffman and Reiter (Hoffman and Reiter 1965). After anesthetizing with ketamine: xylazine mixture (90 mg/kg, i.p. and 10 mg/kg, i.p. respectively) intraperitoneally (Rameshrad, Babaei et al. 2016), A dental drill was used to make an imperfect square cut, and after folding the anterior portion of the skull over the pineal gland, fine-tipped forceps were utilized to incision of superior sagittal venous sinus, and the pineal gland, located underneath the sinus was removed. The scalp was then closed with a steel surgical staple after the skull flap was pushed back and covered with procaine penicillin antibiotic powder, and the skin was sutured by a 3-0 silk suture. The wound was then cleansed with ionophore solution, allowed to dry, covered with procaine penicillin antibiotic powder again, and placed on its side in a warmed recovery cage. Finally, the rats received tetracycline antibiotics in drinking water for one week.

2.4. Isolated aorta preparation

The animals were anesthetized with ketamine: xylazine mixture (90 mg/kg, i.p. and 10 mg/kg, i.p. respectively) intraperitoneally (Rameshrad, Babaei et al. 2016). The chest cavity was opened following anesthesia, and after extra tissue and fat were removed, the aorta was separated and moved to a beaker containing cold Krebs-Ringer solution with the following composition in millimoles per liter: 118 NaCl, 4.7 KCl, 2.5 CaCl2, 1.2 MgSO4, 1.2 KH2 PO4, 25.0 NaHCO3, and 11.0 glucose, aerated with 95% O2 -5% CO2 (pH 7.4), then excess surrounding tissues were removed and 4 aortic rings each about 3 mm in length was prepared.

2.5. Isometric tension recording in isolated aortic rings

Two stainless steel clamps (Tissue clamp, Model Le 0140, Panlab Harvard equipment) were placed on either open side of the aortic ring and quickly passed the isolated thoracic aorta. One of the clamps is fastened to a hook in the organ bath, in contrast, the other clamp is fastened to a thread leading to a force transducer to capture aortic vibrations and vasoreactivity before transmitting them to the computer system software via an amplifier (Quad bridge amplifier power lab 8/35) (LabChart 7.1). The aortic rings were suspended in organ baths containing 10 mL Krebs-Henseleit solution, maintained at 37°C, and bubbled with roughly 95 percent O2 and 5 percent CO2 to maintain the pH at 7.4. The segments were then placed inside the chamber (Automatic organ bath, Panlab Harvard Apparatus, USA). At intervals of 15 minutes (min), the aortas were cleaned. After 60 minutes of acclimatization with a 2 g rest tension. Then, thoracic aorta contractions were brought about by adding a submaximal concentration (60 mM) of potassium chloride (KCl) to the water to test the produced aortic segments' structural integrity. The state of the preparations was deemed to be equilibrium when the amplitude of two consecutive thoracic aorta contractions matched. First, the isolated aorta was contracted with 1 M Phynylphrine (ph), and then

the aorta was relaxed with 1 M Acetylcholine to validate the viability of the used aorta. After that, cumulative ET-1 concentrations were applied to the aortic rings. If the aorta relaxes, it is still alive; otherwise, the aorta is denuded. The cumulative-dose response curves were acquired in 5-minute intervals, and it was left to run until they reached their maximum contraction and plateau.

Statistical analysis

Vascular contraction induced by ET-1 was expressed as a percentage of tension produced by 60 mM of KCl. Two-way analysis of variance (ANOVA) was used for comparison among the studied groups followed by the Sidak post hoc test. The area under the curve was calculated from the DRC of ET-1 for all groups, and a difference AUC was applied. One-way ANOVA was used to compare the Pd_2, $E_{max,}$ and AUC among all studied groups followed by the Tukey post hoc test. Graphpad prism (Version 8) was used Mean ± standard error of the mean is used to express the data. When $p<0.05$, differences are deemed statistically significant.

3. Results

3.1. The effect of Melatonin and Ramelteon on vascular response to ET-1 in continuous light exposure rats

As shown in figure 1, the obtained results showed that continuous light exposure of the rats for 10 weeks significantly shifted the DRC of ET-1 on the right side, which means that the ET-1 induced intact aortic ring contraction markedly reduced and according to two-way ANOVA analysis, such reduction was started from $3*10^{-8}$ mm to $3*10^{-7}$ mm of ET-1 DRC. The efficacy of ET-1 (Emax) was significantly decreased from 131.4-±7.740 in control rats to 89.24-9.830 in the continuous light group. Furthermore, the AUC value was also reduced in the DRC of ET-1 from 117.8±8.422 to 63.9±12.32.

