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ORIGINAL ARTICLE

Effects of Melatonin on Blood Pressure, Oxidative Stress and Placental Expressions of TNFα, IL-6, VEGF and sFlt-1 in RUPP Rat Model of Preeclampsia

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Introduction

Preeclampsia (PE) is known as a major cause of maternal and neonatal morbidity and mortality. Maternal hypertension, proteinuria, placental dysfunction and imbalance of angiogenic factors can be observed in PE (1). The reduction in uteroplacental perfusion can cause ischemia and the ischemic placenta releases soluble factors causing maternal endothelial dysfunction and hypertension (2). These alterations also lead to an increased production of proinflammatory cytokines such as tumor necrosis factor alpha (TNF-α) and IL-6 from the placenta (3). In recent years, a wide body of evidence supports that the imbalance in the soluble fms-like tyrosine kinase I (sFlt-1) or vascular endothelial growth factor (VEGF) and placental growth factor (PIGF) play important role in etiology of PE (4,5). sFlt-1 blood levels and placental expression increase during PE which subsequently falls postpartum and the increased sFlt-1 and decreased VEGF levels are considered together in systemic endothelial dysfunction (6).

PE may be affected by seasonal variations such as temperature, humidity and changes in circadian rhythms and these variations are considered in pathogenesis of PE (7–9). Melatonin is a hormone mainly synthesized and secreted from the pineal gland has antioxidative and antihypertensive effects. In recent years, melatonin, a marker of circadian rhythm, to be involved in normal pregnancy and pathogenesis of PE (10,11). It was reported that human placenta has high affinity melatonin receptors, MT1 and/or MT2 and melatonin promotes placental cell survival through these receptors (11). Furthermore, placenta may produce melatonin as much as pineal gland during pregnancy (12). The studies dealing with the relationship between the circulating levels of melatonin and PE is limited, however, significantly lower melatonin level were obtained in PE compared to normal pregnancy (10,13). Reduction in level of melatonin, downregulation of melatonin receptors in placenta, hypertension and increased oxidative stress observed in PE were considered in potential utility of melatonin in PE (14,15). Despite to the preliminary results, the role of pineal gland secreted melatonin in pathogenesis of PE is remains unclear, and the detailed experimental studies are required. Therefore, in this study, we investigated the effects of melatonin on blood pressure, oxidative stress and the alterations of TNF-α, IL-6, VEGF and sFlt-1 blood levels and placental expression using reduced uterine perfusion pressure (RUPP) model in pinealectomised rats and to discuss the potential role of melatonin in pathogenesis of PE.

Methods

Animals

This study was performed in Canakkale Onsekiz Mart University Experimental Research Center (COMUDAM) by the approval of Laboratory Animals Local Ethics Committee (Protocol number 2013/08-02). Totaly 42 Wistar rats (220–250 g) were used. Rats were maintained in air-conditioned rooms (21 ± 2) with 12 h light-dark cycle and fed *ad libitum* with standart rat chow and tap water.

Pinealectomy Operation

SHAM pinealectomy (PINX) and PINX were performed under general anesthesia (60 mg/kg ketamine and 5 mg/kg xylazine) before the pregnancy according to our previous study (16).

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Three days after SHAM PINX and PINX, rats were mated in individual cages. Melatonin (Absolute GR for analysis, Cas-No-73-31-4, MERCK, Germany) was added in drinking water (1 mg/kg/d freshly prepared every day) until first day of gestation except for SHAM group in order not to cause melatonin deficiency.

Experimental Design

Pregnant rats were divided into six groups.

1st group (SHAM; $n = 7$): SHAM PINX + SHAM RUPP.

2nd group (PINX; $n = 7$): PINX.

3rd group (PINX+PE; $n = 7$): PINX + RUPP.

4th group (PINX+PE+MEL1; $n = 7$): PINX + RUPP + melatonin (sc, 5 mg/kg/d, by the 1st d of gestation).

5th group (PINX+PE+MEL14; $n = 7$): PINX + RUPP + melatonin (sc, 5 mg/kg/d, by the 14th d of gestation).

6th group (PINX+SHAM RUPP+MEL1; $n = 7$): PINX + SHAM RUPP + melatonin (such as in 4th group).

Melatonin (1 mg/kg/d oral and 5 mg/kg/d sc) was dissolved in physiological solution contains 5 % ethanol.

