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Melatonin attenuates caspase-dependent apoptosis in the thoracic aorta by regulating element balance and oxidative stress in pinealectomized rats

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Abstract

The aim of this study is to explain the possible mechanisms by which melatonin deficiency results in cardiovascular injury and to investigate the effects of melatonin administration on important signalling pathways and element equilibrium in the thoracic aorta (TA). For this purpose, we analysed the cellular and molecular effects of melatonin deficiency or administration on the oxidative stress, DNA damage, molecular chaperone response and apoptosis induction in TA tissues of pinealectomised rats using ELISA, RAPD, qRT-PCR and Western blot assays. The results showed that melatonin deficiency led to an imbalance in essential element levels, unfolded/misfolded proteins, increased lipid peroxidation, and selectively induced caspase-dependent apoptosis in thoracic aorta tissues without significantly affecting the BCL2/BAX ratio (PNX: 2.28, PNX+M: 2.73). In this condition, the genomic template stability (80.22%) was disrupted by the significantly increased oxidative stress, and stress-specific HSP70 (20.96-fold), TNF-α (1.73-fold), caspase 8 (2.03-fold), and caspase 3 (2.87-fold) were markedly overexpressed compared to SHAM group. Melatonin treatment played a protective role in cell apoptosis and inhibited oxidative damage. In addition, melatonin increased the survivin level and the regulation of element equilibrium in the TA tissues. The results of the study indicate that melatonin deficiency induces TNF-α-related extrinsic apoptosis signals and that the administration of pharmacological doses of melatonin attenuates cardiovascular toxicity by regulating the increase in the rate of oxidative stress-related cellular and molecular damage-induced apoptosis caused by melatonin deficiency in the TA tissue of Sprague-Dawley rats.

Keywords: Oxidative stress, Intrinsic and extrinsic apoptosis signalling, Caspases, Melatonin
Introduction

Atherosclerosis and atherosclerotic vascular diseases are key components of cardiovascular diseases, which affect the quality of life, and are known to be the most prevalent major causes of death (Favero et al. 2014). Recent studies have demonstrated that apoptosis, lipid deposition, vascular reactive oxygen species (ROS), inflammation, endothelial damage and vascular muscle cell migration are the major causative factors of the progression of atherosclerosis (Son et al. 2007; Tengattini et al. 2008). In the development of atheromatous diseases, vascular calcification occurs in the intima layer of vessels (Wexler et al. 1996). Molecules such as survivin (Blanc-Brude et al. 2007), heat-shock proteins (Madden et al. 2010), TNF-α, Bax, caspases, oxidized low-density lipoprotein, advanced glycation end products (Luu et al. 2007; Kim et al. 2014; Qin et al. 2015), Cu/Zn superoxide dismutase (SOD) and calcium are related to atherosclerosis or vascular calcification (Tang et al. 2007; Wei et al. 2013).

Melatonin is a hormone synthesized and secreted as a major product of the pineal gland and it has different physiological effects on many tissues and organs, including the cardiovascular system, in a receptor-dependent or receptor-independent manner (Reiter et al. 2010; Ovali and Uzun 2017). Melatonin has a protective effect on the cardiovascular system against oxidative damage, endothelial dysfunction, vascular inflammation and systemic hypertension (Hung et al. 2013), which are all triggered by increases in both the tissue and plasma levels of ROS, pro-inflammatory factors and leukocyte adhesion molecules (Paulis and Šimko 2007; Reiter et al. 2009). Stress, hypertension, cancer, obesity, lifestyle and several other factors cause melatonin deficiency in either a chronic or a transient form. Melatonin deficiency affects a broad range of individuals, including the large sector of the population that uses electronic devices such as phones, tablets and computers until morning, shift workers, jet travellers, and older people living in the modern digital world (Turek and Gillette 2004).
Therefore, melatonin use for melatonin deficiency-related stress and disorders has increased significantly in recent years. However, whether that exogenously taken melatonin can reverse the negative micro-environmental conditions that occur in the absence of melatonin has not yet been clearly demonstrated. Recent studies conducted using several cell and animal models reported that the administration of melatonin provides great defence against oxidative stress (Reiter et al. 2005; Galano et al. 2011). Moreover, melatonin is known to induce the expression of antioxidant genes, such as CuZn-SOD, manganese superoxide dismutase (Mn-SOD), glutathione peroxidase and catalase (CAT) and anti-inflammatory pathways (Rodriguez et al. 2004; Mauris et al. 2013). Melatonin prevents mitochondrial membrane disruption and inhibits the release of cytochrome-c to reduce caspase activation and apoptosis. Moreover, melatonin markedly decreases caspase 3 expression, suppresses DNA damage (Zhang et al. 2013; Yang et al. 2014) and reduces cardiomyocyte apoptosis (Asghari et al. 2016).

The effects of melatonin administration on the cardiovascular system have been extensively investigated, but the effects of melatonin deficiency or external melatonin administration on specific cell defence systems, essential element equilibrium and the underlying molecular mechanisms of vascular toxicity and caspase-dependent apoptosis in the thoracic aorta (TA) tissues still need to be determined. This study aims to explain the possible mechanisms by which melatonin deficiency results in vascular injury and to investigate the role of melatonin administration in alleviating TA apoptosis with a specific focus on oxidative stress markers, DNA damage, heat-shock protein families, element equilibrium and both intrinsic and extrinsic apoptosis pathways in pinealectomized rats.
Material and Methods

Experimental study

The experimental study was performed in the laboratories of Canakkale Onsekiz Mart University Experimental Research Center (COMUDAM). Experimental animals obtained from COMUDAM were licensed by the approval of the Canakkale Onsekiz Mart University Animal Care and Use Ethical Committee (Protocol number: 2014/11-10).

