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The effects of melatonin administration on KCNQ and KCNH2 gene expressions and QTc interval in pinealectomised rats

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Abstract: Melatonin is a hormone secreted from the pineal gland and has different cardiovascular effects. KCNQ genes expressed in aorta related with vascu- lar tone and KCNH2 gene characterised in left ventricle associated with QT duration. The aim of this study was to investigate the effects of melatonin on the regulation of the blood pressure and the relationships between the expressions of aorta KCNQ1-5, left ventricle KCNH2 genes and the QTc interval. For that purpose, 42 male adult Sprague-Dawley rats were devided into six groups; SHAM, SHAM+L-NAME, PLT, PLT+L-NAME, PLT+MEL and PLT+L-NAME+MEL. Pinealectomy operation was applied in PLT groups. L-NAME was added in drinking water (40 mg/kg/day) and melatonin was given subcutanously (5 mg/kg/day). The blood pressure, heart rate (HR) and QTc interval values were recorded on 0, 1st, 7th, 14th and 21st days of experiment. Left ventricle and thoracic aorta samples were obtained to investigate the changes of gene expression levels of KCNQ1-5 and KCNH2, respectively. The increased blood pressure and HR were observed in SHAM+L-NAME, PLT, and PLT+L-NAME groups compared to MEL and SHAM groups ($p<0.05$). On the other hand, the long QTc interval was recorded in PLT and all L-NAME groups compared to others ($p<0.05$). The decreases in KCNH2 gene expression levels were observed in groups have QTc prolongation. In conclusion, PLT operation could cause an increasing in blood pressure, HR and QTc duration, melatonin was able to prevent these increasings and could change KCNQ and KCNH2 gene expresion profiles. Further molecular studies are required to evaluate these effects.

Key words: Blood pressure; KCNQ; KCNH2; Melatonin; Rat.

Introduction

Melatonin is a hormone synthesized and secreted from the pineal gland as the major secretory product and it's receptors are located in three distinct regions of cell including membrane, cytoplasm and nucleus. Melatonin exerts receptors dependent or independent different physiological effects in many tissues and organs including the cardiovascular system (CVS) (1-3).

In recent years, besides the effects of a lot of parameters that regulate the blood pressure, researchers focus on to the Kv7 channels encoded by KCNQ1-5 genes. The KCNQ1-5 genes expressed in many blood vessels and have been shown to be effective on the vascular contractility in some experimental study (4-7). However, KCNQ1-5 genes response to melatonin is not clear.

The QT interval represents time of ventricular electrical activity and reflects the depolarization and repolarization time of the myocardium. Prolongation of QT interval may be encountered *torsade de pointes* type arrhythmias and ventricular fibrillation (8). The role of potassium currents are very important in the length of the QT interval. Kv11.1 channel which is one of the cardiac K^+ channels that is expressed by the gene KCNH2 (hERG: human ether go go related gene). If this channel is blocked by some drugs or a disruption in the activity of the potassium current, polymorphic ventricular tachycardia could be occured (9,10).

Abnormal heart rate (HR) reflects an autonomic nervous system imbalance and elevated or insufficient HR increases cardiovascular risk. Melatonin is a promising candidate in the struggle against elevated HR and hypertension in terms of melatonin production of the pineal gland depends on the sympathetic stimulation or melatonin inhibits the sympathetic system activitiy. The HR-reducing action of melatonin was also observed in experimental hypertensive rats (11). The length of the QT interval is influenced by alterations in HR and autonomic nervous activity (12). Murakawa et al (13) reported that change in sympathovagal balance was responsible for the diurnal variation in QT interval. QT interval parameters have mainly been associated with left ventricular mass in patients with hypertension (14). Pinealectomy caused a relative deficieny of melatonin and hypertension in rats (15). However, there is no sufficient information about the relationship between melatonin-QT interval and KCNH2 gene expression. The present study was designed to investigate the effect of melatonin on the regulation of the blood pressure and the relationships between the expressions of aorta KCNQ1-5, left ventricle KCNH2 genes and the QTc interval.

