Melatonin Alleviates Cardiovascular Inflammation in Sedentary or Exercised Postmenopausal Rats by Upregulating SIRT1

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Research Article

Keywords: Melatonin, hormone replacement therapy, exercise, cardiovascular disease, sirtuin-1.

Posted Date: March 21st, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1449661/v1

Abstract

We aimed to evaluate the impact of hormone replacement, melatonin, or exercise alone or their combination on oxidative damage and functional status of heart, brain, and aorta of postmenopausal rats and to determine whether the signaling pathway is dependent on sirtuin-1 (SIRT1). Ovariectomized Sprague Dawley rats were orally given either a hormone replacement therapy (1 mg/kg/day17β estradiol; HRT) or melatonin (4 mg/kg/day) or HRT + melatonin treatments or tap water, while each group was further divided into sedentary and exercise (30 min/5 days/week) groups. After the heart rate measurements and memory tests were performed, trunk blood was collected at the end of the 10th week to determine metabolic parameters in serum samples. Tissue samples of abdominal aorta, heart and brain were taken for biochemical measurements and histopathological evaluation. Heart rates and memory performances of the postmenopausal rats were not changed significantly by none of the applications. Melatonin treatment or its co-administration with HRT upregulated the expressions of IL-10 and SIRT1, reduced the expressions of IL-6 and TNF-α and reduced DNA damage in the hearts and thoracic aortae of non-exercised rats. Co-administration of melatonin and HRT to exercised post-menopausal rats reduced inflammatory response and upregulated SIRT1 expression in the aortic and cardiac tissues. The present study suggests that melatonin treatment, either alone or in combination with exercise and/or HRT, alleviates oxidative injury and inflammation in the hearts and aortas of postmenopausal rats via the activity of SIRT1 signaling. Melatonin should be considered in alleviating cardiovascular disease risk in postmenopausal women.

Introduction

Cardiovascular disease (CVD), which was accounted for 35% of total women deaths in 2019 [1, 2] is globally the primary cause of mortality in women, while ischemic stroke is the second cause [3–5]. Although CVD is less common in premenopausal women, its incidence abruptly rises after menopause [6], which could be partly associated with changes in the levels of sex hormones [7, 8]. Despite that women have a greater antioxidant capacity than men, this high antioxidant capacity gradually decreases as estrogen levels decrease in the postmenopausal period [9, 10]. Lower estrogen concentrations were suggested to be involved in a higher CVD risk in postmenopausal women by causing autonomic impairment and endothelial dysfunction due to the lack of the modulatory effects of estrogen on cardiac myocytes and fibroblasts, vascular smooth muscle cells, and endothelial cells [8, 11, 12]. However, randomized controlled trials, which were conducted to study primary preventive potential of estrogen replacement during postmenopausal period paradoxically revealed a slight increase in CVD risk, while secondary prevention using estrogen plus progestin in women with CVD had no beneficial effect in reducing cardiac events [13]. Thus, menopausal hormone therapy is not indicated for either primary or secondary prevention of CVD. On the other hand, early treatment with low-dose estrogen is currently recommended for the management of postmenopausal symptoms in women with low CVD risk [14]. As an alternative non-pharmacological option, exercise has been suggested to ease menopausal symptoms, including psychological, vasomotor, somatic and sexual symptoms [15]. Moreover, clinical and

experimental studies have shown that aerobic exercise training has a positive effect on cardiovascular adaptation in hypertensive postmenopausal women [16]. However, the synergistic effect of estrogen treatment and exercise in postmenopausal cardioprotection was not elucidated yet.

Recent studies have investigated the protective effects of melatonin on menopause-related sleep disorders, bone loss and cancer development during the postmenopausal period [17–19]. Pineal gland hormone melatonin, which can cross all biological membranes with its amphiphilic nature, is well known to regulate circadian rhythm, immunomodulation, reproduction, and to protect tissues against oxidative damage by its extensive radical scavenging capability [20–25]. On the other hand, aging and various age-related diseases were associated with reduced plasma levels of melatonin [26–28], while exogeneous administration of melatonin in aged postmenopausal rats was shown to decrease oxidative stress and inflammation [29]. Similarly, melatonin supplementation in ovariectomized rats was shown to reverse metabolic dysregulation more potently than estrogen replacement, suggesting that melatonin could be an alternative therapy to treat postmenopausal symptoms [30]. Moreover, several experimental studies have also demonstrated that melatonin is protective against myocardial and cerebral injury with beneficial effects on ischemic cerebral arteries [31–35]. Recently, a double-blind randomized clinical trial conducted in patients with heart failure demonstrated that a 6-month melatonin intake improved echocardiographic indexes, reduced hospitalization and mortality, and provided a positive impact on overall well-being of the patients [36]. Moreover, a recent systematic review of the medical literature has revealed that oral administration of melatonin in menopausal women improved climacteric symptoms and resulted in several health benefits, which makes melatonin to be considered as an appropriate treatment choice in menopausal women [37]. In light of the aforementioned studies, we aimed to evaluate the impact of hormone replacement, melatonin or exercise alone or their combination on oxidative damage and functional status of heart, brain and aorta in postmenopausal rats. In addition, we aimed to determine whether the signaling pathway dependent on sirtuin-1 (SIRT1), a class III histone deacetylase with verified antioxidant and antiaging effects [38], plays a regulatory role in the putative protective effects of exercise or supplementation with estrogen or melatonin.

Materials And Methods

Animals

Sixty-four female Sprague-Dawley rats (250–300 g, 8–10 weeks old), supplied by the Marmara University (MU) Animal Center (DEHAMER), were housed in an air-conditioned room with 12 h–12 h light–dark cycles, relative humidity (65–70%) and constant temperature (22 ± 2°C). Rats were fed with standard rat pellets and water *ad libitum*. The experiments were performed in compliance with the Turkish law on the use of animals in experiments, and the principles and guidelines developed by the New York Academy of Sciences were followed. All experimental procedures were approved by the MU Animal Care and Use Committee (approval code:100.2018.mar).

Surgery and Experimental Design

Under anesthesia induced with intraperitoneal injection of ketamine and xylazine (100 mg/kg and 10 mg/kg), all rats underwent ovariectomy (OVX) by excision of ovaries through a small midline incision [39]. After the closure of the muscle and skin incisions, the rats were returned to their home cages and rats treated for three days with subcutaneous injection of acetaminophen (Perfalgan; Bristol Myers Squibb; 0.1 mg/kg/day) for analgesia. Starting immediately after surgery, all rats were randomly divided into four groups and they were given either tap water or they received in their drinking water either 17β estradiol (1 mg/kg/day, Bayer Turk İlaç Sanayii, İstanbul) as a hormone replacement therapy (HRT) or melatonin (4 mg/kg/day, Sigma, St Louis, MO) or HRT + melatonin for ten weeks (Fig. 1). The rationale for the selected doses of estradiol and melatonin was based on previous studies reporting their efficiency in inflammatory models [25, 40]. Water bottles were freshly prepared each day and wrapped with aluminum foil to protect from light. Amount of water remaining in the bottle of each cage (with 4 rats) was daily measured for two weeks and water intake of the experimental groups were not significantly different. After the two-week postsurgical recovery period, each of the groups was further divided randomly into sedentary and exercise groups and treatments were continued throughout the following 8 weeks.

Heart rate measurements and memory tests (object recognition and passive avoidance tests) were performed within the last week of the 10-week protocol (Fig. 1). On the 65th day of the protocol, needle electrodes were placed on the extremities of the rats under isoflurane anesthesia, and standard limb lead II on electrocardiogram was monitored using a computerized data acquisition system (Power Lab; ADInstruments, Radon Medical Ltd, Ankara, Turkey), and heart rates were calculated. At the end of the 10th week, following the second session of the passive avoidance test, rats were euthanized by decapitation and trunk blood was collected for the measurement of metabolic parameters in serum. Tissue samples of abdominal aorta, heart and brain were taken for biochemical measurements and histopathological evaluation. Protein levels of inflammatory cytokines tumor necrosis factor (TNF-α) and interleukin (IL)-6, anti-inflammatory IL-10 and silent information regulator type 1 (SIRT1) were determined using western blotting, while DNA damage was evaluated by measuring 8-hydroxy-2'-deoxyguanosine (8-OHdG) levels in the cardiac and aortic samples.

Exercise Program

During the second week of the postsurgical recovery period, all rats were adapted to swimming training for 5 days (15 min/day) in the cylindrical glass containers (50 x150 cm) that were filled with lukewarm water (28–32°C) at a 35-cm depth. In the exercise groups, swimming exercise was performed for 30 min in these glass containers, while sedentary rats were placed in containers filled with shallow water (5 cm; 32 – 28°C) just enough to wet their feet for 30 minutes, and these sessions were repeated 5 days a week for eight weeks. The intensity of this swimming exercise is accepted as a moderate level of exercise [41, 42].