Animal material and study design

Fifteen adult male Sprague-Dawley rats, weighing 200–250 g, were used as animal subjects. The animals were housed in standard cages with food and water ad libitum, under a 12:00 L-12:00 D cycle (8:00 am to 8:00 pm) and controlled temperature of 22 ± 2 °C, and were randomly divided into three groups: Control (SHAM; n=5), pinealectomy (PNX; n=5), and pinealectomy+melatonin (PNX+M; n=5). After one week of adaptation, ten animals were subjected to a pinealectomy according to Canpolat et al. (2001), and the remaining animals received a sham operation. After three days of recovery, vehicle solution (physiological saline containing 5% ethanol) was injected into the animals of the SHAM and PNX groups, and melatonin (absolute GR for analysis, Merck, Germany) was injected into the animals of the PNX+M group (5 mg/kg, subcutaneously). Melatonin was dissolved in physiological saline containing 5% ethanol and freshly prepared and applied every day to the PNX+M group at 10:00-10:30 a.m for 21 days (Ovali and Uzun 2017). Experimental procedures were performed in all groups at the same time and under the same conditions. Thoracic aorta (TA) tissue was harvested from euthanised rats on the last day of the experiment (day 21) at 10:00 a.m. and was washed with sterile physiological saline solution before being placed into
cryotubes. The operation was performed under sterile conditions to minimise any contamination risk. The cryotubes were frozen in liquid nitrogen and then stored at -80 °C until experimental analysis was performed.

**Determination of the Melatonin Level in TA Tissue**

For each group, five thoracic aorta samples, which were frozen in liquid nitrogen, were lysed in cold conditions (4 °C) with a TissueLyser LT lysing system (TissueLyser LT, Qiagen, USA) in a 2-mL extraction tube for 2 min. Afterwards, approximately 50 mg of powder, which was crushed with tungsten carbide beads, was mixed with 500 µL of extraction solvent (methanol/ultrapure water, 20:80 (v/v) with 0.2% formic acid) in an Eppendorf tube. This mixture was vortexed immediately with cooling and shaking at 1250 rpm (Bioneer Mixing Block MB-102) for 10 min under dark conditions. After centrifugation (15000 rpm for 10 min at 4 °C), the supernatant was filtered through a 0.22-µm PTFE filter. Samples (100 µL) were analysed by a MicroLC-ESI-triple quadrupole TOF system (API 4600 Q-TOF; ABI Sciex, USA). During this analysis, melatonin standards (BML-NS520-000, Enzo Life Sciences) were individually loaded into the Q-TOF system to determine the fragments and analysis conditions. Afterwards, mixtures of the five amines were prepared with concentrations ranging from 0.5 to 100 µg/kg to set up six points of calibration. All data files were opened with PeakView (ABI-Sciex, USA) software, and the mass and fragment results were checked with Master View (ABI-Sciex, USA) options. The mass and specific fragments were exported to MultiQuant software (ABI-Sciex, USA) to generate calibration curves for each type of biogenic amine. Samples (2 µL) were analysed by MicroLC-Q-TOF using an Eksigent MicroLC 200 Plus system coupled to an Applied Biosystems 4600 Triple Quadrupole TOF mass spectrometer. Chromatographic separation was carried out on an Eksigent 2.7 µ*3 cm C18 halo column at 30 °C. The isocratic flow was as follows: 20% A (99.8% UPW:0.2% formic acid) to 80% B (99.8% acetonitrile:0.2% formic acid) over 3 min. Biogenic amine
analyses were performed with a DuoSpray source and electrospray ionization (ESI) probe. An IDA method with a TOF-MS survey of 70 ms and up to 20 dependent TOF-MS/MS scans of 25 ms accumulation time was used, the mass range was set to 100-960 Da for MS, and the product ion mass range was set to 30-960 Da for MSN. The curtain gas was set at 20 a.u., the source temperature was 400 °C, and ion source gases 1 and 2 were both 30 a.u. The declustering potential was set at 100 V. The source voltage was 5500 V. A collision energy (CE) of 20 V and collision energy spread (CES) of 15 V were used. The main and precursor ion and calibration curve of melatonin were 232 \[174\] and \( y = 92.13602x + 1302.73990 \), \( R^2 = 0.9966 \), respectively.

**Determination of elements**

The 20 mg of powder obtained from the previous method used for the thoracic aorta samples from each group was digested with 5+1 mL Suprapur 65% nitric acid solution and ultrapure 99% \( \text{H}_2\text{O}_2 \) with a CEM Mars 6 (CEM Corporation, USA) microwave digestion system (power: 1600 W; time: 15 min; temp: 180°C; pressure: 200 bar; animal tissue procedure, CEM application method). Digested solutions were diluted with ultrapure water, and the final volume of the solution was adjusted to 100 mL. A calibration curve was generated in eight points between 0.100 and 800 µg/kg using Agilent ICP-MS certified mixture standards (part number: 5183-4688). The amounts of Li, Mg, Ca, Mn, Cu and Zn were determined by inductively coupled plasma mass spectrometry (ICP-MS, 7700 xx, Agilent Technologies, USA)

**Determination of Malondialdehyde (MDA)**

The MDA level of TA tissues was determined by the thiobarbituric acid (TBA)/trichloroacetic acid (TCA) reactive substance assay, as described by Kheradmand et al. (2009). The absorbance of the final supernatant was read at 532 nm after subtraction of the non-specific
absorption at 600 nm, and the MDA concentration was calculated using 155 mM$^{-1}$ cm$^{-1}$ as the extinction coefficient. The MDA levels were given as nmol per gram of TA tissue.

**DNA extraction procedures and RAPD assay**

Genomic and mitochondrial DNA were extracted using a DNeasy Tissue Blood Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The total genomic DNA was diluted with nuclease-free water to a concentration of 25 ng/µL, and the diluted DNA was used as the template DNA for the PCR reaction. Standard 50 µL PCR reactions were performed using 2 µL (50±10 ng) of template DNA, 1 µL of each primer, 25 µL of DreamTaq Master Mix (Thermo, K1071) and nuclease-free water (Sigma, W4502). The primer names and sequences and the PCR conditions are given in Table 1. After amplification of the DNA, 15 µL of product with 3 µL of loading dye (R0611, Fermentas) was loaded onto a 2% agarose gel with ethidium bromide in 2 × TAE (Tris 1.6 M, acetic acid 0.8 M, and EDTA 40 mM) buffer. The molecular weight standard Gene Ruler 100 bp plus DNA (Thermo, SM0241) was used according to the manufacturer's instructions. The DNA bands were visualised with a UV transilluminator (Vilber Lourmat, Quantum, France), and the sizes of all of the bands were calculated with the BIO-PROFIL, BIO-1D++ programme. Genomic template stability (GTS (%)) was calculated using the following equation: GTS % =100-(100a/n), where n is the number of bands detected in control DNA profiles and a is the average number of changes in the DNA profiles.