RUPP Operation

The RUPP rat model of PE was adapted by Li and colleagues and surgical procedures were completed with rats under isoflurane anaesthesia (17). On the 14 d of gestation, pregnant rats underwent clipping, and a silver clip (0.203 mm ID) was placed around the abdominal aorta above the iliac bifurcation. Another silver clips (0.100 mm ID) were also placed on branches of both right and left ovarian arteries that supply the uterus. These procedures reduce uterine blood flow in the pregnant rats by 40%. If the clipping procedure resulted in reabsorption of all fetuses, those rats were excluded from the experiment.

Determination of Urinary Protein and Creatinine

On the 12 and 19 d of gestation, 12 h urine samples were collected in metabolic cages. The urinary protein and creatinine measurements were determined using by turbidimetric and colorimetric methods, respectively (ROCHE Diagnostics GmBH).

Blood Pressure Measuring

On the of 20 d, the rats were underwent invasive blood pressure recording under inhalation anaesthesia. The *a. carotis* was isolated, and following the cannulation the rats were connected to the device (Biopac MP35 Systems INC, USA). Blood pressure values were recorded for 1 hour and no handled during recording procedure (18).

Biochemical Analysis

Intracardiac blood samples were collected into EDTA containing tubes for plasma separation in the 20th d of gestation. The TNF-α (Bender MedSystems, Vienna, Austria), VEGF-C (Bender MedSystems, Vienna, Austria), VEGF-A (Thermo Scientific, USA), sFlt-1 (MyBioSource, San Diego, CA, USA), IL-6 (Bender MedSystems, Vienna, Austria) and Malondialdehyde (MDA) (MyBioSource, San Diego, CA, USA) concentrations were measured using by ELISA. The VEGF level referenced of VEGF-C, because VEGF-A levels were observed undetectable limits. Total antioxidant status (TAS) and total oxidant status (TOS; Rel Assay Diagnostics, Gaziantep, Turkey) levels were estimated spectrophotometrically by using commercial kits. Oxidative stress index (OSI) value was calculated by using TAS and TOS measurements.

Fetus and Tissue Harvesting

On the 20th d of the study, rats were anaesthetized by 2% isoflurane and the uterines were taken out from the abdomen. The fetuses located on the proximal and middle of the uterine were harvested and the gestational sac were isolated. Placental tissue samples were harvested in *DNAse/RNAse free* tubes and stored in −80°C until analyses.

Genetic Analyses

After homogenization of tissues total RNA isolation was performed manually (PureLink RNA Mini Kit, Life Technologies), and the RNA concentrations were evaluated by Qubit® Fluorimeter (Invitrogen, USA). Following manually synthesising cDNA (High Capacity cDNA Reverse Transcription Kit ABI) qRT-PCR procedure was started.

Statistical Analyses

Biochemical and genetic values were evaluated by IBM SPSS Statistics for Windows, Version 16.0 (Armonk, New York, USA: IBM Corp.) Groups were compared by One Way Anova and Man Whitney U test, and $p < 0.05$ was accepted as statistically significant. To evaluate the expression levels of the genes $2^{-\Delta\Delta Ct}$ method was used [ΔΔCt (Ct Target gene- Ct reference gene)].

Results

General Values

The mean body, fetal and placental weights, individual fetus number and placental index values were recorded in the 20th d of gestation. RUPP operation affected the fetus number and weights, however melatonin had limited effect on these values. The dramatically decreasing of urine

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Table 1. General values of rats and fetuses

