Original Article

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Oxidative stress in the cerebellum of pinealectomized rats and its correlation with GFAP expression

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ABSTRACT

Aims: This study aims to investigate the results of the lack of melatonin in the cerebellar tissue of pinealectomized Wistar albino rats using immunohistochemistry and biochemistry.

Methods: Control, pinealectomy, and sham pinealectomy groups were designed for the experiment (n=8). Pinealectomy and sham pinealectomy surgery were employed. At the end of 30 days, cerebellum tissue was used for histopathological, glial fibrillary acidic protein (GFAP) immunostaining, and biochemical (oxidative stress markers) analyses. Purkinje cell counts and cerebellar layer thickness in the cerebellum were also measured within the scope of histometrical analyses.

Results: The study revealed that melatonin deficiency (pinealectomy) adversely impacts the overall histological structure of the cerebellum, leading to heightened immunoreactivity to GFAP antibody, elevated malondialdehyde levels, and reduced glutathione and superoxide dismutase levels in comparison to control and sham pinealectomy groups ($p<0.05$). This study has, for the first time, elucidated the amounts of oxidants and antioxidants, GFAP immunoreactivity, Purkinje cell counts, and cerebellar layers thicknesses in the cerebellum of a pinealectomized rat model. This study is the inaugural investigation to elucidate the association between melatonin and the cerebellum, a topic hitherto overlooked in the literature, thereby establishing a significant foundation.

Conclusion: Lack of melatonin can be a reason for neurodegeneration and oxidative stress in the cerebellum. Pinealectomy surgery was found to be a reason for the elevation of oxidative stress, deterioration of the histological architecture, and increase of GFAP expression in the cerebellar tissue of the Wistar albino rats.

Keywords: Cerebellum, GFAP, oxidative stress, pinealectomy

INTRODUCTION

The circadian rhythm, governed by the pacemaker in the hypothalamic suprachiasmatic nucleus (SCN), governs daily physiological processes and behaviors to sustain melatonin production by the pineal gland and ensure optimal performance.[1,2] Melatonin, synthesized by the pineal gland at night, is integral to the circadian rhythm and serves as a neurohormone that significantly regulates physiological systems and aids in adapting to environmental changes.[3] Disruption of the circadian rhythm exacerbates stress in the brain and several bodily systems, influencing the onset and progression of numerous diseases that induce oxidative damage to cellular components due to the overproduction of free radicals.[4]

Free radicals, perpetually generated as metabolic wastes,[5] oxidize unsaturated fatty acids in membranes via lipid peroxidation. The elevation of free radicals results in the excessive synthesis of malondialdehyde (MDA), a byproduct of lipid peroxidation and an indicator of oxidative stress.[6] Nevertheless, several antioxidant enzymes, including superoxide dismutase (SOD), catalase (CAT), and reduced glutathione (GSH), are molecules capable of stabilizing or neutralizing free radicals prior to cellular harm.[7,8] Numerous studies demonstrate melatonin's preventive role against free radical-induced oxidative alterations in brain tissue by enhancing antioxidant enzyme activity and diminishing lipid peroxidation.[9-11] Conversely, research involving experimental melatonin deprivation (pinealectomy) indicates that pinealectomy exacerbates tissue damage induced by reactive oxygen species and cerebral damage resulting from localized ischemia and excitotoxic convulsions.[12] Pinealectomy concurrently disrupts the antioxidant system by elevating SOD activity in the frontal brain and hippocampus while diminishing GSH levels. Numerous studies indicate a substantial rise in oxidative and structural alterations in the

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tissues and organs, including the liver, kidneys, testes, uterus, and lungs of rats following pinealectomy, which also results in a notable elevation of MDA levels and a reduction in GSH levels.[13]

