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Original article

Melatonin prevents nicotine-induced hepatotoxicity by modulating apoptosis and histopathological changes in rats

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Abstract

Nicotine, the main toxic component of tobacco, directly or indirectly causes adverse effects on the liver metabolism. Melatonin, secreted by the pineal gland, has anti-apoptotic activity as well as antioxidant activity. The aim of this study was to reveal the antiapoptotic effects of melatonin in rats with experimentally induced chronic liver damage with nicotine. In this study, 32 male Wistar albino rats were divided into four groups: control, melatonin, nicotine and nicotine+melatonin. During the experiment, nicotine (1 mg/kg) and melatonin (10 mg/kg) were administered daily intraperitoneally for 56 days. At the end of the study, the liver tissues were taken for histopathological, immunohistochemical and molecular analysis. The administration of melatonin was determined to partially alleviate histopathological changes in the liver tissue induced by nicotine, such as hepatocyte degeneration, vascular dilatation and congestion, and leukocyte infiltration. It was observed that there was a significant decrease in Bax expression levels and a significant increase in Bcl-2 expression levels in the nicotine+melatonin group when compared to the injury group. On the other hand, it was determined that melatonin administration reduced the Bax/Bcl-2 ratio, which was significantly higher in the nicotine group compared to the other groups, to a level close to the control group. Additionally, as a result of immunohistochemical evaluation, it was observed that decreased Bax expression and increased Bcl-2 expression in hepatocytes in the nicotine+melatonin group were at a level close to the control group. Our results revealed that melatonin is a hepatoprotective and effective antioxidant by suppressing cell apoptosis and increasing the rate of healing after damage at both the immunohistochemical and molecular levels.

Keywords: apoptosis, hepatotoxicity, immunohistochemistry, melatonin, nicotine, protein expression

Introduction

The consumption of tobacco, whose main toxic component is nicotine, is a global health problem since it is responsible for the pathogenesis of many important diseases (El-Sherbeeny et al. 2016, Jalili et al. 2017). Today, it has become one of the most important causes of increasing mortality (Azab and Albasha 2015). Nicotine is the most active alkaloid in tobacco that plays a role in the prevalence of various diseases such as cardiovascular disorders, neurological disorders, chronic obstructive pulmonary disease, various lung diseases, various types of cancer, and many other diseases (Chattopadhyay et al. 2010, El-Sherbeeny et al. 2016, Jalili et al. 2017). During tobacco consumption, nicotine has various toxic effects on organs that are not directly exposed to smoke, such as the liver (El-Sherbeeny et al. 2016). The ingested nicotine is mainly exposed to biotransformation reactions in the liver and converted into several metabolites. The main product released as a result of nicotine metabolism is cotinine (Jalili et al. 2017). The hepatotoxic effect resulting from exposure to nicotine and its metabolites and the biotransformation processes occurring in the liver are attributed to the conditions mentioned (Jalili et al. 2017, Dey et al. 2023). The direct effect of nicotine on liver cells causes the formation of reactive oxygen species (ROS) by suppressing antioxidant enzymes (Jalili et al. 2017). Increased ROS causes DNA damage, leading to oxidative tissue damage and the induction of apoptosis in liver cells (El-Sherbeeny et al. 2016, Jalili et al. 2017). Due to the suppression of antioxidant defense mechanisms by nicotine, several antioxidant substances are used to minimize or prevent the toxic effect of nicotine in the cells (Mahmoud and Amer 2014, Albasha and Azab 2016, Ateyya et al. 2017). In our study, melatonin, which has potent antioxidant properties, was used.

Melatonin (N-acetyl-5-methoxytryptamine) is a hormone that is mainly synthesized and secreted by the pineal gland, which can easily cross barriers in the body and has free radical scavenging properties (Kurus et al. 2009). It is also known to be synthesized in different tissue sections of the body besides the pineal gland (Sainz et al. 2003). In addition to its antioxidant activities, it has many functions, such as regulating circadian rhythm and sleep pattern, sexual behavior and reproductive functions, modulating immunity, and anti-jetlag effect (Sainz et al. 2003, Carpentieri et al. 2012). Its effective role in free radical scavenging, prevention of oxidative damage as a result of its support for antioxidant defense mechanisms, and the changes it makes in different ways in the progression of apoptotic mechanisms that occur in tissue damage and cancer situations are among the reasons why melatonin is preferred primarily in treatments (Sainz et al. 2003, Kurus et al. 2009). This study aimed to determine the possible protective effects of melatonin against hepatocellular apoptosis induced by chronic nicotine administration in rats.