Materials and Methods

Experimental conditions and groups

The animal experiments of this research was performed in the laboratories of Canakkale Onsekiz Mart University Experimental Research Center (COMUDAM). Animals were maintained from COMUDAM and used with the approval of the Canakkale Onsekiz Mart Uni-

versity Animal Care and Use Local Ethics Committee (2013/02-13).

In this study, we used 42 Sprague Dawley male adult rats weighing 200–250 g. Animals were housed according to 12-h light–dark cycle and 22 ˚C room temperature. All rats fed ad libitum, and the groups are designed after recording the weights and blood pressure values.

-SHAM group (n=7) rats were SHAM operated.

-SHAM+L-NAME group (n=7) rats were SHAM operated and L-NAME (CAS 51298-62-5: NL'-nitro-L-arginin metil ester, Sigma-Aldrich) was added to the drinking water (40 mg/kg/day).

-PLT group (n=7) rats were pinealectomised.

-PLT+L-NAME group (n=7) rats were pinealectomised and L-NAME was added to the drinking water (40 mg/kg/day).

-PLT+MEL group (n=7) rats were pinealectomised and melatonin was injected subcutanously (sc, S 4858937 244, MERCK; 5 mg/kg/day, at 10 am).

-PLT+L-NAME+MEL group (n=7) rats were pinealectomised, L-NAME was added to the drinking water (40 mg/kg/day) and melatonin was injected sc (5 mg/kg/day).

L-NAME and melatonin application

L-NAME was applied in dose of 40 mg/kg/day with drinking water (16). The waters containing L-NAME were renewed every day. Melatonin was dissolved in physiological solution contains 5 % ethanol (Absolute GR for analysis, MERCK, Germany) and administered sc in 5 mg/kg/day dose (17). Melatonin was applied at the indicated doses that was prepared freshly every day and applied without delaying.

Pinealectomy

Pinealectomy operation was performed under general anesthesia (60 mg/kg ketamine and 5 mg/kg xylazine). A surgical incision was done to the sculp and the dorsal surface area of the brain was exposed. Dura mater was ruptured by micro drill in diameter 3-4 mm. Then fine forceps inserted to enclose the pineal gland from stalk with the angle of 60^{o} (18). In order to stop the possible bleedingstamp was applied by small sized cottons. At the end of the operation the incision was sutured with sterile 3/0 absorble suture.

Blood pressure and ECG recording

Prior to taking the blood pressure measuring rats were allowed to get used to device and procedure to be applied. Systolic, diastolic and mean arteriel blood pressure (MAP) values were obtained from tail artery using the *tail cuff* method before starting the application (day 0) and in the 1st, 7th, 14th and 21st days of the experiment (MAY, NIBP200-A, Noninvasive blood pressure measuring system, Biopac Systems INC, USA). Electrocardiographic procedure was performed as reported by Uzun et al (19). Alligator clips were attached to four limbs. The derivation I, II, III, aVR, aVL and aVF were recorded under slight anaesthesia (20), and ECG was standardised to 1 mV=20 mm, speed 75 mm/sec and the filter (35 Hz;Poly-Spectrum 12 channel ECG-System, Poly-Spectrum-8, Neurosoft, 5, Voronin str., Ivanovo, Russia). The HR and QT values were evaluated from ECG records in all groups at 0, 1st, 7th, 14th and 21st days. The QT interval was corrected for heart rate with the formula used by Fridericia.

Quantitative Real-Time PCR

At the end of day 21 heart left ventricle and arcus aorticus were harvested from all rats and put into the DNAse/RNAse free tubes, stored at -80 ^{O}C until analysis. Left ventricle was stored for expression analyses of KCNH2 and arcus aorticus for KCNQ.

RNA isolation from tissue samples taken from the rats of each group was done at the end of the experimental period. For this purpose, about 25 mg harvested tissue was homogenized (Qiagen Tissue Lyser, Hilden Germany). Total RNA isolation was made manually by using RNA isolation Kit (Ambion PureLink RNA Mini-Kit, Life Technologies, USA) cDNA synthesis was performed manually (High Capacity cDNA Revere Transcription Kit, 200 reaction, USA, Table 1-2).

