REVIEW

Effects of Sedentary Lifestyle and Physical Activity in Gaming Disorder

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Abstract: We are exposed to Nonylphenol which is an endocrine disrupting chemical used in the plastic industry, in our daily life without realizing it. The aim of this study was to evaluate the effect of Nonylphenol (NP) on heart tissue and the protectiveness of melatonin treatment on the heart damage with histopathological and immunohistological parameters. Twenty-one female rats were randomly divided into three groups: Control, NP, and Melatonin+NP. Heart tissues were removed two weeks later in all groups. Histopathological evaluation was performed with Hematoxylin&Eosin staining. IL-1β, IL-6, and Caspase-3 expression in heart tissues were detected immunohistochemically. Melatonin had a protective effect on heart tissue against NP-induced histopathological changes such as necrosis, hemorrhage, and inflammation. According to our results, the immunoreactivity intensity of inflammation markers such as IL-1β and IL-6 was significantly lower in the Melatonin+NP group than in the NP group. Finally, we investigated the pro-apoptotic marker caspase-3 immunoreactivity, which may explain the apoptotic effects of NP on the heart. According to our findings, melatonin significantly reduced the caspase-3 expression induced by NP. Our results showed that pretreatment with melatonin is beneficial to protect from NP-induced heart damage. These findings reinforce the protective effects of melatonin on heart tissue and more clinical studies are needed.

INTRODUCTION

Nonylphenol (NP) is known as an air pollutant that has estrogenic, carcinogenic, and toxic effects. It is xenoestrogen classified as an endocrine disrupter capable of interfering with the hormonal system of various organisms[1]. Generally, nonylphenol is widely used as a pure substance for manufacturing products such as cleaning and surface-active agents, plastics, pesticides, paper and rubbers[2]. In recent years, increasing demand and production for NP-containing products, has led to the release of NP emissions to the environment[3]. NP's original chemical form makes it durable to biological, chemical and physical degradation, which may allow staying longer in the environment[4]. Due to environmental pollution caused by these chemicals, living organisms that are exposed to them, have health problems such as diabetes mellitus, obesity, cardiovascular disease, and liver damage in terms of human health[5, 6]. In recent years, interest in environmental endocrine disruptors (EEDs) such as NP is increasing and recent studies have indicated that environmental exposure causes cardiovascular diseases to be accepted as epidemiological diseases, and NP is closely related to the occurrence and progression of it [7-10].

Melatonin, also known as N-acetyl 5-methoxy tryptamine hormone is secreted by the pineal gland and the synthesis of it is markedly increased in darkness. At the same time, it was reported that intake of foods containing melatonin could markedly increase the melatonin hormone concentration in serum. This also shows that melatonin could provide beneficial effects through foods on health[11]. As the biological effect of melatonin were widely studied, the identified therapeutic effects and the health profits of melatonin could include a wide range. Melatonin regulates sleep rhythm and body temperature, supports the immune system, shows anti-inflammatory, anti-aging and antioxidant effects[12-14]. In addition, melatonin shows neuroprotective effects, facilitates the control of chronic diseases, such as diabetes, obesity and cardiovascular disease[15-17]. It was reported that melatonin with its antioxidant capacity could reduce blood pressure and heart rate, regulate the vascular tone and cardiac rhythm via neurohumoral regulation[18,19]. Moreover, it was indicated that melatonin protected cardiomyocytes and blood vessels against necrosis and vasculitis in radiation-induced heart damage [19].

As melatonin has excellent antioxidative effects both directly and indirectly, there is a possibility that melatonin would preserve against NP toxicity caused heart damage. This study was aimed to show the efficacy of melatonin in protection against NP-induced heart damage, its antioxidant effects

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on the rat heart tissue with histopathologic and immunohistological methods.

MATERIALS and METHODS

Ethics and Animals

For this study, 18/155 numbered approval of Erciyes University Animal Experts Local Ethics Committee was received on December 2018 and was supported by A total of 21 Wistar albino adult female rats weighing 200 – 250 g were used for the experiment. Rats were kept in different cages with four rats in each cage and provided access to food (libitum) and water in appropriate laboratory conditions (22 ± 2ºC, 12 hours light/dark) during the experiment.

Study Design and Animal Treatment

The 21 adult Wistar albino rats were randomly divided into 3 groups (n = 7): Control group: No application was done during the experiment. Nonylphenol (NP) group: A single dose of 50 µl NP dissolved in 10 µl corn oil was given by gavage. Melatonin + NP group: 100mg / kg Melatonin was injected intraperitoneally (ip) for 15 days. On the 16th day, a single dose of 50 µl NP dissolved in 100 µl corn oil was given by gavage. Rats in the Control group were sacrificed together with experimental groups without any application. Rats were sacrificed 1 day after NP was applied to the NP group. The rats in the Mel + NP group were sacrificed on the 17th day following the application of Mel for 15 days and NP on the 16th day. Sacrification was performed by anesthetizing the rats with ketamine hydrochloride (75 mg/kg) and xylazine hydrochloride (10 mg/kg).