Materials and Methods

Chemicals and reagents

Nicotine hydrogen tartrate salt and phosphate buffered saline (PBS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Melatonin was obtained from Alfa Aesar (Karlsruhe, Germany) and stored at -20°C. Absolute ethanol (96%) and entellan were purchased from Merck (Darmstadt, Germany). Streptavidin-peroxidase (catalog no: TS-015-HR), blocking solution (catalog no: TA-015-UB), and diaminobenzidine (DAB) (catalog no: TA-001-HCX) were purchased from Thermo Fisher (Fremont, CA, USA). Ethylenediamine tetraacetic acid (EDTA) solution (pH: 8.0, catalog no: ab93680) was obtained from Abcam. Bcl-2-associated X protein (Bax) (catalog no: sc-20067), B-cell lymphoma 2 (Bcl-2) (catalog no: sc-7382), and ß-actin (catalog no: sc-47778) antibodies were obtained from Santa Cruz Biotechnologies (Santa Cruz, CA, USA). Secondary antibodies were purchased from BioLegend. All the chemicals and reagents used in this study were of analytical grade.

Animals and experimental procedure

32 healthy male Wistar Albino rats, approximately 2 months old, weighing 180-250 g, were used in the study. The rats were obtained from Hatay Mustafa Kemal University (HMKU) Experimental Research Application and Research Center. During the study, the animals were provided with standard chow and water *ad libitum*. Experimental procedures were carried out in accordance with the permission of the Local Ethics Committee for Animal Research at Hatay Mustafa Kemal University, number 2020/03-12. During the study, the rats were kept in well-ventilated plastic cages in a room with a constant temperature of 22-25°C, under a 12-h light/12-h dark cycle. All applications were performed 2 h before lights-off (El-Sokkary et al. 2007). After an adaptive period of 7 days, the rats were randomly divided into four groups, with each group containing eight animals.

Group I (control group): Animals received an injection intraperitoneally with normal saline (0.9% NaCl) for 56 days. Group II (melatonin group): Animals were treated by intraperitoneal injection with melatonin

Table 1. Histopathologic scoring results obtained from rats.

Groups	Hepatocyte degeneration	Vascular dilatation and congestion	Leukocyte infiltration
Control	0.00 (0.00-1.00)	0.00 (0.00-1.00)	0.00 (0.00-1.00)
Melatonin	0.00 (0.00-1.00)	0.00 (0.00-1.00)	0.00 (0.00-1.00)
Nicotine	3.00 (2.00-3.00)*	2.00 (2.00-3.00)*	2.00 (1.00-2.00)*
Nicotine+Melatonin	1.50 (1.00-3.00)**	1.00 (1.00-3.00)**	0.50 (1.00-3.00)**

Data are means ± median (min-max). * $p<0.05$ nicotine group compared to control group. ** $p<0.05$ nicotine+melatonin group compared to nicotine group.

(10 mg/kg) once a day for 56 days. The melatonin was dissolved in ethanol (0.9%) (Othman et al. 2020). Group III (nicotine group): Animals were injected intraperitoneally with nicotine (1 mg/kg) once a day for 56 days. The nicotine was dissolved in normal saline (0.9% NaCl) (Jalili et al. 2017). Group IV (nicotine+melatonin group): Following intraperitoneal nicotine administration, melatonin was administered intraperitoneally 30 minutes later (Mercan and Eren 2012). The dose of nicotine (Azab and Albasha 2015) and melatonin (Oztopuz et al. 2020) were selected based on published studies.

At the end of the experiment, the rats were kept fasting overnight. 24 h after administration of the last dose, the rats were sacrificed under anesthesia. Liver tissues were then collected. The tissues were divided into two parts and washed with normal saline. One part of the tissue was then stored at -80°C until used for molecular analyses, while the other part was fixed in 10% neutral buffered formalin solution for histopathological and immunohistochemical assessments.