The current study's findings revealed that melatonin and its agonist (Ramelteon) for 10 weeks markedly shifted the DRC of ET-1 to the left side. Interestingly, supplementation of melatonin at 10mg/kg body weight of rats exposed to bright light significantly ($P< 0.01$) increased ET-1 Emax from 89.24±9.830 to 158.5-6.619, also Ramelteon reduced Emax significantly ($P <0.05$). On the other hand, the AUC values of both melatonin and ramelteon groups compared to the continuous light group was significantly ($P< 0.05$) elevated from 63.9±12.32 in a continuous light group to 109.3±10.41 and 113.3-18.92 in melatonin and ramelteon group respectively (Figure: 1, Table: 1).

Table 1: The maximum response (Emax) and the potency (pD2) of the Impact of Melatonin and Ramelteon on Endothelin-1 Reactivity in Continuous Light of rat isolated aorta (Mean ± SEM)

Groups	**n**	***Emax* %**	**pD2**	**AUC**
Control	8	131.4 ±7.740 *	-7.152±0.1135	117.8±8.422 **
Continuous light	8	89.24±9.830	-6.665±0.5593	63.9±12.32
Continuous light+ Melatonin	8	158.5±6.619 ***	-5.719±1.309	109.3±10.41 *
Continuous light+ Ramelteon	8	139.1±19.22 *	-7.083±0.3306	113.3±18.92 *

The star (*) sign indicates the comparison between the continuous light group with the other studied groups. * means ($P<0.05$) ** means ($P<0.001$), *** means ($P<0.0001$).

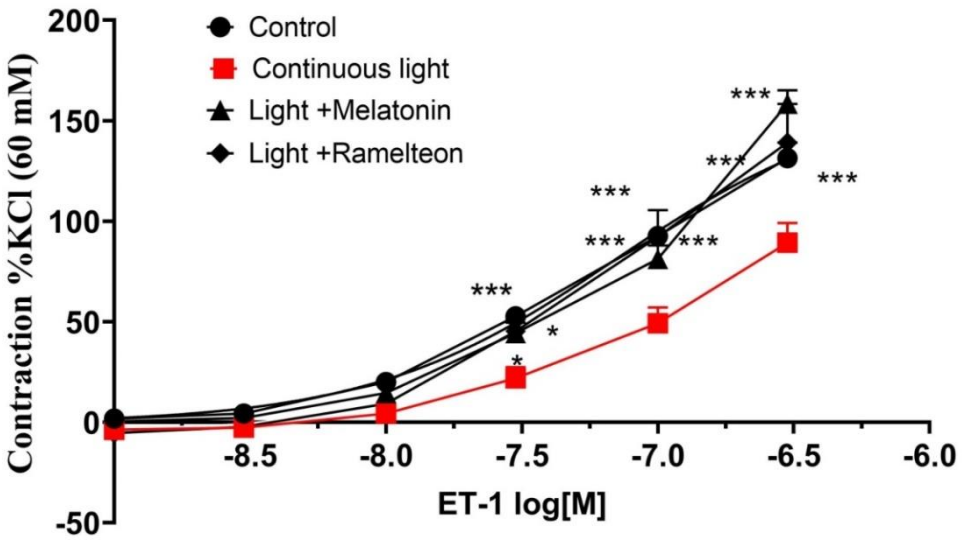

Figure 1: Effect of melatonin and ramelteon on ET-1 dose-response curve in continuous light exposure rats

3.2. The effect of Melatonin and Ramelteon on vascular response to ET-1 in pinealectomized rats

After pinealectomy of rats for 6 weeks, ET-1's DRC has been pointedly moved to the right side, which indicates a significantly ($p < 0.01$) decreased intact aortic ring contraction. The results of the two-way ANOVA analysis showed that such a reduction started from 10-8 mm to 3*10-7 mm of ET-1 DRC. Besides, rats with pinealectomies showed a slight decline in the efficacy and AUC value of ET-1 (141.0±5.558, 90.1±10.5) compared with the control rats (157.9 ±8.447, 133.1±9.528) but efficiency was increased from -7.103±0.0891 to -6.766±0.1588. Additionally, pinealectomy with continuous light exposure rats shifted the ET-1 contraction response curve to the right side too, but not as effective as the pinealectomy procedure alone analysis showed ET-1 E_{max} elevated significantly ($p < 0.001$) and ET-1 efficiency and AUC slightly increased (-5.595±1.208, 117.5±9.256 in pinealectomy with light exposure group compared to the pinealectomized group).