Memory tests

Object recognition test was performed to evaluate short memory, while passive avoidance test was used for the assessment of long-term memory. A day before the object recognition test, rat was placed in the empty test box (50×50×30 cm) for 5 minutes to adapt to the new environment [43]. Then, on the 66th day of the protocol, rat was put into the same box to explore the two identical objects for 3 minutes and returned to its cage. One hour later, one of the objects was changed (new object) keeping one familiar object, and the interest of rat to the new object was recorded for 3 minutes by a video camera. After each test, the box and the objects were cleaned with 70% alcohol. Recordings were analyzed, and the ratio of the time spent with new object to the sum of time spent with both objects was determined as object recognition (%).

Passive avoidance test was accomplished using an electrical apparatus, composed of two sections (each 20×20×20 cm) separated by a gate (Northel, Istanbul). For the acquisition trial performed on the 67th day, rat was placed in the illuminated section. When the rat instinctively entered the dark section through the open gate, the gate was automatically closed and a brief electrical foot shock was given for 5 seconds (0.3–0.6 mA). Seventy-two hours after the first session, the rat was placed again at the illuminated site, and the time delay to enter the dark section was recorded for evaluating memory recall. If the rat has avoided entering the shock-given dark section within 300 sec (cut-off point), it was considered as normal memory performance, but entering the dark section with a shorter latency was regarded as memory dysfunction [44]. After each test, the apparatus was cleaned with 70% alcohol to remove odors. Immediately after the passive avoidance test, rats were decapitated to obtain blood and tissue samples.

Measurement of Myeloperoxidase Activity, Malondialdehyde and Glutathione in the Cardiac and Cerebral Tissue Samples

Myeloperoxidase (MPO) is an enzyme present in the azurophilic granules of polymorphonuclear leukocytes. MPO activity level was measured in the cardiac and cerebral tissues by detecting H_2O_2-dependent oxidation of o-dianizidine, and it was used as an indicator of tissue neutrophil infiltration, which was shown to correlate with the histochemically determined amount of polymorphonuclear leukocytes in the examined tissues [45]. One unit of MPO activity was defined as the amount of MPO present per gram of tissue weight that caused a change in absorbance of 1.0 min^{-1} at 460nm and 37°C.

In order to determine malondialdehyde (MDA) and glutathione levels, cardiac and cerebral samples were homogenized with ice-cold 150 mM KCl. The MDA levels were measured for products of lipid peroxidation by monitoring thiobarbituric acid reactive substance formation and expressed as nmol/g tissue. Glutathione (GSH) levels were determined with a spectrophotometric measurement based on the modified Ellman procedure and expressed as µmol/g tissue [46].

Chemiluminescence Assay in the Cardiac and Cerebral Tissue Samples

Levels of reactive oxygen species (ROS) were measured by the non-invasive chemiluminescence (CL) assay using luminol and lucigenin as enhancers. Generation of ROS was quantitatively measured using a luminometer (Junior LB 9509, EG&G Berthold, Germany) with the addition of the lucigenin (bis-Nmethylacridiniumnitrate, Sigma) or luminol (5-amino-2,3-dihydro-1,4- phthalazinedione, Sigma) probes to the samples. Luminol specifically detects hydroxyl, hydrogen peroxide and hypochlorite radicals, while lucigenin selectively indicates the presence of superoxide radicals. The results are expressed in relative light units (rlu) per mg of tissue.

Measurement of 8-hydroxy-2'-deoxyguanosine Levels in the Cardiac and Aortic Tissue Samples

Genomic DNA was isolated in the heart and abdominal aorta samples for the measurement of 8-hydroxy-2'-deoxyguanosine (8-OHdG) levels by using extraction kit (Invitrogen, USA). The assays were performed using kits, as stated in the manufacturer's instructions (Cell Biolabs, USA).

Western Blot Analyses in the Cardiac and Aortic Tissue Samples

The protein expression levels of TNF-α, IL-6, IL-10 and SIRT-1 were measured by the Western blotting method. Heart and abdominal aorta tissue samples were homogenized by using RIPA cell lysis buffer (89900, Thermo) and Bradford method used for the determination of the protein concentrations. All the samples of the same experimental group were pooled together and a 20 µg was taken from each sample. Protein expressions were determined with Western blot of three independent experiments, where all the experimental samples of the same group were pooled together. The samples were resolved by 4–12% sodium dodecyl sulphate–polyacrylamide gel electrophoresis and were transferred to polyvinylidene fluoride membrane, which was then blocked with 5% non-fat skim milk powder (Sigma, 70166) in Tris-Buffered saline (TBS). The membrane was washed twice in TBS containing Tween-20 (0.1%) and incubated overnight. (1:500 dilution anti-TNF-α sc-1351, anti-IL-10 sc-32815, anti-IL-6 sc-7920 and anti-SIRT1 sc-15404, Santa Cruz Biotechnology, Heidelberg, Germany) and washed with TBS containing Tween-20 (0.1%). The membrane was washed and incubated with horseradish peroxidase conjugated secondary antibody (anti-mouse) sc-2060 or anti-rabbit; sc-2004, Santa Cruz Biotechnology) for 2 h. The blots were developed with chemiluminescence reagents (sc2048, Santa Cruz Biotechnology, Texas, USA) and analyzed with chemiluminescent imaging systems (Syngene, Cambridge, UK). "Image J Programme Optical Density Analysis Software" (NIH) was used to analyze data by measuring each band three times for quantification.

Histopathological Analysis of the Cardiac, Cerebral and Aortic Tissue Samples

For light microscopic analysis of abdominal aorta, heart and brain tissues were fixed in 10% neutral formaldehyde solution and routine paraffin embedding processes were done. Hematoxylin and eosin (H&E) staining was applied to approximately 5 µm-thick paraffin sections of samples and examined by

digital camera (Olympus C-5060) attached photomicroscope (Olympus BX51, Tokyo, Japan). In all groups, aortic tunica intima and tunica media layers were measured from 3 different regions by using the Image J program, and the mean value for each rat was calculated.

Statistical Analyses

Statistical analyses were done by GraphPad Prism 9.2 (GraphPad Software, San Diego; CA; USA). All data are expressed as means ± S.E.M. Three-Way ANOVA was used in analysis of data. $p < 0.05$ was considered as statistically significant.

Results

The four subgroups of sedentary and exercised postmenopausal rats that have not received estradiol supplementation demonstrated a > 30% increase in body weight by the end of the 10th week (Table 1). However, in the other sedentary or exercised groups that were treated with HRT, weight gain was abolished ($p < 0.001$), and melatonin treatment had no additional effect on weight changes. Heart rates of the postmenopausal rats recorded on the postsurgical 10th week were similar in the sedentary or exercised groups that have received either HRT, melatonin or their combination. Object recognition and passive avoidance test results revealed that short- and long-term memory performances of the postmenopausal rats were not changed significantly by none of the applications (Table 1). However, some rats (2 of 8 rats in each group) in the tap water given groups, except for the estradiol-treated exercised group, demonstrated a disturbed long-term recall on passive avoidance test, while all rats in the melatonin-treated groups presented with normal memory performances. Serum levels of glucose, LDL, VLDL, total cholesterol and triglyceride were similar in all the postmenopausal rats that have received different treatment modalities (Table 2).

Table 1
Weight change, heart rate, aortic wall thickness and results of memory tests (object recognition and passive avoidance) in the experimental groups of rats (each n = 8).

		Weight Change (%)	Heart Rate (bpm)	Aortic Wall Thickness (mm)	Passive Avoidance (sec)	Object Recognition (%)
HRT (-)						
Sedentary	Tap water	38.7 ± 8.3	407.2 ± 22.4	98.6 ± 11.8	229.0 ± 46.4	27.1 ± 23.4
	Melatonin	30.8 ± 8.2	422.7 ± 20.1	103.6 ± 8.9	300.0 ± 0	-5.3 ± 17.6
Exercise	Tap water	29.0 ± 7.9	429.5 ± 9.0	105.5 ± 4.8	236.0 ± 42.1	20.4 ± 20.0
	Melatonin	30.7 ± 7.9	413.8 ± 14.7	122.4 ± 13.2	300.0 ± 0	-12.4 ± 20.9
HRT (+)						
Sedentary	Tap water	-7.3 ± 4.6 *	440.8 ± 13.5	109.6 ± 8.1	232.7 ± 44.2	24.7 ± 14.0
	Melatonin	-1.4 ± 3.6*	442.8 ± 6.3	110.6 ± 5.9	300.0 ± 0	4.8 ± 19.1
Exercise	Tap water	-8.5 ± 5.4*	417.6 ± 15.8	114.7 ± 4.4	300.0 ± 0	9.3 ± 14.2
	Melatonin	-5.6 ± 5.2*	396.1 ± 13.3	110.9 ± 9.2	300.0 ± 0	15.7 ± 21.3

Hormone replacement therapy (HRT, 17-β estradiol, 1mg/kg/day) and melatonin (4 mg/kg/day) were continued for 10 weeks. Swimming exercise (30 min/day, 5 days/week) was performed for 8 weeks. bpm: beats per minute.