Histopathological examination

The liver tissue was kept in a 10% neutral buffered formalin solution for 48 h, then cleared by passing through alcohol and xylol series and embedded in paraffin blocks. Sections of 5 µm thickness were taken from the paraffin blocks with the help of a microtome (Olympus Cut 4060, Germany). They were deparaffinized in a hot water bath and placed on slides. The tissue sections were stained with hematoxylin and eosin and microphotographs were taken under a light microscope (Zeiss Imager A2, USA) to observe the microscopic changes. Histopathologic scoring was performed based on the method reported by Durgun et al. (2023). Ten fields from each specimen per group were analyzed by two expert pathologists. The average score was calculated for each group. Histopathological alterations were scored as: 0: none, 1: low, 2: medium, 3: high. Results were given as medians (Table 1).

Immunohistochemical analysis for Bax and Bcl-2

Immunohistochemistry analysis was performed to determine Bax and Bcl-2 proteins in the liver tissues. For this purpose, sections kept at 60°C for 6 h were first dehydrated by passing through xylene and then decreasing alcohol series. The sections were then kept in distilled water for 5 minutes and washed three times with PBS for 5 minutes each. The sections were put into EDTA solution for the antigen retrieval process and heated in a microwave oven for 3x6 minutes. The sections were washed again with PBS and exposed to 3% hydrogen peroxide for 5 minutes to inhibit endogenous peroxidase activity. The sections were then washed with PBS for 3x5 minutes, and blocking solution was applied for 7 minutes. They were then incubated overnight at 4°C with Bcl-2 and Bax primary antibodies. Biotinylated secondary antibody (ThermoFisher, Fremont, CA, USA) was dropped on the sections washed with PBS and incubated for 14 minutes and the washing process was repeated. Streptavidin-peroxidase was then applied for 15 minutes and DAB was dropped on the sections and the color change was observed. The sections were washed with PBS for 3x5 minutes and counterstained with Harris hematoxylin. The sections were washed under tap water and cleared by passing through increasing alcohol series and then xylene. Entellan was dropped on the sections, covered with a coverslip, and evaluated under a Zeiss Imager A2 light microscope.

Western Blot analysis

Liver samples were removed from the -80˚C storage and crushed in liquid nitrogen. Cold RIPA buffer containing protease-phosphatase inhibitor cocktail and nuclease (Thermo Scientific, USA) was then added to the sample. The total cellular protein concentration of lysates was determined using a bicinchoninic acid (BCA) protein assay kit (TaKaRa, Japan). Total cellular proteins (20 µg) were separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and the separated proteins were trans-