Glial fibrillary acidic protein (GFAP) is an intracellular protein exclusive to central nervous system astrocytes and serves as a marker for astrocyte activation.[14] Alterations in GFAP expression and/or phosphorylation in brain injury or various neurological disorders may indicate aberrant synaptogenesis and neurotransmission, potentially correlating with neurodegenerative diseases.[15,16] The reactive alterations in astrocytes inside the hippocampus and cerebellum correlate with neuronal degeneration.[17] Research indicates that GSH levels are markedly diminished in the brains of rats subjected to continuous light, while total and degraded GFAP content is elevated; also, melatonin treatment results in reduced degraded GFAP content. Moreover, melatonin markedly diminishes lipid peroxidation in neural tissue, whereas prolonged light exposure substantially enhances lipid degradation in the brain.[18]

The cerebellum exhibits unique binding sites for melatonin,[19] however, the impact of pinealectomy on cerebellar tissue remains inadequately established. This investigation was predicated on the deficiency of melatonin and its potential impact on cerebellar tissue, considering the underlying causes of oxidative stress, given the possible antioxidant and therapeutic properties of melatonin. In our study, oxidative and structural changes in the cerebellum after pinealectomy were demonstrated by SOD, GSH, and MDA levels, and data supporting their relationship with GFAP were presented.

METHODS

Ethics Statement and Animal Care

Approval from the Karabük University Rectorate Animal Experiments Local Ethics Committee for Animal Experiments was secured for the study (Date: 26.12.2024, Decision No: 2024/12/32) and conducted following its requirements. All procedures were carried out in accordance with the ethical rules and the principles. Twenty-four male Wistar Albino rats, each weighing at least 150 g, were obtained from the Experimental Medicine Research and Application Center of Karabük University. The rats were housed in rooms with constant temperature and humidity, maintained at 22±1°C, and subjected to a 12-hour light-dark cycle, with unrestricted access to water and food.

Experimental Design and Group Allocation

The rats were randomly separated into three groups 8 rats each. Groups were named as follows:

Control (Co): The rats in this group received no treatment.

Pinealectomy (PNX): The rats in this group underwent pinealectomy surgery.

Sham pinealectomy (PNX-sham): The rats in this group underwent sham pinealectomy surgery. All the procedure for PNX surgery was applied (as described in the "2.3. surgical pinealectomy" title) except that the pineal gland was not removed.

Surgical pinealectomy

The PNX procedure was carried out as the authors mentioned before.[20]

Experiment Termination and Tissue Sample Collection

On the 30th day post-PNX and PNX-sham surgery, rats from all groups were sedated, and beheaded, and the excised cerebellum tissues were taken. Half of the cerebellum tissue was resected and immersed in 10% formalin for histological examination. The remaining portion was employed for biochemical research.

Histometric Analysis of Cerebellar Cortex Layers and Purkinje Cell Quantification

The thicknesses of the molecular layer, granular layer, and total cortex were measured from a minimum of 10 distinct regions across 3 serial sections obtained from the midline of the cerebellar tissue of each animal; Purkinje cell counts were similarly conducted in various regions across 3 serial sections, each with a total length of 1 mm. Serial sections were obtained at roughly 30 µm intervals, omitting 5 sections with each extraction. Sections were evaluated semiquantitatively over 10 distinct fields at 40X magnification.

Histopathological and Immunohistochemical Analysis

Following their removal, cerebellar tissues were submerged in 10% neutral buffered formalin. Following fixation, the tissues were washed in running tap water for 24 hours. The tissues were subsequently dehydrated in ascending alcohol concentrations (from 70% to 100%), clarified with xylene, and embedded in paraffin. Five-micrometer sections were cut from the paraffin-embedded specimens for light microscopic examination. The overall histological architecture was analyzed utilizing hematoxylin-eosin (H&E) staining, while cresyl-violet (C-V) staining was employed to visualize the Purkinje Cells. Leica® DM2500 LED microscope was used to investigate all the slices stained. For immunohistochemical analyses, serial paraffin sections, each 5 µm thick, were placed on positively charged glass slides. Sections were subjected to deparaffinization and hydration. Ten percent hydrogen peroxide was employed to inhibit endogenous peroxidase activity for 10 minutes. Sections were subjected to 0.01 mol/l citrate buffer (pH=6) in the microwave for 5 minutes to expose the antigenic sites. To prevent nonspecific background staining, the slides were initially rinsed in phosphate-buffered saline (PBS) at pH 7.4 for 5 minutes, followed by incubation in 1% bovine serum albumin (BSA) in PBS for 30 minutes at 37°C. Except for negative controls, the sections were treated with two drops of a ready-to-use primary antibody of GFAP (GeneTex-GTX108711) at room temperature overnight. After a PBS rinse, several drops of biotinylated goat polyvalent secondary antibody were applied to the slides and incubated for 10 minutes. The slides were dried, cleaned, and mounted with dibutylphthalate polystyrene xylene (DPX) mounting medium following a 15-minute incubation in 3,3-diaminobenzidine (DAB) to assess the reaction.