*$p < 0.001$; compared with the HRT (-) sedentary group that have received only tap water (3-way ANOVA).

Table 2
Serum glucose, LDL, VLDL, HDL, total cholesterol and triglyceride levels in the experimental groups of rats (each n = 8).

		Glucose (mg/dL)	LDL (mg/dL)	VLDL (mg/dL)	HDL (mg/dL)	Total Cholesterol (mg/dL)	Triglyceride (mg/dL)
HRT (-)							
Sedentary	Tap water	140.9 ± 3.88	17.86 ± 3.47	11.75 ± 2.11	48.63 ± 3.14	77.38 ± 5.00	66.63 ± 12.13
	Melatonin	135.8 ± 1.52	19.14 ± 3.13	13.13 ± 2.75	47.13 ± 3.24	76.13 ± 5.15	65.25 ± 13.54
Exercise	Tap water	152.3 ± 4.51	14.25 ± 1.72	9.250 ± 1.43	40.00 ± 0.96	63.50 ± 1.15	47.63 ± 7.06
	Melatonin	140.0 ± 3.42	15.75 ± 2.39	8.625 ± 1.03	38.88 ± 1.32	63.25 ± 1.15	43.50 ± 5.48
HRT (+)							
Sedentary	Tap water	135.1 ± 4.25	9.286 ± 1.94	13.75 ± 1.99	41.25 ± 4.94	62.63 ± 6.40	68.75 ± 9.77
	Melatonin	143.5 ± 3.03	15.00 ± 3.16	11.88 ± 1.98	42.25 ± 4.17	64.63 ± 6.41	65.25 ± 13.54
Exercise	Tap water	136.4 ± 2.13	13.00 ± 1.41	19.67 ± 3.37	37.59 ± 1.42	59.22 ± 5.56	98.89 ± 17.03
	Melatonin	138.4 ± 4.30	12.57 ± 3.82	18.13 ± 2.81	38.2 ± 0.69	60.63 ± 5.54	90.50 ± 14.57
Hormone replacement therapy (HRT, 17-β estradiol, 1mg/kg/day) and melatonin (4 mg/kg/day) were continued for 10 weeks.							
Swimming exercise (30 min/day, 5 days/week) was performed for 8 weeks. No significant differences were present among the							
experimental groups (3-way ANOVA).							

Both the MDA level, indicative of lipid peroxidation, and MPO activity, showing neutrophil infiltration, were similar in the heart tissues of all the postmenopausal rats that have either exercised or remained sedentary, received either melatonin, estradiol or their combination (Fig. 2). Similarly, ROS generation, as detected by luminol or lucigenin chemiluminescence was not significantly altered in the cardiac tissues of postmenopausal rats of different subgroups. Indicative of its depletion, cardiac GSH content was significantly lower in all the HRT-treated rats, as compared to the sedentary group with no treatments ($p < 0.05 – 0.001$). In parallel with the minimal oxidative changes in the cardiac tissue, histological analysis revealed that mild irregularities in the organization of myofibrils in cardiomyocytes and vascular

congestion were present in the sedentary groups treated with or without melatonin. On the other hand, irregularities in the myofibril organization were observed only in a few cardiomyocytes of the exercised groups (Fig. 3).

Similar to the cardiac tissue, brain MPO activity levels were not different among the experimental groups (Fig. 4). However, in the HRT-treated exercised rats, brain MDA level was significantly higher than that of the non-treated sedentary rats ($p < 0.01$), while melatonin administration abolished this elevation. Unlike the cardiac tissue, cerebral GSH content was not changed significantly by any of the treatment modalities, but a statistically non-significant elevation was observed in both of the HRT-treated exercised rats. Lucigenin-detected superoxide ($O_2.^-$) generation in the brain tissues was higher in the exercised rats without HRT ($p < 0.05 – 0.01$), while the $O_2.^-$ levels were not elevated when postmenopausal rats were treated with estradiol. Histological analysis of the hippocampal sections, which are closely related to memory function, showed that the numbers of damaged neurons in cornus ammonis (CA) 1 and damaged granular cells in dentate gyrus (DG) were higher in the non-treated sedentary group (Fig. 5). However, in either estradiol- or melatonin-treated sedentary groups, as well as in the non-treated exercise and estradiol-treated exercise groups, damaged neurons in CA1 and damaged granular cells in DG were at a moderate level. In the sedentary group treated with the estradiol and melatonin combination and in both of the melatonin-treated exercise groups, a mild degree of neuronal degeneration was evident.

As compared to sedentary non-treated postmenopausal rats, protein expression of the proinflammatory cytokine TNF-α was significantly down-regulated in the cardiac tissues of melatonin- and/or estradiol-treated and/or exercised rats ($p < 0.01 – 0.001$), and the lowest cardiac TNF-α expression levels were observed in the exercised or sedentary rats that have received melatonin plus HRT and in exercised rats that were given only HRT (Fig. 6). Cardiac IL-6 expression in the non-HRT groups was reduced by melatonin in both sedentary or exercised rats ($p < 0.001$) or by exercise alone ($p < 0.001$; Fig. 6). Similarly, supplementing with HRT in sedentary rats also downregulated IL-6 expression in the heart tissue ($p < 0.001$). On the other hand, exercise added to co-administration of HRT and melatonin resulted in the overexpression of cardiac IL-6. The expression of the anti-inflammatory cytokine IL-10 in the hearts of sedentary postmenopausal rats was not changed by estradiol supplementation alone (Fig. 6). However, melatonin treatment or exercise or their combination significantly upregulated cardiac IL-10 expression in both HRT and non-HRT groups ($p < 0.01 – 0.001$). In parallel with the changes in cytokine expressions, cardiac level of 8-OHdG, an indicator of DNA oxidation, was decreased in melatonin-treated estrogen-depleted sedentary group as compared to non-treated sedentary group ($p < 0.001$), but addition of exercise to melatonin treatment reversed the reduction in 8-OHdG (Fig. 6). On the other hand, in the absence of melatonin treatment, cardiac 8-OHdG levels were significantly elevated in the HRT-treated sedentary or exercise groups ($p < 0.001$), while melatonin suppressed DNA oxidation in both sedentary and exercised groups treated with HRT ($p < 0.001$).

In the abdominal aortae of sedentary rats that were not supplemented with HRT, administration of melatonin significantly suppressed TNF-α expression ($p < 0.001$), but aortic TNF-α expression was further elevated in the exercised rats with no HRT ($p < 0.001$), and melatonin did not change the elevated TNF- α

expression (Fig. 7). Supplementing with estradiol in either sedentary or exercised rats reduced the aortic expression of TNF-α ($p < 0.001$), while addition of melatonin to HRT and exercise further depressed TNF-α expression ($p < 0.001$). In the non-HRT groups, exercise *per se* significantly reduced the expression of aortic IL-6 ($p < 0.001$; Fig. 7). Compared with the sedentary group with no treatments, aortic IL-6 expression was increased by 3 folds in the sedentary rats supplemented with estradiol ($p < 0.001$), but this elevation was reduced by the addition of melatonin to HRT in sedentary rats ($p < 0.001$). However, melatonin and HRT co-administration resulted in an elevated IL-6 expression in the aorta of exercised rats ($p < 0.001$). In sedentary rats, treating only with estradiol had no impact on aortic IL-10 expression (Fig. 7). In the absence of estradiol treatment, IL-10 expression in the aorta was significantly increased by exercise as compared to untreated sedentary rats ($p < 0.001$), but conversely exercise and melatonin combination reduced IL-10 expression ($p < 0.05$). However, in the HRT-supplemented rats, melatonin given to sedentary rats ($p < 0.001$), as well as exercise with or without melatonin further increased IL-10 expression, reaching to a 4-fold increase by the combination of exercise, melatonin and HRT ($p < 0.001$). The 8-OHdG levels measured in the aortic tissues of rats treated with either melatonin or HRT or their combination were significantly lower in both the exercised and sedentary groups, as compared to that of the non-treated sedentary rats ($p < 0.001$), and these depressed levels of 8-OHdG were similar in all the treatment modalities.