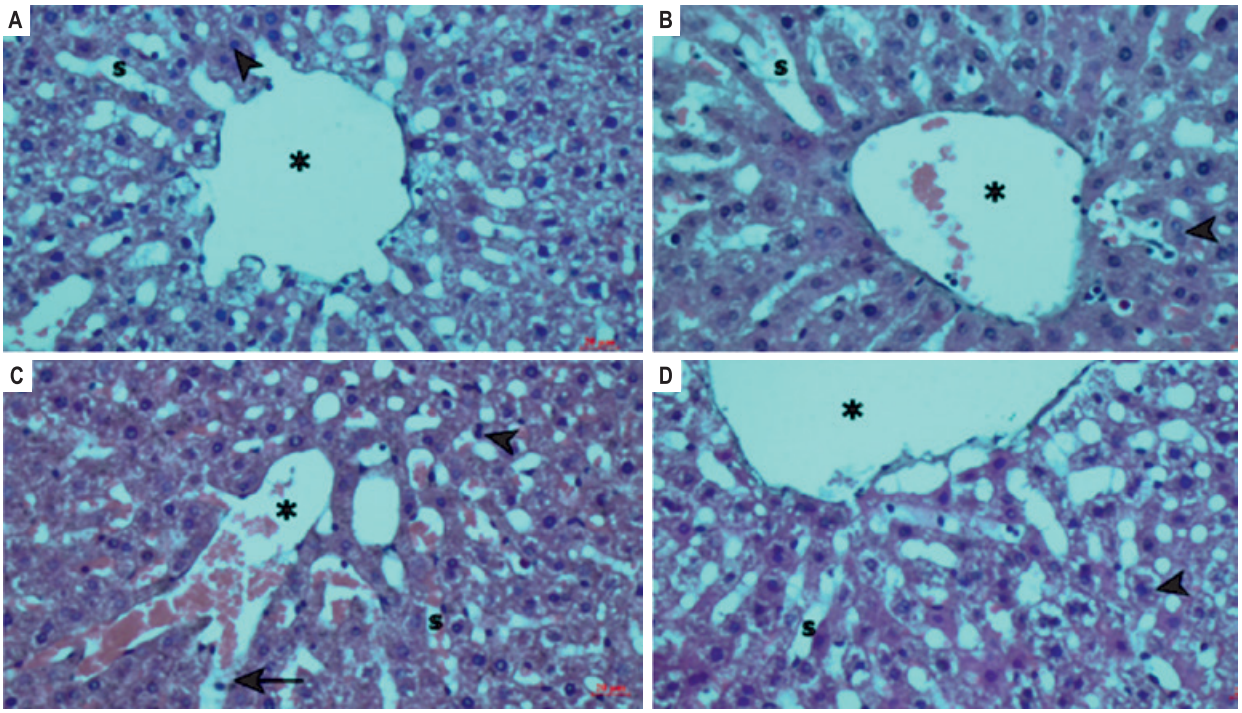

Fig. 1. Hematoxylin and eosin staining of transverse sections of the rat liver tissue. A) control group: Normal liver histology; B) melatonin group: Histological appearance similar to the control group; C) nicotine group: Degenerated hepatocytes, dilated and congested central vein and sinusoids; D) nicotine+melatonin group: Reduced dilatation and increased hepatocyte regeneration, arrowhead: hepatocytes, asterisk: central vein, s: sinusoid, arrow: leukocyte (bar=20 µm, Hematoxylin-Eosin stain: A-D; x40).

ferred to a polyvinylidene fluoride (PVDF) transfer membrane (Bio-Rad, USA). The membranes were incubated with anti-Bax antibody and anti-Bcl-2 antibody for 2 h at room temperature. β-actin was used as the loading control. Appropriate HRP conjugated secondary antibodies were used to visualize the specific bands using ECL (LI-COR Biosciences, USA) and the images were taken using G:Box Chemi XRQ (Syngene, USA).

Statistical analysis

Statistical analysis was performed using an SPSS® 11.5 (SPSS Inc; Chicago, IL, USA) program. Normally distributed data among the multiple groups was analyzed with the one-way ANOVA test and the Tukey HSD test was applied for post hoc analysis. $p<0.05$ was considered as statistically significant.

Results

Histopathological findings

Histopathological changes observed in the liver tissues are presented in Fig. 1. In the sections from the control group, which had a normal microscopic appearance, the central vein was located in the center with hepatic cords radiating around it. The liver cells were observed to be polygonal and binucleated. No dilatation or congestion was observed in the liver sinusoids (Fig. 1A). The melatonin group exhibited an appearance similar to that of the control group. No pathological changes were detected in this group (Fig. 1B). Chronic nicotine administration was found to cause significant histopathological changes compared to the control group. In the nicotine group, dilatation and congestion were observed in the central vein and sinusoids. Disruptions in the radial arrangement of hepatic cords and a pyknotic appearance in hepatocytes were found (Fig. 1C). It was observed that the majority of the histopathological changes induced by nicotine damage were largely corrected by melatonin administration, resulting in an appearance similar to that of the control group. The pathological changes in the central vein and sinusoids were repaired, and the hepatocytes were seen to regenerate and align radially (Fig. 1D).

Hepatocyte degeneration, vascular dilatation and congestion, and leukocyte infiltration were significantly greater in the nicotine group than in controls ($p<0.05$). However, damage for the nicotine+melatonin group was significantly less than for the nicotine group ($p<0.05$).