**Isolation of total RNA, cDNA synthesis and Quantitative real-time PCR (qRT-PCR) analysis**

Total RNA was extracted using a PureLink® RNA Mini Kit (Life Technologies, USA) according to the manufacturer's instructions. The extracted RNA concentrations were measured by the Qubit® Fluorometer (Life Technologies, USA). The concentration of total
RNA was adjusted to 100 ng/µL for the synthesis of the first strand of cDNA using a High-Capacity cDNA Reverse Transcription Kit (Life Technologies, USA). cDNA synthesis was performed using an Applied Biosystems® Veriti® thermal cycler (Step 1: 25°C, 10 min; Step 2: 37°C, 120 min; Step 3: 85°C, 5 min). The cDNA was stored at -20°C for subsequent steps of the analysis procedure.

Expression levels of the antioxidant system components [CuZn-superoxide dismutase (CuZn-SOD), Mn-superoxide dismutase (Mn-SOD), catalase (CAT) and glutathione synthetase (GS)], heat-shock proteins (HSP27, HSP60, HSP72 and HSP90) and apoptosis pathway components [tumour necrosis factor alpha (TNF-α), B-cell lymphoma 2 (BCL2), BCL2-associated X (BAX), apoptotic protease-activating factor 1 (APAF 1), cytochrome complex (Cyt-C), caspase 3 and caspase 8] in thoracic aorta tissues of control (SHAM), pinealectomy (PNX) and pinealectomy plus melatonin (PNX+M) rats were analysed by qRT-PCR using SYBR® Select Master Mix (Life Technologies, USA) on an ABI Step One Plus Real-Time PCR system with the primer pairs and PCR conditions shown in Table 1. Gene expression levels were determined as the relative fold change compared to the control and normalised to the GADPH mRNA expression. The comparative cycle threshold (2-ΔΔCt) method (User Bulletin 2, Applied Biosystems, CA) was performed to analyse the expression levels of mRNAs.

**Protein extraction procedure and Western blot assay**

Three 30-40 mg thoracic aorta pieces, which were frozen in liquid nitrogen, from each group were used in cold conditions (4 °C) with the TissueLyser LT lysing system (Qiagen, TissueLyser LT, USA) in a 2-mL extraction tube for 2 min. Afterwards, approximately 20 mg of powder, which was crushed with tungsten carbide beads, was mixed with 500 µL of RIPA (25 mM Tris/HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, and 0.1% SDS) lysis buffer containing a protease inhibitor cocktail (Santa Cruz Biotechnology, Santa
Cruz, CA). The lysates were centrifuged at 14,000 rpm for 30 min at 4°C. Fifty-microgram protein samples, which were measured at A280 using a nanodrop spectrometer (Optisen Nano Q, Mecasys, Korea), were loaded on a NuPAGE® Bis-Tris polyacrylamide gel (10%), electrophoresed, and transferred to PVDF membranes (Life Technologies, USA) after denaturation. The membranes were incubated in 5% milk in TBS buffer. Primary antibodies, including antibodies against HSP70, TNF-α, caspase 8, caspase 3 and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA), were diluted to 1:100-1:1000 in antibody binding buffer overnight in a dark room and then incubated in a secondary antibody solution containing goat anti-mouse IgG-HRP and goat anti-rabbit IgG-HRP for 1 h. The washed and enhanced protein bands of immunoblots were observed with a chemiluminescence gel imaging system (Bio-Rad ChemiDoc MP System, USA).

**Statistical analyses**

The differences in the melatonin, element, and MDA levels, the relative protein density and the relative fold change in gene expression due to melatonin deficiency were compared using the t-test and analysis of variance (ANOVA) with Duncan's separation of means test using SPSS 20 (IBM, USA) software with significance set at the level of $P \leq 0.05$. Correlations between melatonin-gen expressions and melatonin-tissue element contents were analysed by bivariate correlation test with Pearson correlation coefficient and a two-tailed test of significance at significance levels of $P \leq 0.05$ and 0.001. The array analyses were performed with Array Mining software (Glaab et al. 2009) to compare the relative fold change of both physiological and molecular data to the respective control (Control=1) with normalisation to Log10(x+1), and the eBayes and Pearson correlation options were used for hierarchical clustering and correlation analysis, respectively.

**Results**
**Effects of pinealectomy and melatonin treatment on the tissue melatonin level of TA**

In this study, we determined the TA melatonin level in the control and experiment groups using a MicroLC-ESI-triple quadrupole TOF system (MicroLC-Q-TOF). The melatonin level significantly decreased in the PNX group ($0.44 \pm 0.60$ ng mg$^{-1}$), compared with the SHAM group. Compared with that in the PNX group, melatonin administration (PNX+M) increased the melatonin level in the TA tissue by threefold ($1.40 \pm 0.16$ ng mg$^{-1}$), but the amount of this melatonin was less than the level in the SHAM group ($6.28 \pm 0.74$ ng mg$^{-1}$) (Table 2).

**Effects of pinealectomy and melatonin treatment on the element contents of TA**

Inductively coupled plasma mass spectrometry (ICP-MS) analysis showed that both PNX and melatonin treatment induced important and variable changes at the tissue element levels. Whereas Mg, Ca, Cu, Zn and Mn concentrations were significantly increased by pinealectomy, melatonin treatment to PNX animals resulted in a significant decrease at the tissue level of elements compared with the SHAM ($P<0.05$) (Table 2). However, the melatonin treatment (PNX+M) could only stabilise the Zn and Ca element levels compared with the SHAM in the TA tissues (Table 2). The values of all the element contents of PNX+M animals receiving melatonin were significantly low in the PNX (t-test;$P<0.05$). Nevertheless, no significant correlation was found between the tissue element contents and the melatonin levels.

**Effects of PNX and melatonin treatment on the oxidative stress parameters in the TA tissue**

The MDA level in the rat TA tissue is shown in Figure 1. Melatonin deficiency had a significant effect on the MDA content. The mean level of MDA in the TA was approximately two-fold higher in the PNX group ($12.14 \pm 1.63$ nmol g$^{-1}$) than in both the SHAM and PNX+M
groups. In the TA tissues, PNX caused a significant increase in the expression of CuZn-SOD, GS, Mn-SOD and HSP70 (5.94-, 1.99- and 5.82- and 20.96-fold, respectively) in comparison with the SHAM group. Although, a significant reduction was observed in the relative expression of Mn-SOD and HSP 70 in the melatonin-treated (PNX+M) groups compared with the PNX animals, these expressions were still higher than those in the SHAM.