Histopathology

A morphological overview of the structure of the heart tissue was obtained using routine histological methods. Specimens were taken into 10% formaldehyde solution for fixation. After fixation, the tissues were dehydrated by passing through increasing graded alcohol series (50%, 70%, 80%, 96%, 3x 100%) and after being transparent with xylol they were embedded in paraffin by transparent with xylol. Heart tissue samples were taken in 5 µm sections on poly L-lysine coated slides. For histopathological examination, sections were stained with Hematoxylin-Eosin (H&E) and Masson's trichrome (MT). Briefly, the sections were removed from paraffin with xylol and passed through decreasing graded alcohol (100%, 96%, 80%, 70%, 50%) batches and washed in running water. After staining, it was passed through graded alcohol series, passed through xylol, and covered with a coverslip using entellan. The preparations were examined under a light microscope (Olympus BX51).

Immunohistochemistry

Immunohistochemical staining was performed on sections to evaluate (Interleukin-1 beta) IL-1β Antibody (Santa Cruz Biotechnology, sc-52012, USA, 1/500), (Interleukin-6) IL-6 Antibody (Novus Biologicals, NBP2-25275, USA, 1/200) and Cleaved Caspase-3 (Asp175) Antibody (Cell Signaling Technology, 9661S, USA, 1/200) activity using avidin-biotin-peroxidase method (Thermo Scientific, Waltham, MA). Briefly, sections were deparaffinized and rehydrated through decreasing alcohol batches. The sections were incubated with 3% hydrogen peroxide for 10 minutes for endogenous peroxidase activity. The samples were washed with PBS and microwaved in 0.01 M sodium citrate buffer for antigen recovery. Sections were incubated with IL-1β, IL-6 and Caspase-3 primary antibodies at 4 °C overnight. The next day, the sections were washed with PBS. Then, biotinylated secondary antibodies were applied in a humid environment for 15 minutes. After washing with phosphate-buffered saline (PBS), it was incubated with 3,3 3 p-diaminobenzidine tetrahydrochloride (DAB) (Thermo Scientific, Waltham, MA) for 5 minutes at room temperature. Sections washed with deionized water were stained with Gill's hematoxylin. After washing with tap water, it was covered with entellan. Olympus BX51 microscope (Olympus Corp. Tokyo, Japan) was used to examine and photograph the stained samples. Photographs were taken from at least ten different areas of each tissue in a 40X magnification. Using the Image J Software program, the immunoreactivity densities in each area were calculated and the data were analyzed statistically.

Statistical Analysis

Graphpad PRISM (Graphpad Software Inc., Version 8.0d) program was used for statistical analysis. Raw data are presented as group means ± SEM (standard error of the mean). Scoring and immunohistochemical immunoreactivity densities between groups were analyzed by Student's t-test method. Statistical analysis was considered significant if $p < 0.05$.

RESULTS

Morphological Appearance of Heart Tissue

Histological analysis of the rat heart was evaluated with (H&E) and (MT). The microphotographs of the sections are given in Fig. 1. In the samples of the control group, the myocardial structure (endocardium, myocardium, and epicardium) of Wistar rats were similar to the standard parameters of the rats. In the heart tissues taken, myocytes and blood vessels were normal in size and visualized. However free erythrocytes reflecting passive hyperemia findings, as well as vacuolization and edema were observed in the NP group, which had the most severe pathological findings. Areas of focal necrosis, including interstitial hemorrhage and inflammation were identified with H&E. In addition, perivascular and myocardial fibrosis was observed in NP group with MT. Partial degenerative changes in the myocardium and focal hemorrhagic areas were determined in samples from the hearts of the Mel+NP group. In the Mel+NP group, degenerative changes were observed to decrease compared to the NP group.

Immunohistochemical Evaluation of the Change in the Heart

Cardiomyocyte apoptosis density in the myocardium was measured immunohistochemically by looking at the activity of caspase -3, a proapoptotic factor. According to statistical analysis by counting positive cells in the areas, caspase-3 activation was significantly increased when the NP group was compared with the NP-Mel group, while it was low in the Control group ($p < 0.05$).

The levels of proinflammatory cytokines IL-1β and IL-6 were significantly increased in the NP group (Fig. 2). Since IL-1β and IL-6 levels are known to stimulate apoptotic cell death in fibroblasts of animals, they are elevated similarly to caspase-3 activity[20]. When the Melatonin + NP group was evaluated in terms of IL-1β and IL-6 levels, it was significant that it was lower than the NP group ($p < 0.05$). On the other hand, it was significant that IL-1β and IL-6 levels in the Control group were higher ($p < 0.05$). These values showed that the myocardial inflammatory response of the Mel + NP group decreased.