Cardiac SIRT1 protein expression was significantly reduced by the 8-week exercise in postmenopausal rats that had no hormone replacement as compared to sedentary ones ($p < 0.001$; Fig. 6). However, when non-HRT groups were treated with only melatonin ($p < 0.001$) or when melatonin was added to exercise ($p < 0.01$), cardiac SIRT1 expression was significantly upregulated. Similarly, estradiol supplementation in postmenopausal rats significantly increased the expression of SIRT1 in both exercised ($p < 0.01$) and sedentary ($p < 0.001$) rats, while co-administration of melatonin with HRT further upregulated SIRT1 expression in both the sedentary and exercised rats ($p < 0.001$). Aortic SIRT1 expression in postmenopausal rats was increased by all the treatment modalities ($p < 0.01 – 0.001$), while the highest expression levels were observed in the exercised rats that have received HRT (Fig. 7).

Under the light microscope, wall thickness of the abdominal aorta was observed to be lowest in the sedentary group and highest in the melatonin-treated exercised and HRT-treated exercised groups (Fig. 8), but no statistical difference was present between the wall thickness measurements of the experimental groups (Table 1). Histopathological evaluation revealed a normal morphology in the non-treated sedentary group, which demonstrated a moderate inflammatory cell infiltration in the tunica adventitia (externa) layer of the aortic wall, while inflammatory cell infiltration was mild in all the other groups. Except for the melatonin-treated exercised group, which showed a normal aortic wall morphology, all the other groups demonstrated a mild endothelial cell separation.

Discussion

The experiments described here demonstrated that daily melatonin treatment or its co-administration with HRT for 8 weeks starting by the second week of surgical menopause, upregulated the expressions of

IL-10 and SIRT1, reduced the expressions of the pro-inflammatory cytokines and reduced DNA damage in the hearts and thoracic aortae of non-exercised rats, showing a significant improvement in the inflammatory status of the cardiovascular system. Similarly, co-administration of melatonin and HRT to exercised post-menopausal rats also resulted in a reduced inflammatory response in the aortic and cardiac tissues. When taken together, these results implicate that melatonin treatment by itself upregulates SIRT1 expression and reduces the intensity of menopause-associated cardiovascular inflammation more potently than either exercise or HRT alone, while addition of melatonin to HRT or exercise alleviates upregulation of the inflammatory cascades induced either by HRT, exercise or HRT plus exercise.

Since the aging process in women overlaps with the period of reduced ovarian hormone production, age-related morphological, autonomic, biochemical and functional changes in the cardiovascular system are exacerbated by menopause, increasing the incidence of CVD [47]. Accumulating evidence has revealed that depletion of ovarian hormones results in increased sympathetic tonus, oxidative stress, and impaired endothelial function, and thereby has a dominant impact on the occurrence of atherosclerotic processes, vascular damage and cardiovascular dysfunction [9, 47–50]. Although estrogen supplementation in OVX-rats [51–55] or in younger postmenopausal women [56] was shown to be effective in delaying atherosclerosis and reducing the CVD risk [57, 58], randomized controlled trials were not able to confirm the CVD-preventive effect of postmenopausal hormone therapy alone [13]. Our findings demonstrated that estradiol treatment for 8 weeks decreased TNF-α expression and elevated IL-10 expression in both the cardiac and aortic tissues, reduced IL-6 expression in the cardiac tissue and decreased oxidative DNA damage in the aortae of the non-exercised postmenopausal rats. Moreover, HRT resulted in the upregulation of SIRT-1 expression in both tissues, demonstrating the possible involvement of SIRT1 pathway in HRT-induced amelioration of menopause-associated cardiovascular inflammation. Despite these improvements reached by estradiol treatment, aortic IL-6 expression was increased, while DNA damage was elevated and GSH levels were depleted in the hearts of estradiol supplemented non-exercised postmenopausal rats, showing some augmentation in cardiovascular inflammation by HRT. In parallel to our findings, a randomized, placebo-controlled study conducted in healthy normotensive postmenopausal women has demonstrated that a short-term estradiol treatment initially increased the plasma levels of C-reactive protein (CRP), which was associated with the increased risk of cardiovascular events due to HRT [59]. Similarly, it was demonstrated that the inflammatory markers CRP and IL-6 are predictive of incident vascular events among otherwise healthy postmenopausal women, and long-term use of HRT elevates CRP [49]. Thus, combining estrogen replacement with other pharmacological treatment modalities would be a meaningful strategy to reduce the ongoing inflammation. Accordingly, our findings revealed that addition of melatonin to HRT, as well as melatonin *per se* improved all oxidative and inflammatory parameters in the non-exercised rats.

As a non-pharmacological therapeutic approach, exercise was demonstrated to have beneficial effects in attenuating cardiometabolic syndrome, improving antioxidant status, reducing proinflammatory cytokine levels and ameliorating cardiovascular dysfunction in OVX rats [60–64]. Clinical studies verified the advantageous effects of exercise training on metabolic and lipid profiles and cardiorespiratory fitness of

postmenopausal women [16, 65, 66]. On the other hand, it was reported that running exercise for a 6-week period either alone or in combination with phytoestrogen treatment had no positive effect on OVX-induced deterioration of cardiovascular functions in rats [67]. Another study demonstrated that estradiol treatment has not prevented cardiac dysfunction in OVX-rats with myocardial infarction, while adding estradiol treatment to exercise has abolished the positive effects of 8-week treadmill exercise in infarcted postmenopausal rats [68]. Similarly, estrogen-supplemented OVX rats have presented a decrease in cardiac IL-6 levels, while this decrease in IL-6 was abolished by an 11-week treadmill program [69]. We have previously demonstrated that 4 weeks of swimming exercise prior to myocardial injury has reduced the plasma TNF-α level in the postmenopausal rats as compared to sedentary rats, but the elevated plasma IL-6 and IL-8 levels were not reduced by exercise [70]. Our current findings revealed that regular swimming exercise alone for 8 weeks, downregulated IL-6 and upregulated IL-10 in both the cardiac and aortic tissues of the postmenopausal rats, while aortic TNF-α expression was further increased by exercise. Despite the elevation in aortic SIRT1 expression showing the beneficial effect of exercise on the vasculature of the non-HRT-treated rats, cardiac SIRT1 was conversely downregulated. However, exercise accompanied by HRT upregulated SIRT1 expression in both tissues, but the elevations observed in cardiac 8-OHdG and aortic IL-6 expressions of only HRT-given rats were not changed by the addition of exercise. On the other hand, except for some ongoing elevations in IL-6 and TNF-α melatonin intake in the postmenopausal exercised rats resulted in elevated SIRT1 expression and reduced DNA damage in both tissues, demonstrating the ameliorative effect of melatonin on the additive cardiovascular oxidative injury triggered by exercise.

Since postmenopausal hormone therapy has been linked to an increased risk of several female cancers [71], postmenopausal use of estrogen has been restricted and an alternative to estrogen has become crucial. Accordingly, a recent systematic review has reported that oral melatonin administration improves hemodynamic measures, glucose metabolism, bone density, sleep quality and climacteric symptoms, suggesting that melatonin could be considered as an effective treatment option for menopausal women [37]. Clinical studies have indicated that melatonin improves lipid metabolism and protects against the atherosclerotic changes, and thereby could have benefits in the treatment or prevention of cardiovascular diseases [36, 72–74]. In support of these clinical outcomes, melatonin supplementation in OVX-rats enhances the antioxidant effects of estrogen and prevented oxidative stress more effectively than estrogen replacement [75, 76]. In agreement with the aforementioned studies, the findings of the present study demonstrated that melatonin treatment alone in postmenopausal rats with no additional treatment strategies elevated the SIRT1 expression and improved the oxidative status of the cardiovascular tissue. Furthermore, our results also showed that HRT- or exercise-induced oxidative cardiac damage and enhanced cardiovascular inflammation were ameliorated when melatonin was added to the treatment regimen.