No significant differences in the relative expression of small HSPs were detected between the SHAM and treatment groups in the TA tissues of rats. Both applications caused a significant decrease in the expression level of other HSPs at 60 kD and 90 kD (Figure 2, Table 2). The Pearson correlation analysis showed that a significant negative correlation was found between the melatonin and expressions of CuZn-SOD (-0.738, P=0.004), CAT (-0.593, P=0.004), Mn-SOD (-0.815, P=0.001), HSP70 (-0.644, P=0.018), and that a positive correlation was observed only in HSP60 (0.732, P=0.004) and HSP90 (0.583, P=0.036).

**Effects of pinealectomy and melatonin treatment on the genomic template stability in the TA tissue** In this study, the effects of PNX and PNX+M on DNA profiles were determined by RAPD analysis. In addition, the appearance/disappearance of bands and the decrease/increase in the intensities of the bands compared with those in the SHAM group were investigated. Of the 20 oligonucleotide primers evaluated to screen the genomic DNA isolated from both TA and rats for polymorphism, 2 primers yielded no band, 6 primers produced inconsistent results, and 12 primers generated consistently positive results. Among the 12 primers, 8 informative and stress-specific primers were selected for use in the analyses of RAPD profiles (Figure 3, Table 3). In total, 47 bands belonging to the control group and 1 new band were observed, and 16 losses of bands belonging to the treated group, ranging in molecular size from 185 (SP1) to 1398 (RAPD6) were amplified by 8 primers in the TA tissue. The highest number of band changes was detected in the PNX animals in the TA.
tissues, and the band patterns observed in the RAPD-PCR exhibited significant differences between the PNX and PNX+M animals, with changes in the band intensity, the loss of normal bands and the appearance of new bands. Genomic template stability (GTS) was used as an indicator to detect the changes in the RAPD band profiles, providing a rapid response under the pressure of stress factors stemming from DNA repair and replication mechanisms in tissues. The GTS (%) of the total DNA of the TA tissue was calculated from the band changes in the eight primers tested. In the TA tissue, pinealectomy operation affected the band profiles, and GTS decreased to 80.22%. In this study, the GTS values were regulated by melatonin treatment in PNX animals, and the value was 96.61% in the TA tissues (Figure 3).

PNX induces TNF-α-related extrinsic apoptosis and melatonin treatment regulates apoptotic cell death

Western blotting and quantitative real-time polymerase chain reaction (qRT-PCR) amplification were employed to measure the expression of intrinsic and extrinsic apoptosis genes and proteins. The qRT-PCR results revealed no signs of Apaf1 and Cyt-c expression during the study. Furthermore, a significantly lower BAX/BCL2 ratio revealed the decrease in mitochondrial apoptosis in the PNX and PNX+M animals in comparison with the SHAM group. Pinealectomy triggered significant changes in the TNF-α (1.73-fold)/caspase 8 (2.03-fold)/caspase 3 (2.87-fold) apoptosis signalling in comparison with their respective controls (Table 2). The apoptosis inhibitor survivin gene was significantly downregulated in PNX in comparison with the SHAM group. Our result demonstrated that the melatonin treatment after PNX could suppress the overexpression of both caspase 3 and caspase 8 in PNX animals. Moreover, the survivin (1.12-fold) gene expression level was the same as that of the SHAM in the PNX+M group (Figure 2, Table 2).
Following gene expression studies, we intended to determine whether some proteins involved in the extrinsic apoptosis signalling were released. Western blot analysis was used to monitor the release of TNF-α, caspase 3, caspase 8 and the stress-specific molecular chaperon HSP70 isolated from the TA tissues. The results indicated that PNX directly caused the upregulation of both caspase 3 and caspase 8 in comparison with that in the SHAM animals. These results provided insight into the mechanism of melatonin action, in which TNF-α/caspase 8/caspase 3 extrinsic apoptosis signalling appeared to be a direct target of melatonin for the induction of caspase-dependent apoptosis (Figure 4).

**Discussion**

Element equilibrium in cells plays an important role in a wide variety of biological pathways in living systems. However, many studies reported an adverse effect of essential or xenobiotic metals at high or inadequate concentrations. These metals could interact with special biological membranous organelles and molecules, thus causing oxidative damage and disrupting the maintenance of several pathways, membrane permeability and redox homeostasis.

The interaction between elements and physiological and genetic systems is well documented, although the molecular mechanisms are not completely understood (Bal and Kasprzak 2002; Chen et al. 2003; Leonard et al. 2004). Metal-melatonin interactions were observed in several cell lines and in rodent and human tissues, generally in neuronal cells. These studies indicated that melatonin plays a protective role against the ROS-mediated neuronal degeneration observed on the serotoninergic and dopaminergic sides of neuron cells (Antolín et al. 2002; Jiménez-Ortega et al. 2010; Lin et al. 2008). The effect of melatonin on redox mechanisms is mainly exerted through the regulation of the gene expression of signalling molecules and enzymes (Jiménez-Ortega et al. 2010). In addition, in vitro studies of cell lines demonstrated that melatonin has a regulatory effect on the production of ROS. This
effect was mainly observed in the presence of specific elements, with effects varying in the order of Fe > Cu > Zn > Mn > Al (Zatta et al. 2003). Researchers reported that melatonin supplements act protectively against Mn-induced hypokinesis and neuronal loss by inhibiting ROS (Deng et al. 2015). Although Cu, an essential element, contributes to several physiological functions (e.g., redox reactions) by binding to specific proteins, an excess concentration of this metal causes the oxidative damage caused by a redox imbalance. Melatonin administration helps to scavenge free radicals or chelate excessive Cu, thus protecting cellular systems from its toxicity (Chen et al. 2003; Galano et al. 2015). Owing to the Cu-chelating function, melatonin inhibits oxidative stress induced by both Cu(II)-ascorbate mixtures and highly toxic hydroxyl anion (·OH) production. Furthermore, the oxidative stress-inhibiting effect of melatonin is also exerted by its metabolites (cyclic 3-hydroxymelatonin, N1-acetyl-N2-formyl-5-methoxykynuramine and N1-acetyl-5-methoxykynuramine). Therefore Galano et al. (2015) reported that melatonin is an extremely efficient antioxidant because of the continuation of oxidative stress inhibitory properties even after their metabolization.