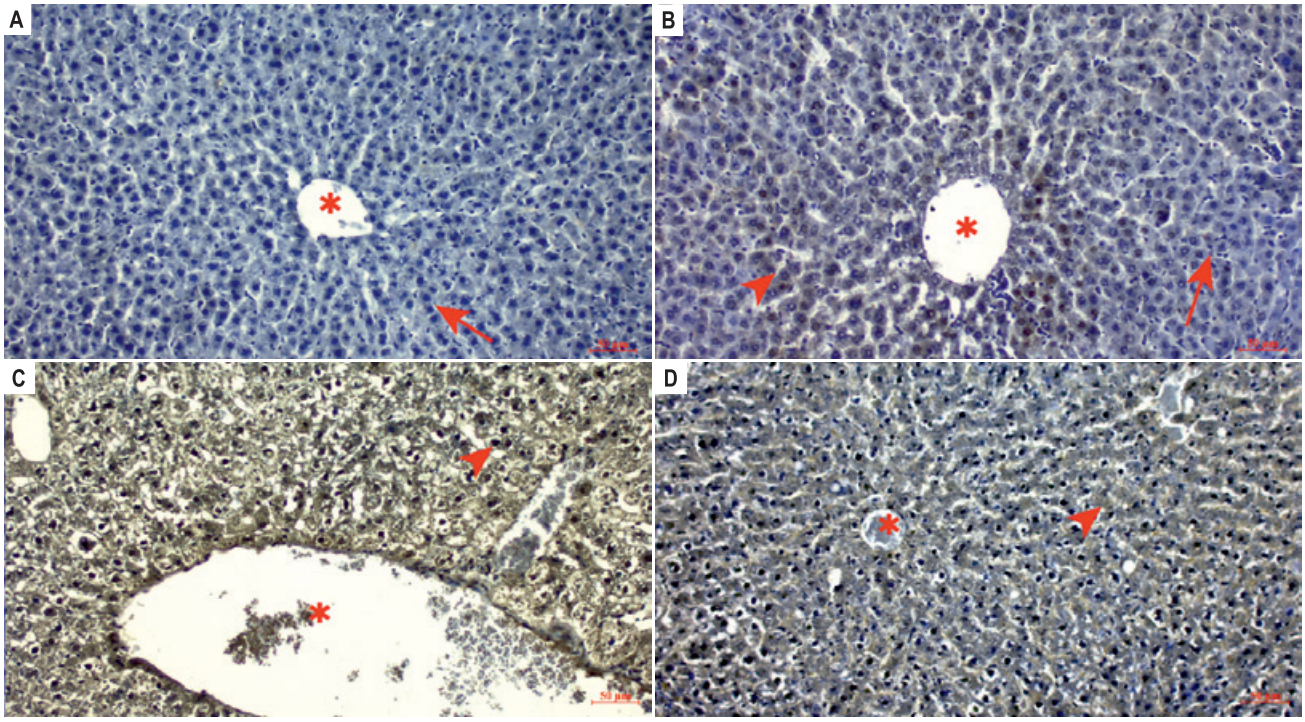

Fig. 2. Transverse sections of the rat liver tissues stained with Bax immunostaining. A) control group: Negative Bax expression in hepatocytes (arrow) and central vein endothelium (asterisk); B) melatonin group: Mostly negative Bax expression in hepatocytes (arrow) and central vein endothelium (asterisk), positive Bax expression in some hepatocytes (arrowhead); C) nicotine group: Very intense Bax expression in liver cells (arrowhead) and central vein endothelium (asterisk); D) nicotine+melatonin group: Decreased Bax expression in liver cells (arrowhead) and central vein endothelium (asterisk) (bar= 50 µm, anti-Bax immunostain: A-D; x200).

Immunohistochemical findings

Immunohistochemical findings of Bax in liver tissues

The Bax immunostaining of liver tissues is presented in Fig. 2. In the control group, mostly negative Bax expression was observed in hepatocytes and the central vein (Fig. 2A). In the melatonin group, a mild level of Bax expression was observed in hepatocytes and around the central vein (Fig. 2B). Nicotine administration increased cell death by inducing apoptosis in the liver tissue and thus Bax expression was observed very intensely in this group (Fig. 2C). In the nicotine+melatonin group, Bax expression decreased in hepatocytes and the central vein endothelium with the effect of melatonin (Fig. 2D).

Immunohistochemical findings of Bcl-2 in liver tissues

The Bcl-2 immunostaining of liver tissues is presented in Fig. 3. In the control group, mostly positive Bcl-2 expression was observed in hepatocytes and the central vein (Fig. 3A). In the melatonin group, a Bcl-2 expression similar to the control group was observed in hepatocytes and around the central vein (Fig. 3B). Nicotine administration suppressed Bcl-2 expression by inducing apoptosis in hepatocytes and increased cell death (Fig. 3C). In the nicotine+melatonin group, Bcl-2 expression increased in hepatocytes and central vein endothelium with the effect of melatonin and reached a level close to the control group (Fig. 3D).

Western blot analysis results

The expression levels of Bax and Bcl-2 proteins in liver tissues were determined using the Western blot method. The expression levels of Bax and Bcl-2 proteins in liver tissues are presented in Fig. 4, and the Bax/Bcl-2 ratio is shown in Fig. 5. In the nicotine-induced damage group, the expression levels of Bax protein were found to be higher, and Bcl-2 levels were lower compared to the control group (Fig. 4). Additionally, the Bax/Bcl-2 ratio in the nicotine group was significantly higher compared to other groups ($p<0.05$) (Fig. 5). However, it was observed that melatonin administration after nicotine-induced damage brought this ratio to a level close to the control group. In contrast, in the treatment group, nicotine+melatonin, a significant decrease in Bax levels compared to the nicotine group and a significant increase in Bcl-2 levels were observed (Fig. 4).