Parameters	Groups					
	SHAM	**PINX**	**PINX+RUPP**	**PINX+RUPP MEL1**	**PINX+RUPP MEL14**	**PINX MEL1**
Body weight (g)	300 ± 7	285 ± 7	234 ± 10	241 ± 6	246 ± 5	300 ± 6
Mean fetal weight (g)	2.4 ± 0.1	2.8 ± 0.2	2.2 ± 0.1[b]	2.1 ± 0.7[a,b,c]	2.2 ± 0.1	2.3 ± 0.1
Placental weight (g)	0.56 ± 0.1	0.55 ± 0.1	0.45 ± 0.1[d,e]	0.48 ± 0.1[b]	0.52 ± 0.1	0.47 ± 0.1[b,e]
Individual fetus number	11	10	3	4	4	12
Placental index (g)	0.23 ± 0.1	0.21 ± 0.1	0.21 ± 0.1	0.23 ± 0.1	0.24 ± 0.0	0.21 ± 0.1
Urine volume (12 d)	16.3 ± 1.8	9.3 ± 1.3[f]	19.1 ± 2.3[g]	17.9 ± 3.1	13.9 ± 0.9	16.3 ± 1.8
Urine volume (19 d)	14.9 ± 1.7	9.7 ± 1.4	13.6 ± 1.5	11.4 ± 2.2	15 ± 1.2	13 ± 2.0
Urine protein/creatinine (12 d, %)	0.91 ± 0.1	0.65 ± 0.1[a]	0.65 ± 0.1	0.83 ± 0.1	0.77 ± 0.1	0.68 ± 0.1
Urine protein/creatinine (19 d, %)	0.87 ± 0.1	0.68 ± 0.1	1.66 ± 0.5[b,h]	0.93 ± 0.1	1.11 ± 0.3	0.82 ± 0.1

[a]compared to SHAM group $p < 0.05$, [b]compared to PINX group $p < 0.05$, [c]compared to PINX+MEL1 group $p < 0.05$, [d]compared to SHAM and PINX groups $p < 0.05$, [e]compared to PINX+RUPP+MEL14 group $p < 0.05$, [f]compared to SHAM, PINX+RUPP, PINX+RUPP+MEL1 and PINX+RUPP+MEL14 groups $p < 0.05$, [g]PINX+RUPP+MEL14 and PINX+MEL1 $p < 0.05$, [h]PINX+RUPP compared to 12[th] d $p < 0.05$.

volumes were recorded in PINX+RUPP and PINX+RUPP+ML1 groups (Table 1, $p < 0.05$).

Blood Pressure Values

The higher MAP values were observed in PINX and PINX+RUPP groups, and PINX+RUPP group has significant MAP records compared to others groups without PINX (Table 2, $p \leq 0.05$).

Biochemical Values

The significant MDA levels were observed in PINX and PINX+MEL1 groups compared to SHAM (Table 2, $p \leq 0.05$). Higher OSI value was observed in PINX group (Table 2, $p \leq 0.05$).

In blood VEGF levels, decreasing in PINX group and increasing in melatonin administrated groups were observed (Table 2, $p \leq 0.05$). There was no significant changes in blood TNF-α, IL-6 and sFlt-1 values in all groups (Table 2). The TNF-α level was recorded as 18.8 pg/mL in SHAM, 35.2 pg/mL in PINX+RUPP and 42.4 pg/mL in PINX+RUPP+MEL14 group but these dramatic changes were not found significant (Table 2).

Placental Gene Expression Results

VEGF expression levels were decreased in all melatonin injected groups compared to the others, but significant decrease was obtained only in PINX+RUPP+MEL1 group (Figure 1, $p < 0.05$). Dramatically decreased sFlt-1 gene expression values were observed in all melatonin administrated groups, however the alterations were not found significant (Figure 2). Significant increasing of the TNF-α expression levels was observed in PINX+RUPP, PINX+RUPP+MEL1 and PINX+RUPP+MEL14 compared to SHAM. On the other hand, significant decreasing was obtained in PINX+MEL1 group compared to SHAM (Figure 3, $p < 0.05$). Placental IL-6 expression levels were found decreased in all melatonin injected groups, furthermore significant decreasing was obtained only in

Table 2. Mean arterial pressure (MAP) and blood MDA, OSI, TAS, TOS, VEGF, sFlt-1, TNF-α and IL-6 levels of all groups