Despite the rich literature on the relation between melatonin and tissue element levels, to the best of our knowledge, research on the connection between melatonin and TA element levels is lacking. In the present study, the TA Mg, Ca, Cu, Zn and Mn concentrations significantly increased in PNX rats, which had a lower melatonin level than did the rats in the SHAM group. In contrast to the Ca and Zn levels, the Li, Mg, Cu and Mn levels in TA were significantly lower in PNX+M than in the SHAM group (Figure 2, Table 2). According to Cunanne et al. (1979), the TA Zn content was increased, whereas serum, pituitary, adrenal, lung and hair Zn contents were decreased in rats eight weeks after pinealectomy. Similarly, a decreased Zn level and an increased Mg level were reported in the serum of pinealectomized chicks (Turgut et al. 2006). Furthermore, the exogenous melatonin (100 µg/mouse for one
month) treatment restored the plasma Zn levels, which were strongly reduced in pinealectomized mice, and Zn deficiency reoccurred when exogenous melatonin was interrupted for one month (Mocchegiani et al. 1996). In another study, pinealectomy caused increases in the element levels of kidney (Mn, Cu, Fe and Zn), liver (Ca, Na, Ni and Mn), heart (Mg, Fe and Zn) and lung (Cr, Mn, Mg, Na, Ca, Fe, Cu and Se) tissues in rats (Koykun, 2012). This researcher also reported that although the melatonin treatment (3 mg/kg/day for four weeks) caused some elements to return to the control levels, others did not return depending on the element and tissue types (Koykun, 2012). Our results demonstrated that the external melatonin dose administered in our experiment was inadequate to regulate the imbalance of ions other than Ca and Zn in the TA tissues of PNX animals. According to the literature and the result of our study, the element content in the serum and tissue was significantly affected by pinealectomy, and the exogenous melatonin could restore the element levels depending on the concentration, treatment time and tissue type (Cunanne et al. 1979; Mocchegiani et al. 1996; Turgut et al. 2006).

Melatonin deficiency causes blood pressure elevation, which is considered to be related to melatonin–nitric oxide association. Another critical mechanism in this relation involves calcium. Calcium is an important molecule that regulates the vascular tone related to vasoactive agents, such as endothelium-derived hyperpolarizing factor, nitric oxide, endothelin and superoxide anion (Calver et al. 1993). The mobilization of Ca from internal and external sources depends on several mechanisms and can be related to the effect of melatonin on the endothelium Ca signalling machinery under physiological and pathological conditions. Moreover, melatonin was found to play a role in the Ca response of vascular endothelial cells and to have the capacity to reverse the inhibitory effect of free radicals on the internal release of Ca (Pogan et al. 2002). Our results showed that increased levels of Ca in the TA due to PNX could be regulated by melatonin treatment. The melatonin concentration
of 6.78 pg/mg in the TA of SHAM-operated animals was higher than the values of 0.44 and 1.40 pg/mg in the PNX and PNX+M animals, respectively. This outcome suggests that external melatonin administration may be inadequate but partly effective in replacing the original pineal melatonin secretion in the present study. Sallinen et al. (2008) administered melatonin subcutaneously, and their results in the left ventricle (LV) were similar to those in our study. In a different study, the same research group found that the melatonin concentration was surprisingly high in the LV, contradicting our results (Sallinen et al. 2007).

In several organisms, melatonin deficiency causes oxidative stress. Increased oxidative stress levels due to an inadequate antioxidant system can lead to toxic effects, such as lipid peroxidation, DNA polymorphisms and gene induction/repression (Ochsendorf 1999). Recent studies have shown that during stressful conditions, differential regulation occurred for genes that encode antioxidant enzymes (SOD, CAT and GS) and HSPs (Doganlar et al. 2014). Glutathione mechanisms depollute toxic xenobiotic and endobiotic electrophiles to metabolize and excrete secondary products (Circu and Aw 2010). HSPs are called cellular stress proteins, and they are important for the stress responses of all types of living organisms. Recent studies have attempted to characterize the gene expression profiles of HSPs during stress responses, such as heat and oxidative stress, as well as heavy metal and pesticide toxicity (Doganlar and Doganlar 2015; Doganlar et al. 2016). However, the HSP species of different sizes exhibit different responses to biotic and abiotic stressors depending on the duration and severity of the stress. In the current study, a significant increase in the relative expression levels of antioxidant enzymes genes (CuZn-SOD, Mn-SOD and GS) was detected in the TA tissues of pinealectomized rats compared with SHAM rats. A recent study has demonstrated that high expression levels of antioxidant genes are biomarkers of cell oxidative cellular oxidative stress (Silins and Högbberg 2011). Therefore, we suggest that the melatonin deficiency present in different tissues causes oxidative stress in the TA, supporting the
previous results. Melatonin is a strong antioxidant that acts as a ROS scavenger (Reiter et al. 2000; Galano et al. 2011; Reiter et al. 2017), and melatonin metabolites also have a similar action (Galano et al. 2013; Janjetovic et al. 2017). Melatonin can stimulate both the content of non-enzymatic antioxidants, such as glutathione and ascorbic acid, and the activity and transcript levels of several important antioxidant enzymes, including peroxidase group enzymes, SOD, glutathione reductase, glutathione-S-transferase and CAT (Banerjee et al. 2017; Sruthi et al. 2017; Dutta et al. 2018, Guclu et al. 2018). However, intraperitoneal melatonin administration downregulates lipid peroxidation in the particular region of the nucleus and prevents apoptosis (Chen et al. 2003). In our study, melatonin deficiency significantly induced the HSP70 expression in the PNX group. The increased expression of HSP70 revealed that cytoprotective mechanisms were involved in the degradation of misfolded proteins. As ROS attacks vital proteins and causes their oxidation or aggregation, low-level melatonin-induced oxidative stress can lead to an augmentation of the expression of antioxidant genes and HSP70. In addition, we propose that the HSP70 synthesis that occurs during oxidative stress may be initiated to prevent the protein damage caused by oxidative stress and to correct and/or degrade misfolded proteins. The modulatory activity of melatonin against oxidative stress is evidenced in our study. In the PNX+M group, the regulator effect of melatonin was able to sufficiently modify the expression of CuZn SOD and Mn-SOD and the tissue MDA level in comparision with the PNX. Moreover, the melatonin treatment could have partially reversed the DNA damage and protein disruption-induced alterations in the HSP70 expression (Table 2, Figure 3).