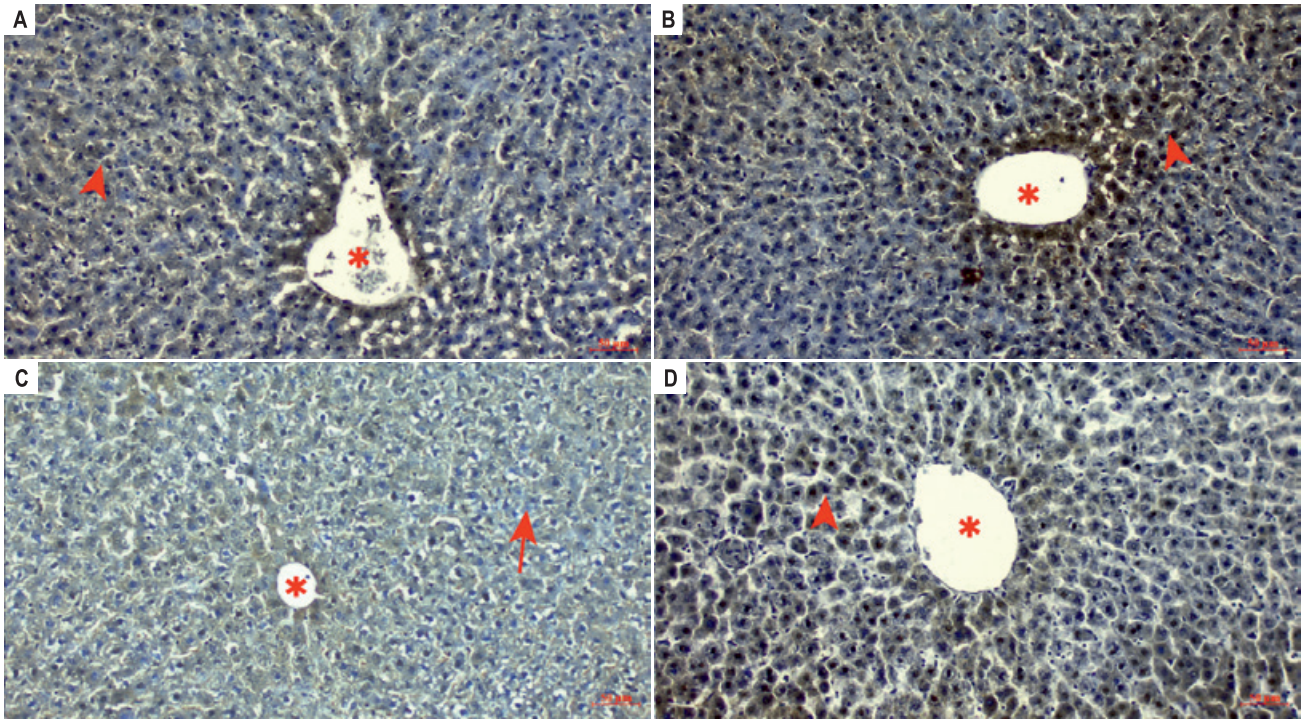

Fig. 3. Immunostaining of transverse sections of the rat liver tissues for Bcl-2. A) control group: Positive Bcl-2 expression in hepatocytes (arrowhead) and central vein endothelium (asterisk); B) melatonin group: Intense positive Bcl-2 expression in hepatocytes (arrowhead) and central vein endothelium (asterisk); C) nicotine group: Decreased Bcl-2 expression in liver cells (arrow) and central vein endothelium (asterisk); D) nicotine+melatonin group: Increased Bcl-2 expression in liver cells (arrowhead) and central vein endothelium (asterisk) (Bar= 50 µm, anti-Bcl-2 immunostain: A-D; x200).