Parameters	Groups					
	SHAM	**PINX**	**PINX+RUPP**	**PINX+RUPP MEL1**	**PINX+RUPP MEL14**	**PINX MEL1**
MAP	83 ± 4	98 ± 7	111 ± 9[a]	83 ± 3	86 ± 3	77 ± 2[b]
MDA	9.7 ± 0.5	12 ± 0.6[c]	11 ± 0.9	11.5 ± 1.1	11.1 ± 0.7	11.9 ± 0.4[c]
OSI	10.4 ± 1.4	14.9 ± 3.4	7.9 ± 1.7	8.5 ± 1.7[d]	7.6 ± 1	9.7 ± 1
TAS	0.7 ± 0.1	0.6 ± 0.1	0.9 ± 0.1	0.9 ± 0.1[d]	1 ± 0.1[d]	0.7 ± 0.1
TOS	6.5 ± 0.4	7 ± 0.5	6.6 ± 1	7.3 ± 1	6.8 ± 0.4	6.2 ± 0.6
VEGF	115 ± 4	108 ± 4[e]	116 ± 4	125 ± 3[f]	125 ± 7	127 ± 7
sFlt-1	2.8 ± 0.1	3.2 ± 0.2	2.7 ± 0.2	3.0 ± 0.3	2.7 ± 0.2	3.0 ± 0.1
TNF-α	18.8 ± 2.9	22.3 ± 4.2	35.2 ± 13	28.9 ± 7.7	42.4 ± 18	34.3 ± 6.3
IL-6	20.5 ± 3.2	27.8 ± 6.3	24.6 ± 3.4	21.5 ± 2.1	23.7 ± 2.9	22.4 ± 1.8

[a]compared to SHAM, PINX+RUPP+MEL1, PINX+RUPP+MEL14 and PINX+MEL1 groups $p < 0.05$, [b]compared to PINX and PINX+RUPP+MEL14 groups $p < 0.05$, [c]compared to SHAM group $p < 0.05$, [d]compared to PINX group $p < 0.05$, [e]compared to PINX+RUPP+MEL1, PINX+RUPP+MEL14 and PINX+MEL1 groups $p < 0.05$, compared to SHAM group $p = 0.05$, [f]compared to PINX group $p < 0.05$, compared to SHAM and PINX+RUPP groups $p = 0.05$.

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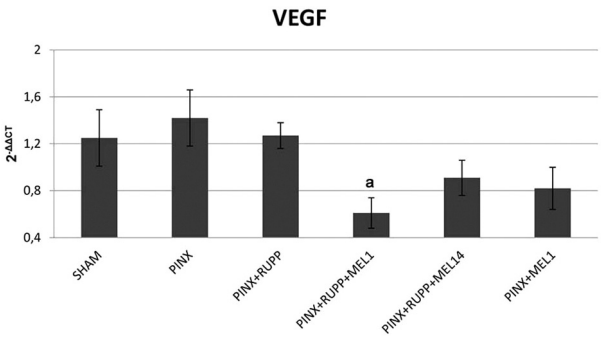


Figure 1. VEGF gene expression levels determined in the placenta ([a]compared to SHAM, PINX and PINX+RUPP groups $p < 0.05$).

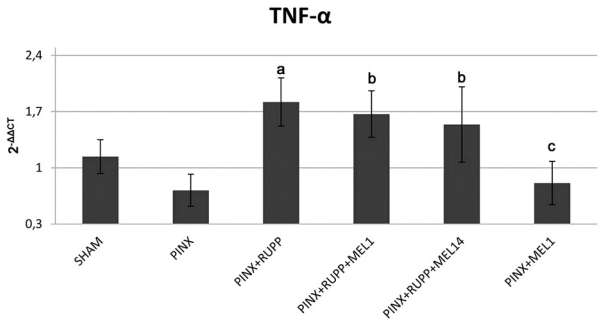


Figure 3. TNF-α gene expression levels determined in placenta ([a]compared to SHAM and PINX groups $p < 0.05$, [b]compared to PINX group $p < 0.05$. [c]compared to PINX+RUPP, PINX+RUPP+MEL1 and PINX+RUPP+MEL14 groups $p < 0.05$).

PINX+MEL1 group compared to SHAM and PINX groups (Figure 4, $p < 0.05$).

Discussion

To the best of our knowledge, the changes of blood pressure, oxidative stress and TNF-α, IL-6, VEGF and sFlt-1 blood levels and placental expressions were firstly investigated in PINX or melatonin administrated RUPP rats with this study.

Hypertension, proteinuria and abnormal renal function could be occurred in preeclamptic women and RUPP model in rats mimics these outcomes. For that reason, the RUPP model used as an animal model in investigation of PE pathogenesis (19).