On the other hand, the DRC of ET-1 shifted to the left side after ramelteon administration (10mg/kg body weight) for 6 weeks in pinealectomized rats. It showed an enhanced intact aortic ring contraction significantly ($P< 0.001$), which increased started from 10^{-8} mm to $3*10^{-7}$mm of ET-1 DRC. In contrast, melatonin administration (10mg/kg body weight) of pinealectomized rats during this period showed a non-significant change in ET-1 DRC without significant change in ET-1 efficacy, efficiency, and AUC value in both melatonin and ramelteon supplementation during the experimental period. (Fig.2, Table 2).

Table 2: The maximum response (Emax) and the potency (pD2) of the Impact of Melatonin and Ramelteon on Endothelin-1 Reactivity in pinealectomized rat isolated aorta (Mean ± SEM)

Groups	**n**	***Emax* %**	**pD2**	**AUC**
Control	8	157.9±8.447	-7.103± 0.0891	133.1± 9.528 **
Pinealectomy	8	141.0±5.558	-6.766 ±0.1588	90.1± 10.5
Pinealectomy + Continuous light	7	185.9±5.650 ***	-5.595 ±1.208	117.5 ± 9.256
Pinealectomy + Melatonin	7	135.1±9.800	-5.666± 1.535	90.55± 8.467
Pinealectomy + Ramelteon	**7**	134.0±6.603	-5.99 ±1.091	115.5± 9.909

The star (*) sign indicates the comparison between the pinealectomy group with the other studied groups. * means (P<0.05) ** means (P<0.001), *** means (P<0.0001).

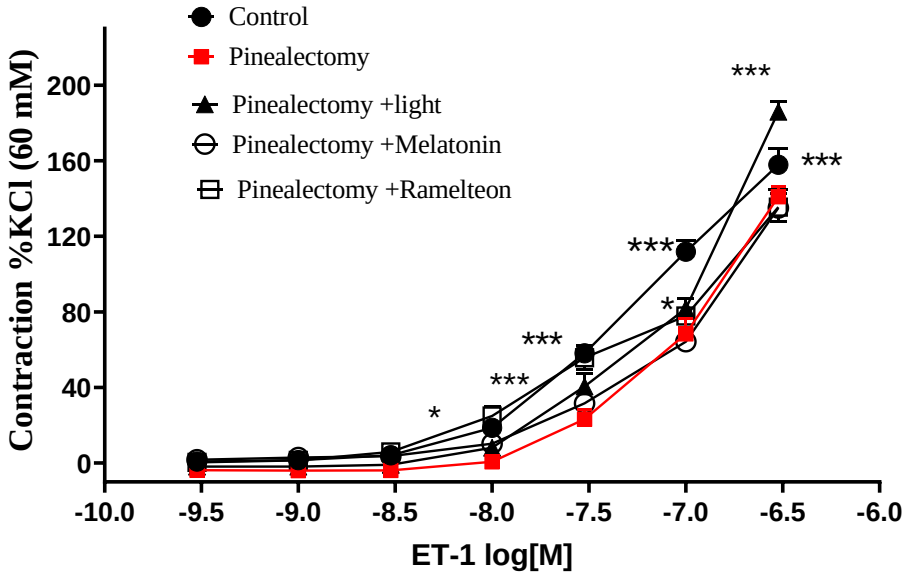

Figure 2: Effect of melatonin and ramelteon on ET-1 dose-response curve in pinealectomized rats.