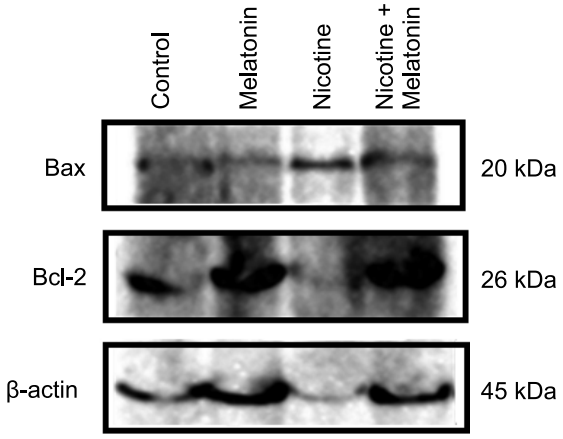

Fig. 4. Bax and Bcl-2 protein expression levels in the rat liver tissues from different experimental groups. Quantification of protein band optical densities to determine Bax, Bcl-2 and ß-actin levels.

Discussion

This study was conducted to investigate the impact of melatonin, a potent antioxidant, on apoptosis in rats with liver damage induced by chronic nicotine administration. In the conducted *in vivo* study, histopathological changes and immunohistochemical as well as molecular alterations of apoptotic proteins (Bax, Bcl-2) were examined. Numerous *in vivo* and *in vitro* studies in this field demonstrate the alterations induced by nicotine in apoptotic protein levels (Dasgupta et al. 2006, Ivey et al. 2014, El-Sherbeeny et al. 2016, Jalili et al. 2017, Khalaf et al. 2017).

Nicotine, the main toxic component of tobacco, is a potent oxidant that disrupts antioxidant defense mechanisms, leading to the release of reactive oxygen species. Suppression of defense mechanisms results in inadequate breakdown of radicals, causing cell damage and giving rise to conditions such as DNA damage, oxidative stress, apoptosis, and tumor formation (Chattopadhyay et al. 2010, Mahmoud and Amer 2014, Jalili et al. 2017). Nicotine causes serious damage

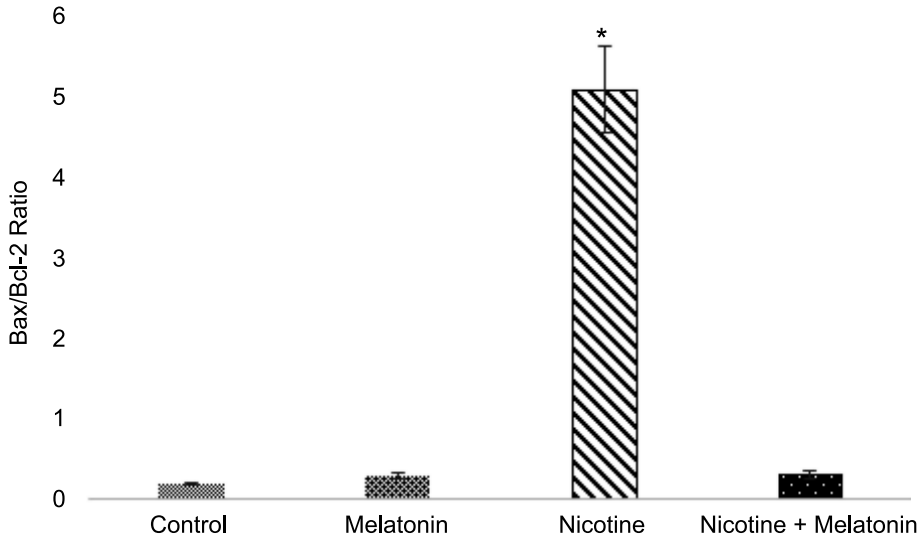

Fig. 5. Bars represent the mean±SE of experiments. Bax/Bcl-2 ratio levels in the rat liver tissues from different experimental groups. (* $p<0.05$, when the Bax/Bcl-2 ratio is compared with control group).