Electrophoretic DNA profiling through the RAPD-PCR technique is used to determine the molecular response of several model organisms to several stress conditions. The constant pattern in the electrophoretic RAPD band profiles using arbitrarily primed PCR reactions provides useful and viewable data on the observable DNA alterations produced by biotic and
abiotic stressors in several cell lines and tissues (Rocco et al. 2014). RAPD band polymorphism may be due to changes in the priming sites of oligonucleotides caused by genome rearrangement. Newly occurring bands may also be caused by the DNA damage induced by stressors (Dogenlar and Doganlar 2015). Moreover, new bands may be the result of genomic template instability. Genetic stability is related to the level of DNA damage and the efficiency of DNA repair and replication mechanisms in the cell. Liu et al. (2005) and Lee et al. (2007) reported that the disappearance of PCR products mainly affected the high-molecular-weight bands because the probability of sustaining DNA damage increased with the length of the amplified fragment. In our study, we observed a decrease in the intensity of nine bands in the TA tissues in pinealectomized rats. However, a decrease in the intensity of four specific bands was found in the TA samples of the PNX+M group (Figure 3, Table 3). The changes in band intensity mainly depend on the genome rearrangement caused by the reactions of enzymes, such as Taq polymerase, and the efficiency of DNA repair mechanisms, particularly single 8-oxo-7’,8’-dihydro-2’-deoxyadenosine lesions. We suggest that the differences in band intensity related to insufficient genome repair mechanisms may be due to the altered molecular composition that is induced by oxidative stress and to the irregular changes in element balance and redox potential sources resulting from melatonin deficiency in cells.

Apoptosis is programmed cell death, and it occurs in several physiological and pathological conditions (Hengartner 2000). The primary mechanisms for the beginning of an apoptosis response upon melatonin deficiency may depend on a distinct stimulus, and these mechanisms have not been clearly identified. Excessive ROS, insufficient antioxidant scavenging, impaired genomic stability, and changes to other vital molecules are accepted as the main initial factors in apoptosis (Rich et al. 2000). Our results revealed that melatonin deficiency causes DNA damage, significantly increases the levels of MDA and both cytosolic and
mitochondrial SOD, serves as a biomonitor of oxidative stress, affects the stress-specific HSP70 gene expression and activates the gene and protein expression of caspase 3 and caspase 8 (Figure 2, 4). The activation of caspases in response to several stressors triggers the activation of both extrinsic and intrinsic apoptosis signalling pathways in mitochondria (Fulda and Debatin 2006). In our study, melatonin deficiency markedly induced the expressions of TNF-α, caspase 8 and caspase 3, downregulated the anti-apoptotic survivin but did not significantly change the ratio of BCL2 to BAX, which are components of the intrinsic apoptosis pathway, in the TA tissues of the pinealectomy group. For this reason, we considered that melatonin deficiency could induce apoptosis through the signalling involving TNF-α/caspase 8/caspase 3, which are the principal components of the extrinsic pathway. The present findings are consistent with those of recent studies reporting that caspase-dependent apoptosis can be initiated through the Fas ligand or TNF-α receptors. The molecules linked to the extrinsic pathway include FADD/TNF-α-connected death domain protein and caspase 8, which activates caspase 3 and induces apoptosis (Sheen et al. 2016). Previous studies demonstrated that melatonin exerts marked anti-apoptotic effects (Govender et al. 2014; Radogna et al. 2015) by preventing the release of cytochrome c from the mitochondria to inhibit caspase 3 activation and DNA damage. Melatonin also acts as an antioxidant and reduces reactive oxygen and nitrogen species, thus alleviating the damage to cell components that triggers apoptosis (Asghari et al. 2016). Our study demonstrated a significant decrease in the levels of TNF-α and the apoptotic genes caspase 3 and caspase 8 in the TA tissues of PNX+M animals compared with those in the PNX group. These gene expression results are supported by the protein expression with a marked correlation coefficient (gene/protein correlations: Tnf-α: 0.842, P=0.04; HSP70: 0.861, P=0.003; Casp8: 0.707, P=0.033; Casp3: 0.927, P<0.001). In addition, the anti-apoptotic gene survivin was significantly regulated by melatonin. This finding is consistent with previous data, which indicate that exogenous
melatonin may be an effective anti-apoptotic agent in cardiovascular cells (Zhang et al. 2013).

Melatonin administration is known to have beneficial effects on the cardiovascular system through oxidative stress inhibition, free radical scavenging, increased antioxidant activities and the prevention of ischemia/reperfusion injury, hypertension and atherosclerosis (Tengattini et al. 2008; Semercioz et al. 2017). In addition, melatonin supplements regulate jet lag, daytime fatigue and insomnia-induced cardiovascular disease (Girish et al. 2013). In the modern world, various factors connected to changes in lifestyle and living conditions may cause melatonin deficiency. Melatonin deficiency causes a number of cellular and molecular damage signals that are initially triggered by increased reactive oxygen species levels in tissues and organs. Oxidative damage to nuclear and cellular nucleic acid molecules and proteins through the overproduction of ROS in cardiovascular cells, such as endothelium, smooth muscle, and adventitia cells, often results in apoptosis. Both oxidative stress and ROS-mediated apoptosis have been directly associated with several cardiovascular diseases, particularly atherosclerosis, ischemia/reperfusion injury, calcification of blood vessels, formation of atherosclerotic plaques, heart failure and hypertension (Reiter et al. 2000; Rodriguez et al. 2004; Dominguez-Rodriguez et al. 2012). In conclusion, our results indicated that melatonin deficiency triggered the extrinsic TNF-α/caspase 8/caspase 3 apoptosis signalling induced by excessive oxidative stress, DNA and protein damage and elemental imbalances. Melatonin administration exhibited significant therapeutic effects on the TA tissues by regulating elemental balance, decreasing oxidative stress, DNA and protein damage, and preventing apoptotic cell death in pinealectomized rats.