Synthesized cDNA samples were used for quantitative Real-Time PCR (lightcycler®480, Roche) study. Gene expression levels were analyzed by using SYBR Green (Power SYBR Green PCR Master Mix, 5 ml, Applied Biosystems, England). Beta-actin was used for normalization of the genes as housekeeping gene. The qRealTimePCR reactions were performed under the conditions as follow, at 95 °C for 10 min, 95 °C for 15 sec, and 60 °C for 60 sec completing 40 cycles and components were given in Table 3. Primer sequences were prepared for mentioned genes and studied after analysis (Table 4).

Data analysis and statistics

The ECG and blood pressure datas were evaluated by the SPSS 20.0 programme, groups were compared by OneWay 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

Table 1. cDNA synthesis reaction components.

Components	Volume for each sample
10x Buffer	2 µl
dNTP	0,8 µl
Random Primer	2 µl
Enzyme	1 µl
Water	4,2 µl
RNA	10 µl
Total	20 µl

Table 2. Thermal cycler conditions for cDNA synthesis.

Temperature	Time
25 ºC	10 min
37 ºC	120 min
85 ºC	5 min

Table 3. Quantitative Real-Time PCR components.

Components	Volume for each sample
SyberGreen Master Mix	5 µl
Forward Primer	0,75 µl
Reverse Primer	0,75 µl
RNAse Free Water	2,5 µl
cDNA	1 µl
Total	10 µl

Table 4. Primers.

GENES	Primers	Tm Value (°C)	Base Pair	GC %
KCNQ 1	Forward: 5'-**TGGGTCTCATCTTCTCCTCC-** 3'	57.83	20	55.00
	Reverse: 5'- **GTAGCCAATGGTGGTGACTG-** 3'	58.55	20	55.00
KCNQ 3	Forward: 5'- **CAGCAAAGAACTCATCACCG-** 3'	57.11	20	50.00
	Reverse: 5'- **ATGGTGGCCAGTGTGATCAG-** 3'	60.03	20	55.00
KCNQ 4	Forward: 5'- **GAATGAGCAGCTCCCAGAAG-** 3'	58.33	20	55.00
	Reverse: 5'- **AAGCTCCAGCTTTTCTGCAC-** 3'	59.04	20	50.00
KCNQ 5	Forward: 5'- **AACTGATGAGGAGGTCGGTG-** 3'	59.10	20	55.00
	Reverse: 5'- **GATGACCGTGACCTTCCAGT-** 3'	59.39	20	55.00
KCNH2	Forward: 5'- **CTCAAAGGCGACCCTTTCCT-** 3'	59.96	20	55.00
	Reverse: 5'- **AATGAGCCAGTCCCACACTG-** 3'	59.96	20	55.00

Tm: Melting Temperature, GC %: Guanin-Cytosin Percentage, BP: Base Pair.

(ΔΔCt (Ct Target gene- Ctreferans gene)) and the results were studied with the GraphPad Prism programme ($p<0,05$ was accepted as statistically significant).

Results

In this study, HR, blood pressure and QTc interval values and KCNQ (arcus aorta) and KCNH2 (left ventricule) gene expression changes were investigated in totaly 42 pinealectomised, L-NAME administrated or melatonin injected rats.

Blood pressure and QT/QTc values

The all MAP values were shown in Table 5. The 21th MAP values were recorded 133, 138, 147 mmHg in SHAM+L-NAME, PLT and PLT+L-NAME groups, respectively. This three groups have higher and significant ($p<0.05$) MAP results compared to others. These results indicated that pinealectomy operation and L-NAME administration increased MAP and melatonin has shown protective effect against this increasing.

The dramatic changes were observed in SHAM+L-NAME and PLT+MEL groups for HR (Table 6). The increased HR values were observed after PLT operation or L-NAME administration and melatonin caused lower HR results in MEL injected groups ($p<0.05$).

The longer QTc values were observed in SHAM+L-NAME, PLT and PLT+L-NAME groups compared to others. QTc duration were recorded 90, 96 and 87 ms in SHAM, PLT+MEL and PLT+L-NAME+MEL groups in 21th day of experiment, respectively. However, 131, 110 and 113 ms QTc interval values were observed in SHAM+L-NAME, PLT and PLT+L-NAME, respectively (Table 7).