to various tissues such as the kidney, testis, lung, and brain (Albasha and Azab 2016, Ateyya et al. 2017, Saad et al. 2020). However, since its metabolism primarily occurs in the liver, these processes are more induced by nicotine in the liver. Excessive reactive oxygen species (ROS) production activates apoptosis (Jalili et al. 2017). Apoptosis, known as a physiological control mechanism of programmed cell death, is a defense mechanism playing a role in essential processes such as fetal development, maintenance of cell homeostasis, and the development and functions of multicellular organisms (Williams 1991, Sainz et al. 2003). It ensures the control of mechanisms involved in the removal of damaged, infected, and DNA-damaged cells, serving as an intermediary in maintaining homeostasis and playing a role in the treatment of diseases. Understanding this process contributes to the emergence of different treatment strategies against diseases (Sainz et al. 2003, Dailah 2022). Apoptosis is regulated through different pathways initiated independently in target cells, both intrinsically and extrinsically (Strasser et al. 2000). In the intrinsic pathway, signals inducing apoptosis, such as oxidative stress, exert their effects by leading to the release of proteins that activate caspase-3. The balance between cell death and survival is controlled by genes with both inductive and suppressive characteristics (Oltvai et al. 1993, Jalili et al. 2017). Members of the Bcl-2 protein family, known as important regulators of the intrinsic pathway, play a crucial role in the control of apoptosis. Pro-apoptotic proteins (Bak, Bid, Bax, Bcl-xs) and anti-apoptotic proteins (Bcl-xl, Bcl-w, Bcl-2) are key players in regulating apoptosis (Jalili et al. 2017). Bcl-2, which prevents cell death, protects cells against apoptosis by binding to Bax and suppressing caspase activity (Williams 1991, Othman et al. 2020). Numerous studies indicate that nicotine induces damage in liver tissue by promoting ROS production and apoptosis through its direct effects (Ivey et al. 2014, El-Sherbeeny et al. 2016, Jalili et al. 2017, Kim et al. 2017). Therefore, our study investigated the effects of melatonin on the levels of Bax and Bcl-2 proteins, which play a role in the regulation of apoptosis, against liver damage induced by nicotine. In our study, it was noted that the administration of nicotine resulted in increased levels of Bax expression and decreased levels of Bcl-2 in liver tissues. Similarly, in a study with a comparable approach, an increase in Bax and Bax/Bcl-2 ratios and a decrease in Bcl-2 expression levels were reported in the liver tissues of mice subjected to nicotine administration (Jalili et al. 2017). A study conducted by El-Sherbeeny et al. (2016) also reported a widespread increase in Bax expression in the liver tissues of rats treated with nicotine, similar to the observations in our study. There are numerous *in vitro* studies demonstrating both pro-apoptotic and anti-apoptotic effects of nicotine (Heusch and Maneckjee 1998, Dasgupta et al. 2006, Kang et al. 2011, Zeng et al. 2012). The reason for these differences could be attributed to the induction of apoptosis through different mechanisms.

The search for new treatment options is rapidly ongoing to prevent damage to the liver, which is directly exposed to many substances since it is at the center of the metabolism process (Zhang et al. 2017). For this purpose, numerous antioxidants have been reported in the literature for their use in studies aiming to prevent liver damage (Bandyopadhyaya et al. 2008, Mahmoud and Amer 2014, Ateyya et al. 2017). Melatonin, secreted by the pineal gland and possessing powerful antioxidant effects, has been reported in numerous studies

on liver damage to have positive effects on the hepatic system by suppressing apoptosis through its anti-apoptotic properties (Tuñón et al. 2010, Liang et al. 2012, Othman et al. 2020, Yang et al. 2021).

There are numerous studies indicating that melatonin, the focus of our research on reducing nicotine-induced cell death, also exhibits therapeutic and protective effects on liver damage induced by various chemicals. In a study conducted by Yang et al. (2021) melatonin administration to rats with induced liver damage reduced caspase-3 activity and Bax expression and increased Bcl-2 protein expression, thereby inhibiting cell apoptosis and demonstrating a hepatoprotective effect. In another study inducing liver damage and administering melatonin to the treatment group, it was revealed that mitochondrial Bax levels in the liver decreased, and Bcl-2 levels significantly increased compared to the damage group, highlighting the impact of melatonin on the translocation of apoptotic proteins (Liang et al. 2012). In a study assessing apoptosis using immunohistochemical analysis, melatonin was shown to increase Bcl-2 protein expression in the treatment group due to its antiapoptotic properties, and the findings were consistent with our study (Othman et al. 2020). Additionally, our results are similar to the findings of Tuñon et al. (2010) who reported that the administration of melatonin to liver cells of rabbits infected with rabbit hemorrhagic disease virus reduced Bax expression and increased the expression of Bcl-2 and Bcl-xl proteins, indicating modulation of the intrinsic pathway.

In conclusion, our study highlights that melatonin administration protects against liver damage induced by nicotine by inhibiting apoptotic processes. In light of this information, our data suggest that melatonin could be considered as an alternative medication for the treatment of liver damage, which may occur with different substances. These findings provide a basis for future research and may contribute to various studies in the field.

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