Considering the diverse functions of melatonin throughout the body, it is expected that the reduction in endogenous melatonin is involved in the pathogenesis of several aging-associated diseases including CVD [77]. Aging and several age-related pathologic conditions are closely linked with reduced secretion of melatonin and diminished SIRT1 activity [28]. SIRT1, which regulates gene expression by deacetylation of

histone proteins and transcription factors, plays an important role in the prevention of oxidative stress and inflammation in several tissues by mediating multiple signaling pathways [78–81]. In accordance with that, melatonin was shown to upregulate SIRT1 in cell cultures and several animal models, while the beneficial effects of melatonin were abolished by inhibition or knockdown of sirtuin, suggesting that the mechanisms contributing to anti-inflammatory effects of melatonin include its facilitatory effect on SIRT1 signaling pathway [82–85]. Both experimental and clinical studies have shown that melatonin facilitates the recovery of osteogenesis and ameliorates postmenopausal bone damage by upregulating SIRT1-mediated antioxidant enzyme capacity [86, 87]. Although the SIRT-1-mediated anti-inflammatory, antioxidant and antiapoptotic effects of melatonin on cardiovascular injury were demonstrated in several animal models [79, 82, 84, 88, 89] the involvement of SIRT-1 signaling pathway in the postmenopausal beneficial effects of melatonin was not elucidated before. A recent study in OVX mice has shown that estrogen-SIRT1 axis has a dominant modulatory role in protecting aortas against the development of atherosclerosis [90]. In accordance with these results, our data demonstrate that administration of melatonin or melatonin plus estradiol to non-exercised postmenopausal rats improved the oxidative and inflammatory parameters via the upregulation of SIRT1, while exercise-induced downregulation of cardiac SIRT1 was reversed by melatonin intake. When taken together, our data implicate that the beneficial effects of melatonin in ameliorating postmenopausal cardiovascular injury involve the upregulation of antioxidant capacity and the stimulation of the SIRT1 activity, making it a safer alternative treatment option as compared to HRT.

Melatonin was shown to exert neuroprotective effects against sepsis-induced oxidative brain injury by decreasing the production of proinflammatory cytokines and MDA, and increasing antioxidant capacity via the SIRT1 activation [85]. In the present study, exercise-induced ROS generation in the brain tissue of non-HRT-treated rats was not altered by melatonin, while increased lipid peroxidation due to HRT in exercised rats was abolished by melatonin. In accordance with the minor changes in the oxidative status of the brain tissue, the short- and long-term memory functions were normal in all groups and not different among of the studied menopausal treatment modalities, suggesting that the changes in the expression of inflammatory mediators in the cardiac and vascular tissues were not reflected in cognitive functional changes yet. It is well known that cognitive decline occurs due to cumulative exposure to cardiovascular risk factors and chronic atherosclerosis that are responsible for cerebral hypoperfusion and reduced brain oxygenation [91, 92]. Thus, it is possible that surgical menopause conducted in the early life of the rats has not yet impaired the memory functions.

In conclusion, the present study suggests that melatonin treatment, either alone or in combination with exercise and/or HRT, alleviates oxidative injury and inflammation in the hearts and aortas of postmenopausal rats via the activity of SIRT1 signaling. Although the mechanistic relation between melatonin and SIRT1 expression has been

reported previously in several publications, this is the first study to demonstrate that the exercise melatonin combination exerts SIRT1-mediated beneficial effects on post-menopausal complications in the cardiovascular system. Thus, our encouraging results implicate that melatonin should be considered

in alleviating CVD risk in postmenopausal women, and further studies are required to elucidate the complex regulatory mechanisms involved in the melatonin-SIRT1 axis.

Declarations

Part of this work was presented at the FEPS 2019-Bologna Congress and published as an abstract (ACTA PHYSIOLOGICA 2019; 227: S718).

Conflicts of interest/Competing interests:

The authors have no relevant financial or non-financial interests to disclose.

Author contributions:

- All the experiments were performed at Physiology (SAT, TA, ME, SK, RGY, ZB, ZSD, AY, BÇY) and Histology & Embryology (HNL, FE) Departments of School of Medicine, Vocational School of Health Sciences (MY), İstanbul; and Biochemistry (MAU, ÖÇ) Department of School of Medicine at Adnan Menderes University, Aydın, Turkey.
- All persons (SAT, TA, ME, SK, RGY, ZB, ZSD, HNL, MAU, MY, ÖÇ, FE, AY, BÇY) designated as authors qualify for authorship.
- All persons who qualify for authorship are listed.
- All authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

• **Study conception and design of the work:** SAT, AY, BÇY.

• **Data acquisition:** SAT, TA, ME, SK, RGY, ZB, ZSD.

• **Analysis and data interpretation:** SAT, TA, ME, SK, RGY, ZB, ZSD, HNL, MAU, MY, ÖÇ, FE, AY, BÇY (all authors).

• **Drafting of the manuscript:** SAT, TA, ME, SK, RGY, ZB, ZSD, HNL, MAU, MY, ÖÇ, FE, AY, BÇY (all authors).

• **Critical revision:** SAT, AY, BÇY.

• **Approval of the final version of the manuscript:** SAT, TA, ME, SK, RGY, ZB, ZSD, HNL, MAU, MY, ÖÇ, FE, AY, BÇY (all authors).

Acknowledgements: The authors are grateful to Professor Goncagül Haklar for her guidance in measuring the metabolic parameters in blood samples.

This study was partly supported by a student grant from The Scientific and Technological Research Council of Turkey (TUBITAK)–program code: 2209, project no: 1919B011900627).

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Figures

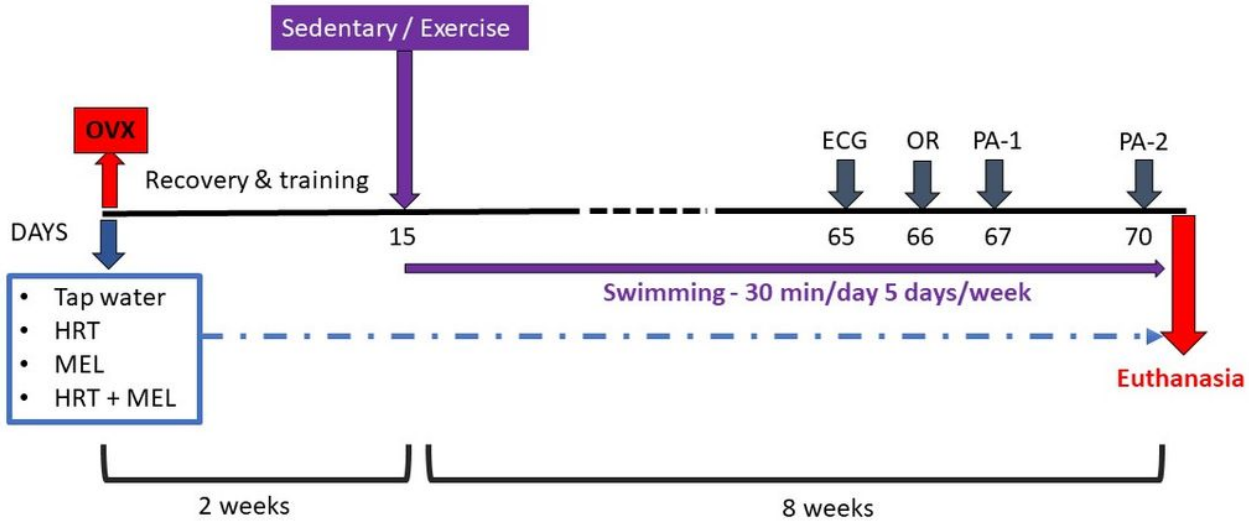

Figure 1

Diagrammatic representation of the experimental protocol. ECG: Electrocardiography; OR: Object recognition; PAT: Passive avoidance test; HRT: hormone replacement therapy (1 mg/kg/day, orally); MEL: Melatonin (4 mg/kg/day, orally); OVX: Ovariectomy. OVX was performed in all experimental groups.