4. Discussion

Results of the current study established that animals with continuous LED exposure for 10 weeks and pinealectomized rats after 6 weeks significantly changed the DRC of ET-1. Reports have suggested that ET-1 produces a vascular contraction in the rat-isolated thoracic aorta (Tykocki, Wu et al. 2015). The most probable mechanism of this action is its endothelin-A (ETA) receptor localization in vascular smooth muscle cells (VSMC) (Schneider, Boesen et al. 2007) Both ETA and endothelin B (ETB) receptors may be connected to phospholipase C (PLC) in the VSMCs through a GTP-binding protein. GTP- binding protein lead to PLC activation, then stimulate ca+2 release from the sarcoplasmic reticulum by inositol trisphosphate (IP3) (Bouallegue 2010). Moreover, the ETA receptor causes Ca2+ influx from the extracellular environment, as a result, VSMCs constrict in response to higher Ca2+ concentrations (Zhang 2016). There is evidence that both melatonin deficiency models, pinealectomy, and continuous exposure to artificial light for 24 hours are linked to elevated blood pressure and vascular

dysfunction (Simko, Reiter et al. 2013). There are relatively few reports on the impact of melatonin insufficiency on the ET-1 DRC. However, a previous study showed that except for the slightly enhanced contractile responses to ET-1 in the endothelium-denuded thoracic aorta, pinealectomy did not affect the vascular reactivity to different vasoconstrictor agents (Kurcer, Sahna et al. 2006).

Because melatonin has strong antioxidant properties (Tykocki, Wu et al. 2015) and melatonin deficiency due to LED light exposure and pinealectomy will reduce the antioxidant status and hence raises the cellular ROS, and the latter mainly causes vascular endothelial dysfunction (Abdraboh, El-Missiry et al. 2022). One hypothesis of reduction of ET-1 DRC might be due to these factors affecting melatonin production and release and hence changing the ET-1 vascular reactivity. Interestingly, recent reports showed that in spontaneously hypertensive rats, artificial light at night reduced the expression of ET-1, sarco/endoplasmic reticulum Ca(2+)-ATPases, which are crucial for controlling cardiac contractility (Sutovska, Miklovic et al. 2021).

Because of its direct vasodilatory effect, melatonin can reduce endothelial dysfunction (Arushanyan and Shchetinin 2015). The new finding of the current study indicated that the administration of melatonin against continuous light significantly changed the dose response of ET-1 compared with ET-1 DRC in LED exposure rats. Another related study on isolated coronary arteries from porcine hearts showed when portions of vessels with an intact endothelium were used to measure the isometric tension of the smooth muscle, they would predictably relax with an increase in NO levels after sodium nitroprusside and other nitrates were added to the medium; however, this relaxation was mysteriously prevented when melatonin was added as a supplement (Tunstall, Shukla et al. 2011).

Additionally, melatonin's capacity to enhance the antioxidant effects of compounds with antioxidant potentials, such as glutathione, vitamin C, and vitamin E, may also help to improve vascular functioning (Paulis, Simko et al. 2012). Moreover, (Zuo and Jiang 2020) concluded that melatonin which represents a potent antioxidant elsewhere could improve vascular ET-1 reactivity through its receptor-independent pathway. But how melatonin causes this modulation of ET-1 in isolated vascular rats, according to a number of investigations, the exact mechanism is not known yet.

Another interesting finding of the present study was a novel melatonin agonist (ramelteon) supplementation against continuous bright light exposure and pinealectomized group rats. Ramelteon had the same impact as melatonin on the ET-1 DRC (Table 1, 2, and Figure 1,2). An earlier study showed that ramelteon has a 3–16 times greater affinity for the MT1 and MT2 melatonin receptors than melatonin, making it a potent and highly selective agonist of these receptors (Kato, Hirai et al. 2005). According to our intense review, this is the first time to investigate how melatonin agonists such as ramelteon modulate ET-1 DRC in the isolated thoracic aorta. However, one report on the isolated aorta concluded that ramelteon affects SMC contraction in the human urinary bladder smooth muscle contraction (Ercan, Hekim et al. 2022). It is noteworthy to mention here that melatonin's main functions attenuating of ET-1 vascular reactivity may be due to its M1 and M2 receptors besides its potent antioxidant activity (Yang, Zhang et al. 2022).

5. Conclusions

Continuous bright light (LED) and pinealectomy caused a marked reduction in vascular ET-1 reactivity in isolated aortic rings. Melatonin and its MT1 and MT2 receptor agonist (Ramelteon) significantly attenuate the ET-1-induced vascular contractility. These results suggested that most of the melatonin actions on vascular modulation are due to its binding to MT1and MT2 receptors.

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Conflict of Interest: The authors declare that there is no conflict of interest

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