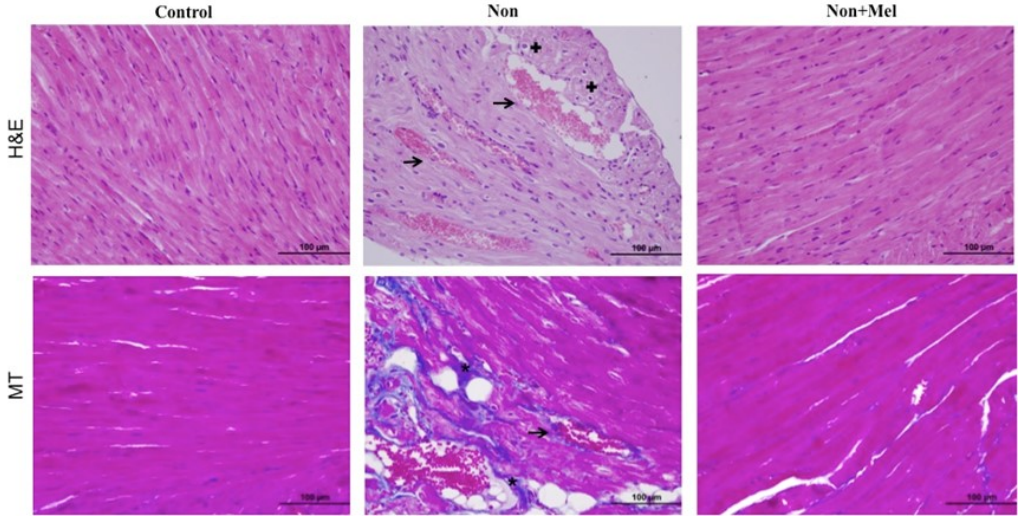

Figure 1. Effects of melatonin on histological changes in the heart tissue affected by nonylphenol. H&E and MT, ×400. Arrow; areas of hemorrhage, star; fibrozis, plus; edema.

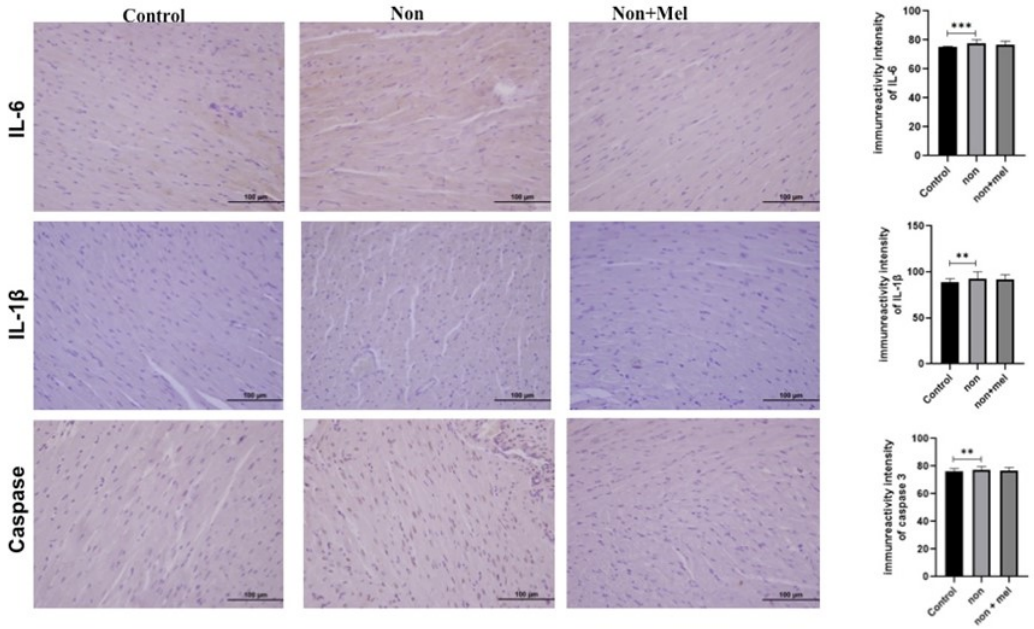

Figure 2. IHC staining of IL-1β, IL-6 and Caspase-3 in rat heart tissue from all experimental groups. IL-1 β and IL-6 expression measurements were made and the statistical difference was shown with a graph. The number of caspase-3 positive cells was counted and the results were graphed ($^{**}p < 0.01$, $^{***}p < 0.01$).