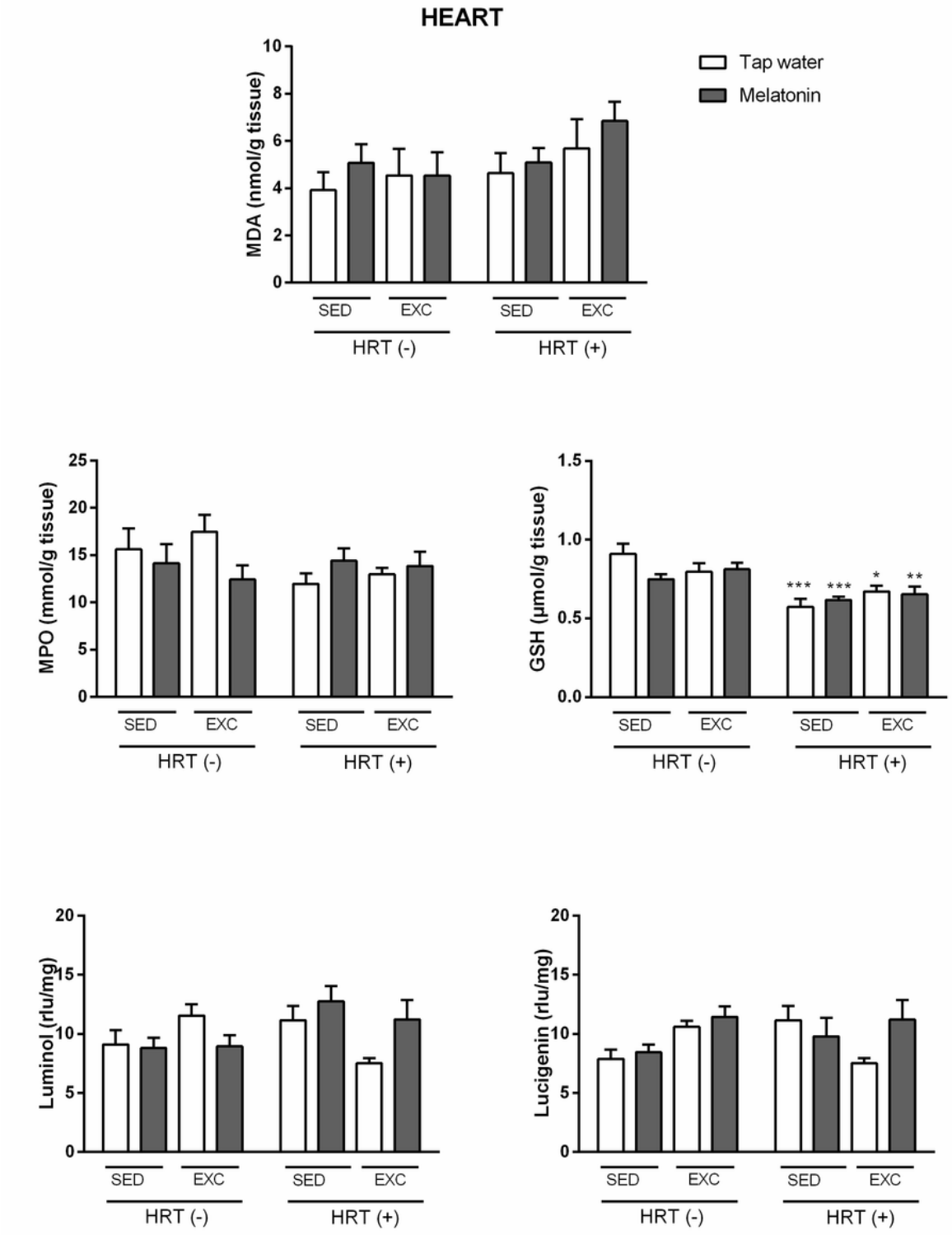

Figure 2

Cardiac levels of malondialdehyde, myeloperoxidase activity, glutathione and chemiluminescence levels of luminol and lucigenin in the sedentary or exercise groups that have received hormone replacement therapy (HRT) or melatonin or normal tap water. Experimental data are presented as mean ± SEM of 8 animals; *p<0.05, **p<0.01, ***p<0.001, compared to tap-water given sedentary control group.

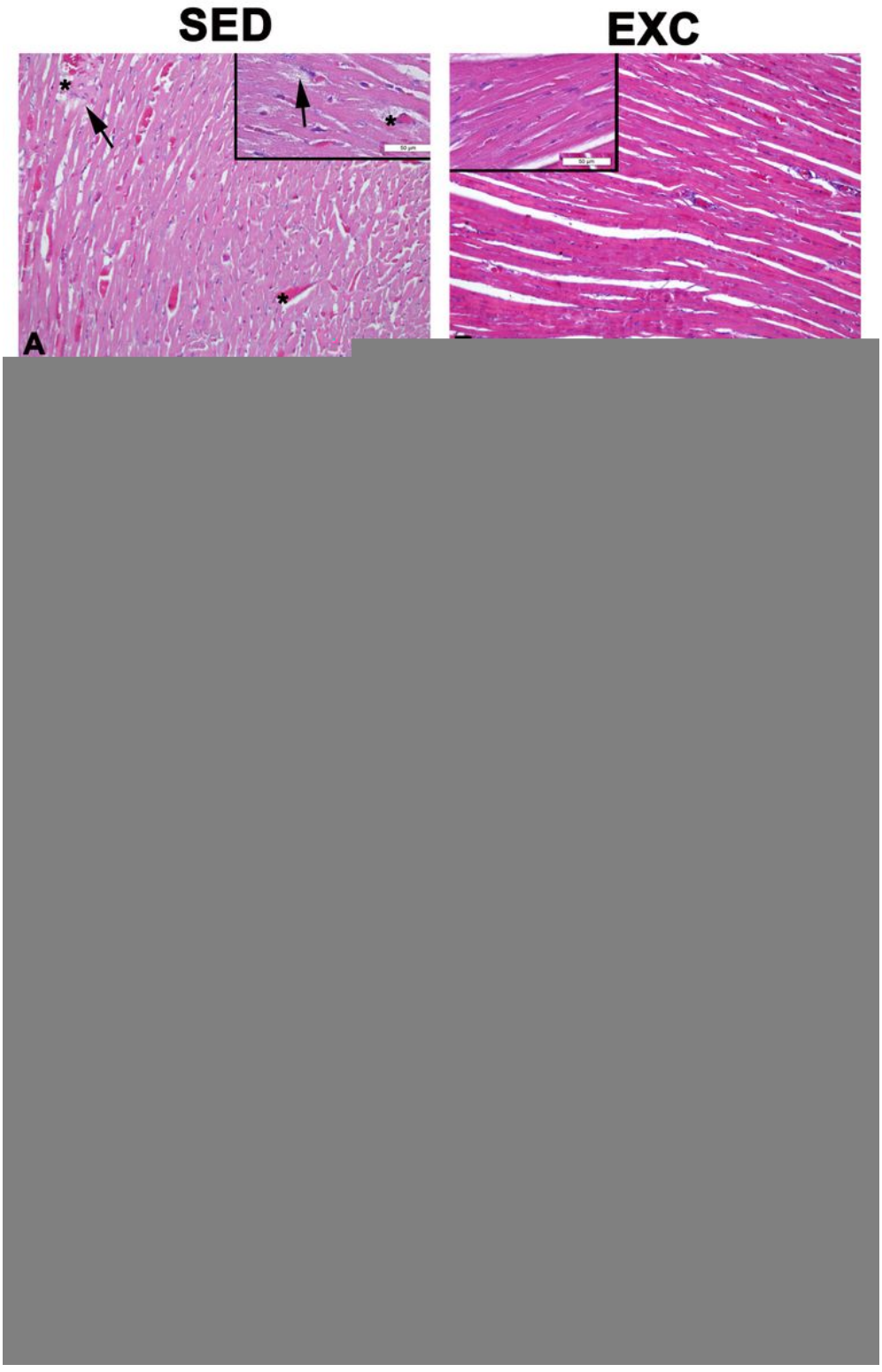

Figure 3

Representative light micrographs of cardiac samples stained with H&E. In the sedentary group (A) vascular congestion (*) and mild cardiomyocyte injury (arrow) are seen. In the groups of sedentary plus melatonin (B), sedentary plus hormone replacement therapy (HRT) (C), Sedentary plus melatonin and

HRT combination (D), exercise (E), exercise plus melatonin (F), Exercise plus HRT (G) and exercise plus melatonin and HRT combination (H); mild vascular congestion (arrow) and very few injured cardiomyocytes (arrow) are seen.