Expression profiles of KCNQ and KCNH2 genes

Expression levels of the genes KCNQ1, KCNQ3, KCNQ4 and KCNQ5 in aorta and KCNH2 in left ventricule were investigated and the results were given in the Figures 1 and 2.

In this study, the statistically significant decreasing were obtained in expression levels of the KCNQ3 and KCNQ5 in SHAM+L-NAME, KCNQ3, KCNQ4 and KCNQ5 in PLT, and KCNQ5 in PLT+L-NAME+MEL

Table 5. Mean arterial pressure values of all groups in the study. Different letters in the same line indicate significant differences among groups in terms of different days ($p<0.05$), * $p<0,05$ refers groups vs SHAM.

Groups	Mean Arterial Blood Pressure (mmHg)				
	0 day	1st day	7th day	14th day	21st day
SHAM	109 ± 1^{a}	107 ± 2^{a}	102 ± 1^{ab}	99 ± 1^{b}	107 ± 2^{a}
SHAM+L-NAME	112 ± 1^{c}	111 ± 1^{c}	119 ± 2^{b}	132 ± 2^{a}	133 ± 2^{a} *
PLT	119 ± 1^{c}	121 ± 1^{c}	116 ± 1^{c}	128 ± 1^{b}	138 ± 2^{a} *
PLT+L-NAME	111 ± 1^{c}	119 ± 2^{c}	138 ± 2^{b}	144 ± 1^{ab}	147 ± 1^{a} *
PLT+MEL	99 ± 1^{b}	118 ± 1^{a}	117 ± 3^{a}	113 ± 1^{a}	114 ± 1^{a} *
PLT+L-NAME+MEL	102 ± 1^{b}	117 ± 2^{a}	117 ± 1^{a}	117 ± 1^{a}	116 ± 1^{a} *

Table 6. Heart rate values of all groups in the study. Different letters in the same line indicate significant differences among groups in terms of different days ($p<0.05$), * $p<0,05$ refers groups vs SHAM.

Groups	Heart Rate (min)				
	0 day	1st day	7th day	14th day	21st day
SHAM	284 ± 17^{ab}	314 ± 8^{a}	321 ± 8^{a}	325 ± 16^{a}	261 ± 6^{b}
SHAM+L-NAME	300 ± 7^{b}	308 ± 24^{b}	329 ± 11^{ab}	329 ± 11^{ab}	382 ± 12^{a} *
PLT	358 ± 13	331 ± 14	332 ± 16	378 ± 14	380 ± 14 *
PLT+L-NAME	358 ± 12	333 ± 12	349 ± 17	344 ± 1	359 ± 4 *
PLT+MEL	360 ± 9^{a}	302 ± 8^{b}	308 ± 14^{b}	307 ± 13^{b}	306 ± 9^{b} *
PLT+L-NAME+MEL	306 ± 8	308 ± 13	309 ± 7	319 ± 10	313 ± 6 *

Table 7. QTcF values of all groups in the study. Different letters in the same line indicate significant differences among groups in terms of different days ($p<0.05$), * $p<0,05$ refers groups vs SHAM.

Groups	QTcF				
	0 day	1st day	7th day	14th day	21st day
SHAM	97 ± 6^{ab}	91 ± 2^{b}	110 ± 3^{a}	81 ± 4^{b}	90 ± 1^{b}
SHAM+L-NAME	95 ± 4^{bc}	96 ± 5^{bc}	78 ± 7^{c}	112 ± 7^{ab}	131 ± 7^{a} *
PLT	95 ± 9	88 ± 3	85 ± 4	110 ± 5	110 ± 5 *
PLT+L-NAME	101 ± 5	95 ± 5	113 ± 5	108 ± 4	113 ± 3 *
PLT+MEL	104 ± 4	95 ± 3	93 ± 5	105 ± 3	96 ± 4
PLT+L-NAME+MEL	102 ± 6	86 ± 5	96 ± 6	94 ± 2	87 ± 6