**Conflict of interest**

The authors declare no conflict of interest.
Acknowledgements

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References


Radogna, F., Albertini, M. C., De Nicola, M., Diederich, M., Bejarano, I., and Ghibelli, L. 2015. Melatonin promotes Bax sequestration to mitochondria reducing cell susceptibility to apoptosis via the lipoxygenase metabolite 5-hydroxyeicosatetraenoic acid. Mitochondrion 21:113-121. doi: 10.1016/j.mito.2015.02.003


## Table 1. Genes, primer sequences and PCR conditions belong to antioxidant systems, HSPs and apoptosis pathway used in expressions studies (qRT-PCR) and RAPD assays

<table>
<thead>
<tr>
<th><strong>qRT-PCR Assay</strong></th>
<th><strong>Antioxidant System</strong></th>
<th><strong>Genes</strong></th>
<th><strong>Primer Sequences</strong></th>
<th><strong>PCR Conditions</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CuZn-SOD</td>
<td>F 5’</td>
<td>GTTCGGTGACAACACCAATG</td>
<td>1 cycle of 2 min at 50 °C and 10 min at 95 °C followed by 48 cycles of denaturation at 95 °C for 15 s, annealing and extension at 60 °C for 1 min</td>
</tr>
<tr>
<td></td>
<td>CAT</td>
<td>F 5’</td>
<td>TACGAGACGCAAGAAGTT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CAT</td>
<td>R 5’</td>
<td>ACCTGTAAGGCCAGTTCAC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>G5</td>
<td>F 5’</td>
<td>TGGGGACGCACTGCACTG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mn-SOD</td>
<td>F 5’</td>
<td>TCTGAGAGGCAAGAAGTT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GSH</td>
<td>F 5’</td>
<td>TGGGACCAGCAAGTAAAAC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>RAPD-PCR Assay</td>
<td><strong>Primer Names</strong></td>
<td>Primer Sequences</td>
<td><strong>PCR Conditions</strong></td>
</tr>
<tr>
<td></td>
<td></td>
<td>D4</td>
<td>5’ CTGTAGCATC</td>
<td>40 cycles of 95 °C denaturation (30 s), 37 °C annealing (30 s) and 72 °C elongation (90 s) with an initial 95 °C</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P2</td>
<td>5’ ATGTAACGCC</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>AP5</td>
<td>5’ TCCCGCTGCG</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>RF1</td>
<td>5’ GTACTCCG</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1247</td>
<td>5’ AAGGAGCGC</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1253</td>
<td>5’ GTTCGGGCC</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>RAPD1</td>
<td>5’ CCGATATCC</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>RAPD2</td>
<td>5’ GGTGTCGCT</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>RAPD3</td>
<td>5’ CCACGATTT</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>RAPD4</td>
<td>5’ CTACTGCGCT</td>
<td></td>
</tr>
</tbody>
</table>
Table 2. Quantitative real time PCR (qRT-PCR) analysis of antioxidant enzymes, heat shock proteins (HSPs), and apoptosis pathway genes and the detected levels of Melatonin, Li, Mg, Ca, Cu, Zn, Mn elements in thoracic aorta tissues of SHAM, Pinealectomy (PNX) and Pinealectomy plus melatonin (PNX+M) treated rats. All transcriptomic data normalized with GAPDH expression and given as relative to control (control=1). mean ± S.E, n:5. Physiological data represent ng mg⁻¹ tissue, mean ± S.E., n:5). ** Green and red colours indicate significantly different values compared to SHAM, analysed by one-way ANOVA, Duncan test (p≤0.05). ** indicate significantly differences values compared to other experiment groups (T*test: p≤0.05).

<table>
<thead>
<tr>
<th>Gen Expression (Fold Change)</th>
<th>SHAM</th>
<th>PNX</th>
<th>PNX+M</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CuZn-SOD</td>
<td>1,02±0.01</td>
<td>5,94±0.26</td>
<td>▲</td>
<td>2,24±0,21**▲&lt;0.0001</td>
</tr>
<tr>
<td>CAT</td>
<td>0,94±0.14</td>
<td>1,25±0.46</td>
<td>▲</td>
<td>1,55±0,09▲ 0.005</td>
</tr>
<tr>
<td>GS</td>
<td>1,12±0.11</td>
<td>1,99±0.77</td>
<td>▲</td>
<td>1,33±0,58▲ 0.003</td>
</tr>
<tr>
<td>Mn-SOD</td>
<td>1,14±0,39</td>
<td>5,82±0.74</td>
<td>▲</td>
<td>3,82±0,21▲ 0.001</td>
</tr>
<tr>
<td>HSP27</td>
<td>1,13±0.53</td>
<td>0,74±0,21</td>
<td>▲</td>
<td>0,64±0,08 ▲ 0.462</td>
</tr>
<tr>
<td>HSP60</td>
<td>0,96±0,26</td>
<td>0,08±0,06</td>
<td>▼</td>
<td>0,16±0,03 ▼ 0.029</td>
</tr>
<tr>
<td>HSP70</td>
<td>1,13±0.07</td>
<td>20,96±4,90</td>
<td>▲</td>
<td>8,16±1,06▲ 0.008</td>
</tr>
<tr>
<td>HSP90</td>
<td>1,08±0.54</td>
<td>0,25±0,02</td>
<td>▼</td>
<td>0,58±0,08 ▼ 0.043</td>
</tr>
<tr>
<td>Survivin</td>
<td>1,18±0.24</td>
<td>0,41±0,04</td>
<td>▼</td>
<td>1,12±0,07▲ 0.0001</td>
</tr>
<tr>
<td>TNF-α</td>
<td>1,06±0.05</td>
<td>1,73±0,10</td>
<td>▲</td>
<td>1,31±0,09▲ 0.045</td>
</tr>
<tr>
<td>BCL2</td>
<td>1,12±0.36</td>
<td>0,57±0,04</td>
<td>▲</td>
<td>0,63±0,04 ▲ 0.111</td>
</tr>
<tr>
<td>BAX</td>
<td>1,04±0.54</td>
<td>0,25±0,03</td>
<td>▼</td>
<td>0,23±0,02 ▼ 0.054</td>
</tr>
<tr>
<td>Apaf1</td>
<td>1,08±0.03</td>
<td>1,34±0,08</td>
<td>▲</td>
<td>1,19±0,07 ▲ 0.720</td>
</tr>
<tr>
<td>Cyt-C</td>
<td>1,12±0.26</td>
<td>1,20±0,08</td>
<td>▲</td>
<td>1,14±0,08 ▲ 0.626</td>
</tr>
<tr>
<td>Caspase 8</td>
<td>0,96±0,26</td>
<td>2,03±0,11</td>
<td>▲</td>
<td>1,29±0,07▲ 0.034</td>
</tr>
<tr>
<td>Caspase 3</td>
<td>1,12±0,14</td>
<td>2,87±0,15</td>
<td>▲</td>
<td>1,40±0,09▲ 0.001</td>
</tr>
</tbody>
</table>