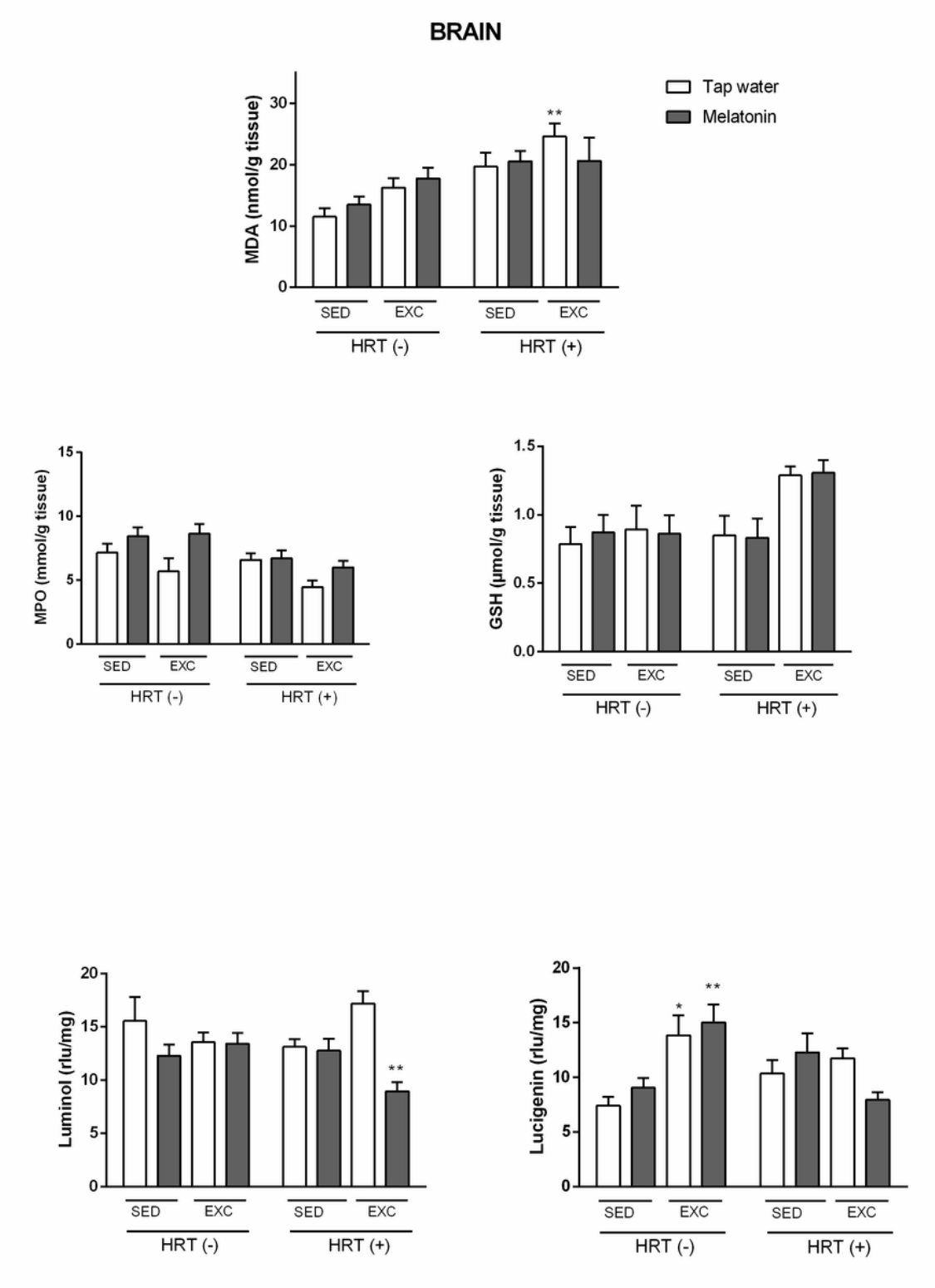

Figure 4

Cerebral levels of malondialdehyde, myeloperoxidase activity, glutathione and chemiluminescence levels of luminol and lucigenin in the sedentary or exercise groups that have received hormone replacement therapy (HRT) or melatonin or normal tap water. Experimental data are presented as mean ± SEM of 8 animals; *p<0.05, **p<0.01, compared to tap-water given sedentary control group.

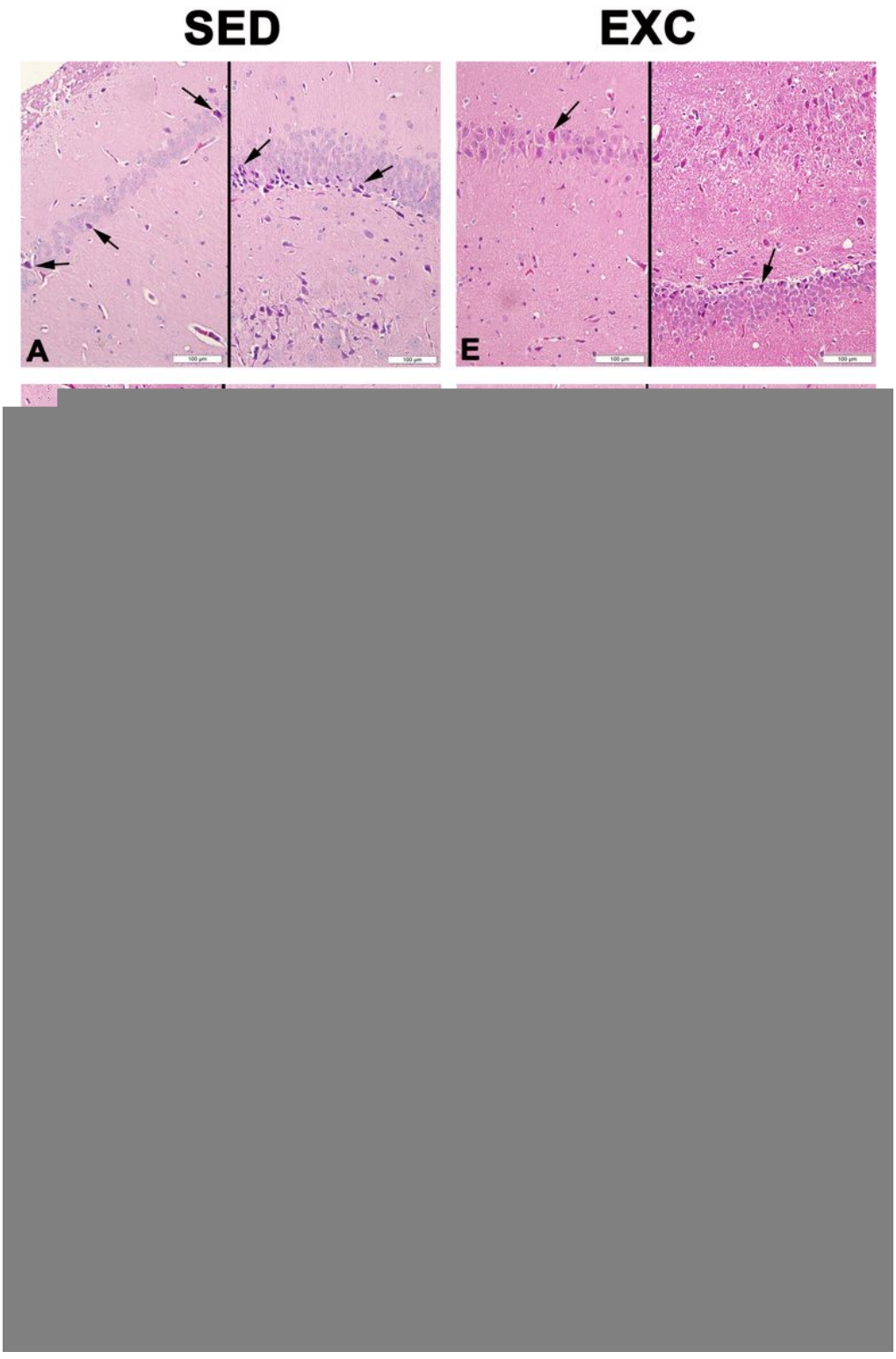

Figure 5

Representative light micrographs of hippocampus samples stained with H&E. In the sedentary group (A), the number of damaged neurons (arrow) in Cornus Ammonis 1 (CA1) and the number of damaged granular cells (arrow) in the dentate gyrus (DG) are higher. The number of damaged neurons (arrow) in CA1 and the number of damaged granular cells (arrow) in DG was observed to decrease in the sedentary plus melatonin (B), sedentary plus hormone replacement therapy (HRT) (C), sedentary plus melatonin and HRT combination (D), exercise (E), exercise plus melatonin (F), Exercise plus HRT (G), and Exercise plus melatonin and HRT combination (H) groups. Left side: CA1 region; right side DG region.