DISCUSSION

NP is accepted as a highly toxic environmental chemical because of its difficult degradability, bioaccumulation, and estrogen activity. In recent years, the number of studies with NP has gained more attention with the expansion of the field of application the EEDs such as NP[21, 22]. The fact that NP is an endocrine-disrupting chemical means that it affects many systems hormonally [23]. NP potentially harms the endocrine, immune, reproductive, and neurological functions of organisms [21,24]. At the same time, it has also been understood that exposure to NP has various harmful effects on the other organs by interfering with the hormonal system. Liu et al. showed that long-term NP exposure causes fibrosis of the myocardial layer and damage to heart function [25]. Recent data suggest that EEDs directly or indirectly contribute to the onset and progression of cardiovascular disease [26-28]. Recent studies have indicated that NP-exposure is closely related to the occurrence and development of CVD [9, 10, 29]. It has been reported to have harmful effects on the heart by blocking calcium channels and reducing myocardial contractility, although the underlying mechanism is still unknown [30]. Gao et al. have reported that NP has an effect on the L-type calcium current (ICA-L) in ventricular myocytes[31]. However, the number of studies showing the mechanism of action of NP on the cardiovascular system is limited. Therefore, in this study, we aimed to investigate the toxic effect of NP on rat heart tissue and the protective effect of melatonin treatment, which is widely studied as an antioxidant. In this paper, in vivo, NP exposed group, the concentration of NP was 50 µl/rat/day consistent with our previous work[32]. Histopathological examination revealed that there were cardiomyopathic changes following the nonylphenol administration such as myocardial degeneration, vacuolization, necrosis, interstitial hemorrhage, inflammation and deterioration of normal histological structure. These findings have been announced as key findings by several researchers who used cardiotoxic agents related to oxidative stress and lipid peroxidation[33, 34].

There is literature information that melatonin provides protection in myocardial pathologies. Melatonin, an antioxidant with protective effects on cardiomyocytes, could be a significant player in therapeutic approaches to damaged heart for various reasons[35, 36]. Sang et all. showed that melatonin modulates interstitial edema, inflammatory cell infiltration, apoptosis and autophagy in coxsackievirus B3-induced myocarditis in mice [37]. Melatonin restricts mitochondrial permeability transition pore opening for reduces mitochondrial dysfunction and reduces lactate dehydrogenase release for attenuating improving heart contractile function in myocarditis with cardiac dysfunction. Additionally, it has been reported to attenuate caspase-9-related apoptosis [38]. It has been proven that NP causes inflammation in the

tissue it affects by many studies[39-41]. For instance, Kim et al. have shown that NP significantly upregulates proinflammatory cytokine gene expression such as IL-1β, IL-6, IL-5, TNFR in U937 cells[42]. Gurses et all. reported that pretreatment of melatonin prevents myocyte necrosis and fibrosis, the intense inflammatory reaction and vasculitis against radiation-induced heart damage in rats[43]. Our finding is consistent with the hypothesis that NP leads to myocardial degeneration, vacuolization, necrosis, interstitial hemorrhage and inflammation in heart tissue. The results of our study established that melatonin treatment reduces vacuolization and inflammation in heart muscle fibers. These results indicate that Melatonin treatment has a protective effect on the heart in accordance with the literature. In the present study, we showed that the expressions of IL-1β and IL-6 inflammatory proteins increased with NP administration, but the expressions of these proteins were found to be lower in the group treated with melatonin.

Abnormalities in the cell apoptosis mechanism can induce tissue damage. A complete completion of apoptosis involves the gradual interaction of a wide array of proteins and cascades of signaling pathways. There are two major apoptosis pathways: extrinsic and intrinsic[44]. Previous studies have demonstrated that NP exposure increases apoptosis in various cell types, such as thymocytes [45], neurons [46], spermatogonia [47], sertoli cells [48], ovarian granulosa cells [49], human epithelial intestinal cells [50] and embryonic stem cells[51]. Studies have demonstrated the cardiomyocyte apoptosis plays an essential role in the pathogenesis of cardiac dysfunction and treatment with melatonin significantly reduced apoptotic index in the myocardium as compared to the group with cardiac dysfunction[52, 53]. However, there is no information on the mechanism by which NP will affect the heart tissue. For this reason, in this study, caspase-3 expression was examined by immunohistochemical method, whether NP would have an apoptotic effect on heart tissue. The results obtained showed that caspase-3 activation was significantly increased when the NP group was compared with the Mel + NP group, while it was low in the control group. Also, we showed that melatonin treatment ameliorates the negative effects of NP on heart injury.

Conclusion

Two important findings were obtained from this study. The first is that NP causes inflammation and apoptosis on the heart tissue, and another important finding is that melatonin administration is protective against the damage caused by NP. Melatonin treatment gives hope in the treatment of complications where cell death is present in NP-exposed rat models.

Conflict of interest

The authors declare that there are no conflict of interests.

Financial disclosure

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