Melatonin is a hormone has role on fertility and gestation and can easily pass through the placental membrane (20). Through the receptors on human and rat placenta melatonin could affect the fetal and placental growth (12,21). The fetal effects of melatonin could be depended on increasing in oxygen utility and nutrient sustaining. Melatonin increases placental antioxidant capacity and vasodilatation of vascular bed by its own receptors (22). Our results indicate that neither PINX nor melatonin could affect number of pups and placental weights however RUPP operation can strongly decrease placental weight. These findings consist with previous studies in terms of pup number and placental weights (23−26). However placental weight values was found to be similar among the SHAM, PINX and PINX+RUPP+MEL14 groups, interestingly. Although any melatonin receptor antagonist was not tested in the present study, this result may be due to the modification of melatonin receptor expression during the pregnancy (21).

In the present study, the MAP values in PINX and PINX+RUPP groups were significantly higher than SHAM and melatonin administrated groups. Other researchers obtained higher MAP values in RUPP rats in several studies (20,23,27). It was obviously observed that melatonin was able to significantly decrease MAP findings not only PINX but also RUPP rats. The blood pressure decreasing effect of melatonin through the NO pathway is described in previous studies in L-NAME induced or genetically hypertensive rats (28,29). Melatonin could also reduce blood pressure by its own antioxidant properties such as decreasing intracellular superoxide anion and renal MDA levels, and elevating erythrocyte glutathione peroxidase activity (30). In previous studies, antioxidant capacity of melatonin was clearly revealed. In our study, we observed that TAS values increased and OSI levels decreased both in first or 14th d of melatonin administration. On the other hand, the highest TAS and lowest OSI values were obtained in PINX group.

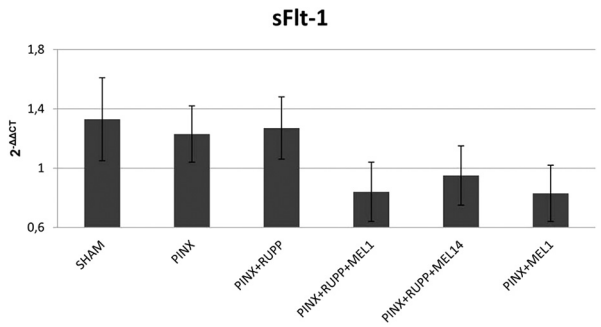


Figure 2. sFlt-1 gene expression levels determined in the placenta.

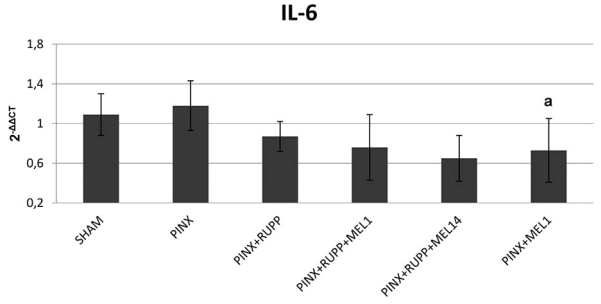


Figure 4. IL-6 gene expression levels determined in the placenta ([a]compared to SHAM and PINX groups $p < 0.05$).

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These outcomes suggested us that melatonin deficiency disrupts oxidative capacity and could facilitate the emerging of PE.

Our results also clearly indicated that melatonin administration significantly decreased blood pressure in RUPP rats. Melatonin may be decreased blood pressure by nitric oxide (NO) pathway. The increased blood pressure value also observed in PINX rat and melatonin was able to significantly decrease this value in PINX+MEL1 group. Melatonin-induced vasorelaxation was observed in myocytes of mesenteric arteries by to its direct or indirect (via MT1/MT2 large-conductance Ca^{2+}-activated K^+ channels) effects (31). Moreover, maternal melatonin could effect the fetal physiology by its receptors and inhibit catecholamine response of the fetal cerebral artery (32). According to recent works, melatonin may effect blood pressure or blood flow both in maternal and fetal cardiovascular systems. The decreasing blood pressure effects of melatonin in our study may be related with large-conductance Ca^{2+}-activated K^+ channels or NO pathway. In recent years, the usage of some agents were discussed to reduce hypertension in RUPP rats (23−27). Our experimental blood pressure results and the previous reports given above may suggest not only melatonin should be considered as a potential agent in therapy of PE and perinatal medicine but also light/dark cycle and sleeping pattern should be considered together in terms of reducing in secretion of melatonin from pineal gland in PE.