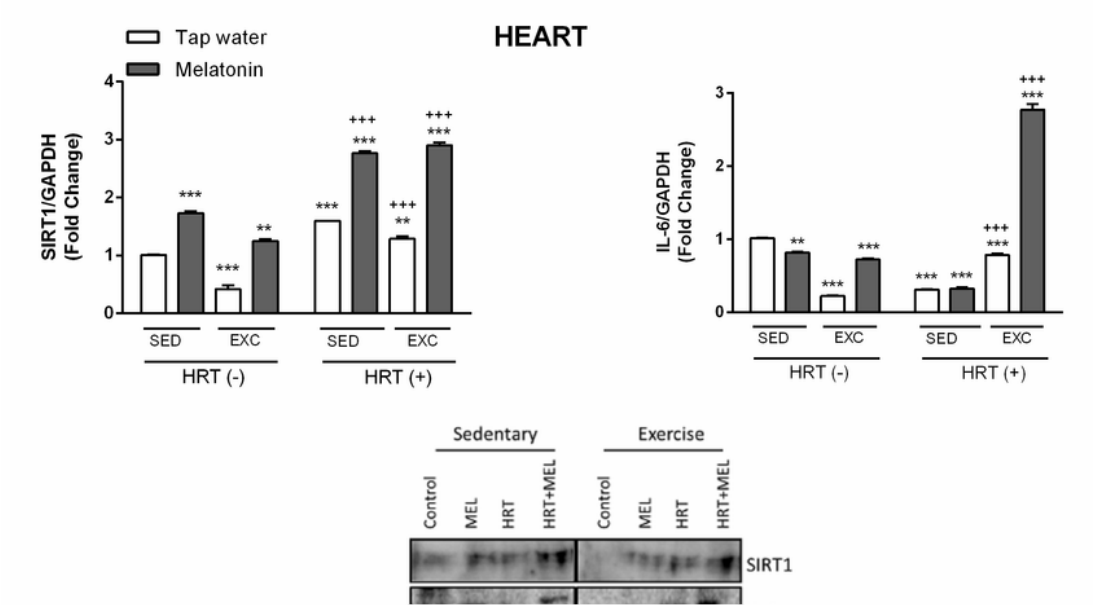

Figure 6

Levels of 8OHdG of cardiac tissues of experimental groups. Western blot results of TNF-α, IL-6, IL-10 and SIRT- 1 levels in the cardiac tissues. Data are presented as mean ± SEM of 3 animals; **p<0.01, ***p<0.001, compared to tap-water given sedentary control group; ++p<0.01, +++p<0.001 compared to HRT-given sedentary control group.

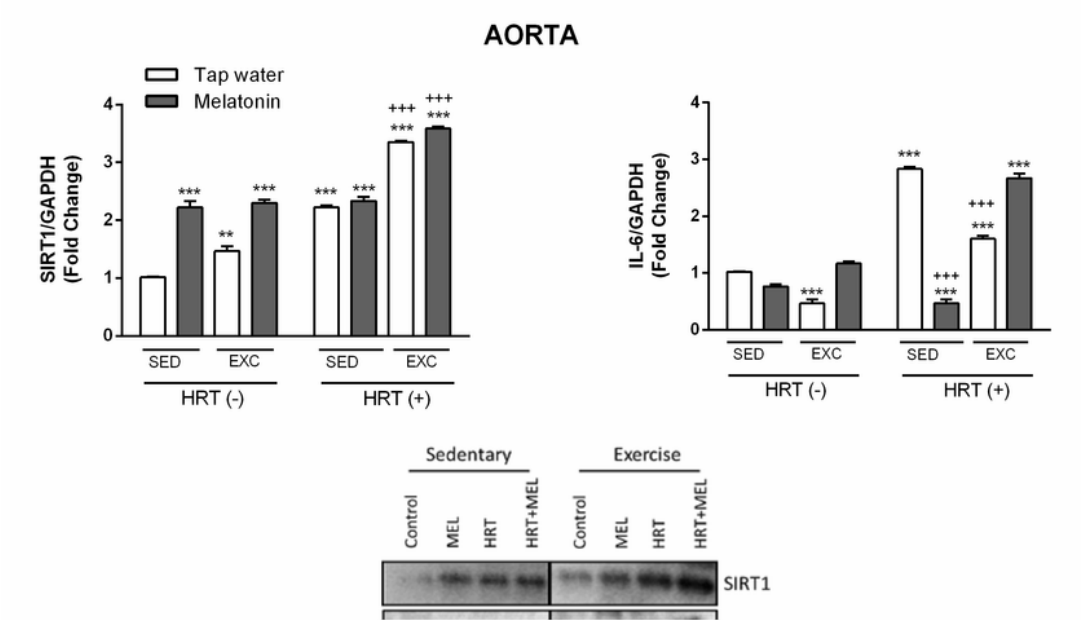

Figure 7

Levels of 8OHdG of aorta tissues of experimental groups. Western blot results of TNF-α, IL-6, IL-10 and SIRT- 1 levels in the aorta tissues. Data are presented as mean ± SEM of 3 animals; *p<0.05, ***p<0.001,

compared to sedentary control group that has received tap-water; +p<0.05, +++p<0.001 compared to HRT-given sedentary control group.

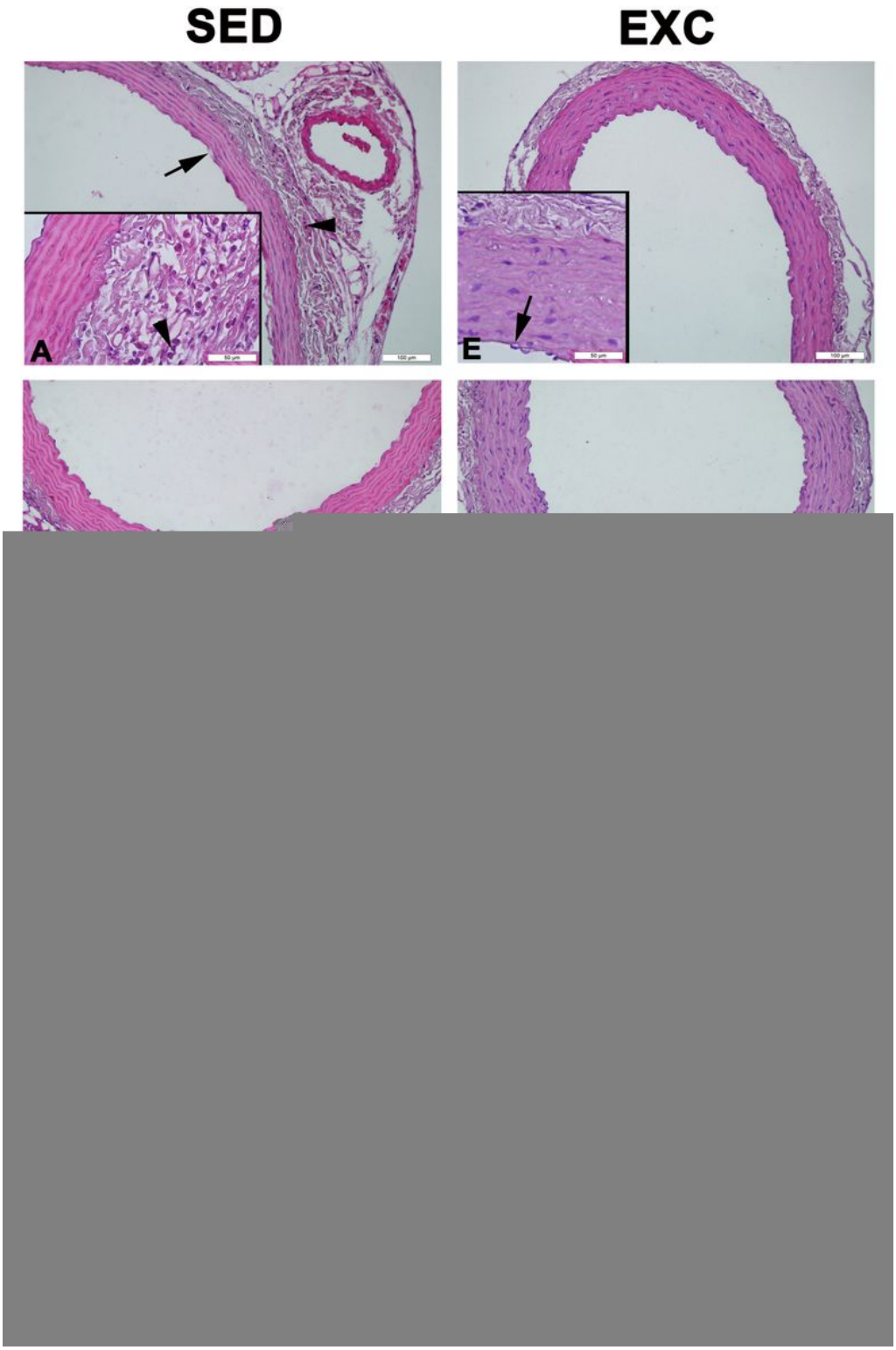

Figure 8

Representative light micrographs of aorta samples stained with H&E. The thinnest aortic wall and dissociation in endothelial cell layer (arrow) and infiltration of inflammatory cells in adventitial layer

(arrow head) were seen in sedentary group (A). Mild endothelial damage (arrow) and a few number of inflammatory cell infiltration in adventitial layer (arrow head) were seen in sedentary plus melatonin (B), Sedentary plus hormone replacement therapy (HRT) (C), Sedentary plus melatonin and HRT combination (D), exercise (E), exercise plus melatonin (F), exercise plus HRT (G) and Exercise plus melatonin and HRT combination (H) groups.