▲ Upregulated ▼ Downregulated ▶ Stable (no significant change)

<table>
<thead>
<tr>
<th>Physiological Parameter (ng mg⁻¹)</th>
<th>SHAM</th>
<th>PNX</th>
<th>PNX+M</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Melatonin</td>
<td>6,78±0,74</td>
<td>0,44±0,60</td>
<td>▼</td>
<td>1,40±0,16**▼&lt;0.0001</td>
</tr>
<tr>
<td>Li</td>
<td>3,01±0,33</td>
<td>3,88±0,14</td>
<td>▲</td>
<td>2,13±0,34▼ 0.004</td>
</tr>
<tr>
<td>Mg</td>
<td>18,10±4,81</td>
<td>23,25±0,76</td>
<td>▲</td>
<td>8,28±0,45▼&lt;0.0001</td>
</tr>
<tr>
<td>Ca</td>
<td>6,25±0,32</td>
<td>13,07±0,19</td>
<td>▲</td>
<td>3,27±0,17▲ &lt;0.0001</td>
</tr>
<tr>
<td>Cu</td>
<td>81,71±9,49</td>
<td>125,83±5,28</td>
<td>▲</td>
<td>44,10±1,77▼ 0.002</td>
</tr>
<tr>
<td>Zn</td>
<td>3,45±0,54</td>
<td>6,33±0,13</td>
<td>▲</td>
<td>2,44±0,15▼&lt;0.0001</td>
</tr>
<tr>
<td>Mn</td>
<td>0,39±0,10</td>
<td>0,44±0,02</td>
<td>▲</td>
<td>0,18±0,06▼&lt;0.0001</td>
</tr>
</tbody>
</table>

▲ Increased ▼ Decreased ▶ Stable (no significant change)
Table 3. Changes in the RAPD profiles based on the appearance (A), disappearance (D) of bands and increase in band intensities (II), decrease in band intensities (DI) with specific molecular sizes (bp) using eight primers in the SHAM, pinealectomy (PNX) and pinealectomy plus melatonin (PNX+M) treated Sprague Dawley rats.

<table>
<thead>
<tr>
<th></th>
<th>SHAM</th>
<th>PNX</th>
<th>PNX+M</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>D</td>
<td>II</td>
</tr>
<tr>
<td><strong>RAPD 6</strong></td>
<td>1297; 1026; 788; 608; 446</td>
<td>1398</td>
<td>608</td>
</tr>
<tr>
<td><strong>RAPD 2</strong></td>
<td>1264; 976; 731; 435; 296</td>
<td>0</td>
<td>976; 731; 435</td>
</tr>
<tr>
<td><strong>SP1</strong></td>
<td>1185; 964; 675; 514; 365; 185</td>
<td>0</td>
<td>1185; 675; 185</td>
</tr>
<tr>
<td><strong>OPU16</strong></td>
<td>1314; 788; 495; 347</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>RAPD 5</strong></td>
<td>1169; 700; 521; 430; 339; 239; 171</td>
<td>0</td>
<td>521; 430; 339; 239; 171</td>
</tr>
<tr>
<td><strong>B18</strong></td>
<td>700; 446; 378; 210</td>
<td>0</td>
<td>700</td>
</tr>
<tr>
<td><strong>OP18</strong></td>
<td>837; 747; 700; 591; 529; 449; 347; 297</td>
<td>0</td>
<td>347; 297</td>
</tr>
<tr>
<td><strong>OPB10</strong></td>
<td>820; 662; 564; 487; 388; 300; 237; 186</td>
<td>0</td>
<td>1061; 904</td>
</tr>
</tbody>
</table>
Figure Legends

**Figure 1.** MDA level of thoracic aorta tissue in the sham operated control (SHAM), pinealectomy (PNX) and pinealectomy plus melatonin (PNX+M) treated Sprague dawley rats (n=5; mean±SE; nmol g\(^{-1}\)). Ns: no significant change * indicates significantly different values compared to other treatment groups (t-test: p ≤ 0.05). ** indicates significantly different value compared to SHAM operated control: PMDA=0.005, (resulted by one-way ANOVA, separated by Duncan test)

**Figure 2.** Heat map analysis using fold change score of gene expressions and physiological parameters of TA tissues of Sprague Dawley rats for control (SHAM), pinealectomy (PNX) and pinealectomy plus melatonin (PNX+M) treatment. All data Log10 (x+1) transformed and eBayes and Pearson correlation options were used hierarchical clustering and correlation analysis, respectively

**Figure 3.** The random amplified polymorphic DNA (RAPD) assay band profiles and genomic template stability (GTS%) of total genomic DNA belongs to thoracic aorta (TA) of Sprague Dawley rats for sham operated control (SM), pinealectomy (PNX) and pinealectomy plus melatonin (PNX+M) treatment. Primers: RAPD6, RAPD2, SP1, OPU16, RAPD5, B18, OPI18, OPB 10. Ladder (L) Geneaid 100-bp DNA ladder (100-3.000 bp).* indicate significantly different values (t-test: p ≤ 0.05)

**Figure 4.** Western blot analysis of Heat shock protein 70 kd (HSP70), Tumour necrosis factor alpha (TNF-α), Caspase 8 and Caspase 3 protein concentrations (n=3; mean±SE) *indicate significantly different values (t-test, p ≤ 0.05).Ns: no significant change, ** indicates significantly different value compared to sham operated control: PTNF-α=0.05, PHSP70=0.002, PCasp8<0.0001, PCasp3= 0.006 (resulted by one-way ANOVA, separated by Duncan test)
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