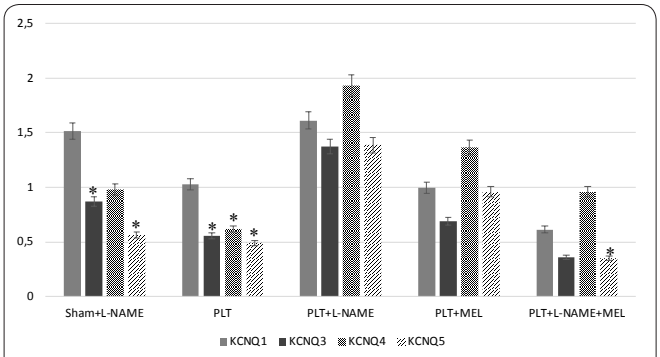

Figure 1. Relative expression levels of the KCNQ1, 3, 4, 5 genes. All data normalized with β-Actin expression and given as relative to SHAM (n=7). * indicate significantly differences values compared to SHAM group ($p<0.05$).

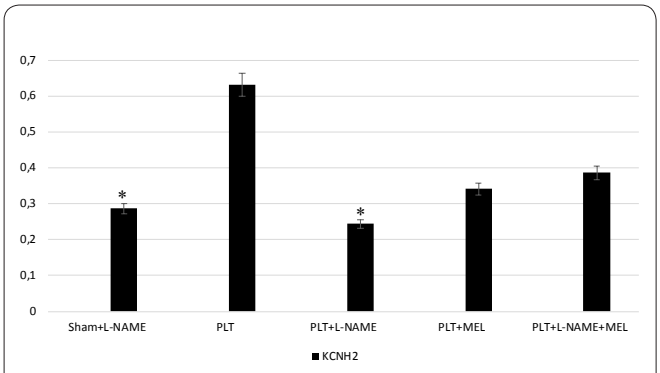

Figure 2. Relative expression levels of the KCNH2 gene. All data normalized with β-Actin expression and given as relative to SHAM (n=7). * indicate significantly differences values compared to SHAM group ($p<0.05$).

groups compared to SHAM group (Figure 1, $p<0.05$).

KCNH2 gene expression level was decreased in all experimental groups compared the SHAM. However the significant change were observed in the groups SHAM+L-NAME and PLT+L-NAME ($p < 0.05$).

Discussion

The effects of melatonin on experimentally hypertensive rats in terms of relationship between the blood pressure, QTc duration, aorta KCNQ1-5 and left ventricular KCNH2 genes expression levels were evaluated.

The CVS diseases have high mortality rate in humans. Hypertension and QT prolon- gation are the most common life-threatening diseases (21, 22). Therefore developing preventive and curative new methods against cardiovascular diseases continues intensively (23-26).

Release of melatonin from the pineal gland was known to have the effect of reducing blood pressure in both humans and experimental animals (27, 28). In this study, the increased blood pressure values were observed both pinealectomised and L-NAME administrated rats. On the other hand, lower blood pressure levels were observed in groups which treated with melatonin. PLT operation could cause melatonin deficiency that could be reason of the increased blood pressure in PLT group. However, melatonin also produced and secreted from the other tissue such as gastrointestinal tract rather than pineal gland. This compensatuary secretion of melatonin could occur in 21th days of experiment but according to blood pressure values observed in PLT groups this compensatur secretion was not able to recover the high blood pressure to basal levels. Whereas the exogenously administered melatonin prevented this increase of blood pressure. The maximum blood pressure increasing is observed in rats that are pinealectomy operated and L-NAME given together. Also, our results indicated that the hypertensive effect of melatonin deficiency in the 21th day, was as strong as the hypertensive effect of L-NAME. When the three hypertensive groups were compared, the most significant increase was observed in the PLT+L-NAME group. This could be resulting from the combination of melatonin deficiency and L-NAME. The increase in MAP levels in PLT group were reduced by melatonin administration. Most of the studies were designed to investigate blood pressure change by the application of either melatonin or melatonin agonist and antagonist drugs (29, 30). Therefore, it is important to know the possible effects of melatonin administration on blood pressure after PLT operated or L-NAME administrated rats.