TNF-α and IL-6 are known as cytokines related with pathogenesis of PE and both are increased in preeclamptic women and experimental RUPP rats. Moreover, ischemic placenta may enhance the synthesis of inflammatory cytokines (33). We observed approximately two fold higher but not significant TNF-α level in RUPP rats compared to SHAM. Also, melatonin was not effective on significantly increased TNF-α gene expression levels in placenta of RUPP rats. As far as increased placental TNF-α expression results in RUPP operated rats, another member of cytokines family placental IL-6 levels emerged distinct findings which were about 50% lower in the same groups. These outcomes show us that melatonin downregulated the placental IL-6 expression with regard to TNF-α in RUPPs rat. On the other hand, any effect of RUPP operation and melatonin application was not observed on plasma IL-6 levels, because IL-6 findings were close to SHAM or experimental groups, surprisingly. Recent studies report that TNF-α and IL-6 are involved in elevated blood pressure in late pregnancy through the inhibition of NO-cGMP mediated relaxation pathway in systemic vessels (34). Also it's declared that TNF-α is an important cytokine mediates endothelial cell activation and hypertension via endothelin-1 (ET-1) in response to placental ischemia in RUPP rat (35).

The roles of VEGF, which facilitates cell migration and angiogenesis through upregulation expression of interstitial collagen and urokinase in endothelial cells, in pathogenesis of PE is well known (36). Hypoxia is an essential factor in stimulating of upregulation of VEGF expression via hypoxia-inducible factor (37). VEGF expressed in cytotrophoblast, syncytiotrophoblast and villus of endothelial cells which regulates vasculogenesis and angiogenesis in early pregnancy during placental formation (38). Placental villous and extra villous trophoblast cells syntheses sFlt-1 which is another molecule has role in pathogenesis of PE in both first and third trimesters of gestation (38). Blood VEGF levels were decreased and sFlt-1 was elevated in both preeclamptic women and experimentally RUPP rats (27,39,40). After VEGF infusion and pravastatin administration in RUPP rats, attenuated hypertension, oxidative stress, increased VEGF and reduced sFlt-1 levels were reported (27,40). VEGF and sFlt-1 level are considered in pathogenesis of PE and current experimental or clinical studies are undergoing on this subject. Endothelial dysfunction, impaired Ach-mediated NO production and vasorelaxation is associated with RUPP hypertension (41). Moreover, sFlt-1 induces reduction in VEGF levels and reveals endothelial dysfunction and hypertension in both renal micro vessels and carotid artery (42,43). In the present study, higher blood VEGF levels and lower MAP values were obtained in melatonin administrated RUPP and PINX rats compared to SHAM, PINX and PINX+RUPP groups. This outcome may be explained by recovery effect of melatonin on impaired NO mediated vasorelaxation or endothelial dysfunction in RUPP rats. The other remarkable result of our study is increased MAP, OSI and placental sFlt-1 expression and decreased blood VEGF, TNF-α and TAS values in PINX rats. Melatonin administration from the first day of gestation reversed these values in PINX+MEL1 group except for TAS. These outcomes are the first experimental results dealing with melatonin deficiency in PINX RUPP rats. However, impaired or decreased melatonin secretion and reduced placental melatonin secretion are related with development of PE in pregnant women (10,44). These results thought us that melatonin deficiency should be considered as a factor in PE pathogenesis.

Melatonin decreased sFlt-1 expression in placenta both in RUPP and PINX rats, however, surprisingly lower VEGF expression levels were observed in these groups compared to SHAM, PINX and PINX+RUPP groups. Moreover, it has reported that melatonin has decreasing effect on VEGF expression and proliferation of cancer cells in cell culture studies (45,46). Higher plasma VEGF levels and mentioned effects of melatonin could cause these outcomes.

In summary, we report that melatonin has powerful decreasing effect on blood pressure, placental sFlt-1 gene expression but moderately oxidative stress levels in both RUPP and pinealectomy operated rats. Moreover, melatonin deficiency may cause increased MAP, OSI and placental sFlt-1 expression and decreased blood VEGF,

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TNF-α and TAS values. Lower maternal blood pressure is important in maternal and fetal health and time of delivery in PE pregnant women. In conclusion, our findings may have essential clinical implication in terms of not only melatonin administration but also melatonin deficiency for continuing phase I clinical trials of melatonin administration in PE (46). Further detailed studies in pregnant women with PE may supply these results and the experimental researches investigating the role of placental melatonin in pathogenesis of PE are required.

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Conflict of Interest

All authors reported no conflict of interest.

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