The KV7 channels have regulatory role in the contraction of smooth muscle of blood vessels in humans and rodents (10, 11). KV7 channels have functional role in thoracic aorta, superior mesenteric artery, coronary circulation and hypertension pathogenesis. KCNQ genes encoding the KV7 channels were compared in normotensive and hypertensive rats and decreasing in KCNQ1 and KCNQ4 and increasing KCNQ5 gene expression levels were reported (31). Although the effects of melatonin on blood pressure levels are clearly observed in our study, it is not possible to say the similar effects on KCNQ gene family. While the gene expressions of KCNQ3 and KCQN5 were decreased in SHAM+ L-NAME group, the expressions of both genes were increased in the PLT+ L-NAME group. In addition, while the expressions of both genes were recovered in the melatonin PLT group, the similar effect was not observed in the PLT+ L-NAME+ MEL group. This

finding claims the hypothesis that these channels were not the only regulators on blood pressure (31). Also, Jepps et al (31) found that Kv7 channel activity and decrease in the expression levels were correlated with blood pressure but suggested that it was not the primary reason for development of hypertension.

The expression profiles of KCNQ4 and KCNQ5 were shown to be more dominant with regards to other KCNQ gene family (31). Similarly, in this study KCNQ4 and 5 were found to have dominant expression profiles. In our study we did not used genetically hypertensive rats, but hypertension in rats in our model was induced by L-NAME application. Chronic L-NAME application which inhibits nitric oxide synthase is widely used in experimental hypertension models (32, 33). Our gene expression profile results were different from Jepps et al.'s (31) results. This suggests that the expression of KCNQ genes could be related with NOS inhibition and melatonin deficiency in our study.

Although QT interval in ECG represents both the depolarization and repolarization phases, prolongation in the QT interval are commonly due to the changes in the repolarization phase (34). Prolongation of OT interval are related with *torsade de pointes* type arrythmias, ventricular fibrillation and sudden cardiac deaths. Potassium flow has an important role in QT prolongation and blockage of the voltage gated channel Kv11.1 are among the most common reasons of this prolongation (12, 13).

The delayed rectifier voltage gated potassium channel Kv11.1 is coded by the KCNH2 gene in myocyte cells (35). Any mutation in the activity of these genes would cause disfunction of the heart and QT prolongation (delayed QT syndrome) by corrupting the action potential and therefore delaying the repolarization period (36). There are some studies investigating the QT interval changes by blocking the hERG channel (37-38). A decrease in KCNH2 gene expressions could lead to a decrease in the hERG channel activity, or vice versa. In our study increased QTc duration was observed in SHAM+L-NAME and PLT+L-NAME groups. However, higher KCNH2 gene expressions levels were observed in PLT group. Neither QTc prolongation nor KCNH2 gene expressions were observed in MEL injected groups. These results indicate that melatonin could be effective on QTc duration. Statistically significant QTc prolongation and decreasing in KCNH2 gene expression levels were observed in the SHAM+L-NAME group. Therefore, the effect of melatonin on KCNH2 gene expression could be related with L-NAME. On the other hand, the normal QTc interval was recorded in PLT+ MEL and PLT+ L-NAME+ MEL groups.

Our results indicate that PLT operation could cause an increasing in blood pressure, like as L-NAME. This findings are important since overexposure to light might cause melatonin deficiency related hypertension, it is also important to define the effects and possible usages of melatonin in cardiovascular diseases. Our study explains the obvious effects of melatonin on blood pressure but we were not observed sufficient outcomes to clarify the effects of KCNQ gene family. Also, this is the first study investigating relationship between melatonin-KCNH2 gene expression and QTc duration. Higher QTc duration and lower KCNH2 gene expressions were observed in PLT operated or L-NAME administrated groups. Melatonin was able to prevent QTc prolongations and decreases in KCNH2 gene expresion level.

In conclusion, further molecular studies are required to evaluate through which pathways melatonin affects the K channels and melatonin-blood pressure-KCNQ-KCNH2 gene families' relationship.

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