All the histological results were obtained using the LAS V4.8 image analysis program on a Leica® DM2500 LED brand research microscope with an MC170 HD model camera attachment.

Levels of MDA, SOD, and GSH in the Cerebellum as Markers of Antioxidants and Oxidants

The cerebellum tissues were homogenized in a 10% phosphate buffer, thereafter centrifuged for 4 minutes at 8,000 rpm and 4°C, and the supernatants were collected to assess oxidative stress indicators. The activities of MDA, GSH, and SOD in the cerebellum were assessed utilizing commercial kits (Rel Assay Diagnostics, Turkiye).

Statistical Analysis

Statistical data analysis was conducted using IBM SPSS Statistics version 25.0 for Windows software. The Kolmogorov-Simirnov test indicated a normal distribution of the data (p>0.05). Multiple comparisons were evaluated using a One-Way ANOVA test with Tukey HSD correction. Results are expressed as mean±standard deviation (SD), with p<0.05 being statistically significant.

RESULTS

Results of Histometrical Measurements of Cerebellar Cortex Layers and Purkinje Cell Quantification

The assessment of molecular, granular, and total cerebellar cortex thickness revealed a substantial reduction in these layers in the PNX group compared to the control and PNX-sham groups (p<0.05). No statistically significant difference was seen between the control and PNX-sham groups for the thickness of the cerebellar cortex layers (p>0.05) (Figure 1).

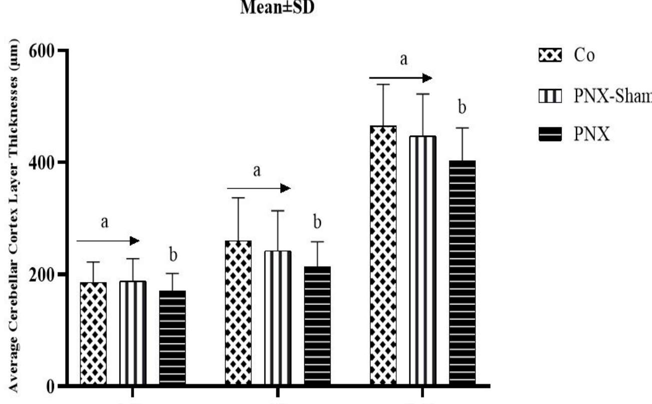

Figure 1. Average cerebellar cortex layers thicknesses

Substantial reduction in the PNX group compared to the control and PNX-sham groups (p<0.05). ML: Molecular layer thickness, GL: Granular layr thickness, TCL: Total cerebellar cortex thickness, Co: Control group, PNX: Pinealectomy surgery group, PNX-sham: Sham-Pinealectomy group. a and b presenting the statistical difference between the groups of the study (p<0.05)

According to the analysis of Purkinje cell counts that revealed the layout in the cerebellar cortex's ganglionary cell layer, the PNX group had a significantly lower number of Purkinje cells than the control and PNX-sham groups (p>0.05). No statistically significant difference was seen between the control and PNX-sham groups for the number of Purkinje cells (p>0.05) (Figure 2).

Histological Results

The H&E and C-V staining results: The H&E and C-V stainings from the control and PNX sham groups revealed distinguishable layers of the cerebellum, which displayed a normal histological appearance (Figure 3 A-D). In the PNX group, pyknotic nuclei, necrosis, and perineuronal vacuolated regions were identified in the Purkinje cells within the ganglion cell layer. Demyelinated and vacuolated regions were particularly observed in the white matter layer (Figure 3 E, F).

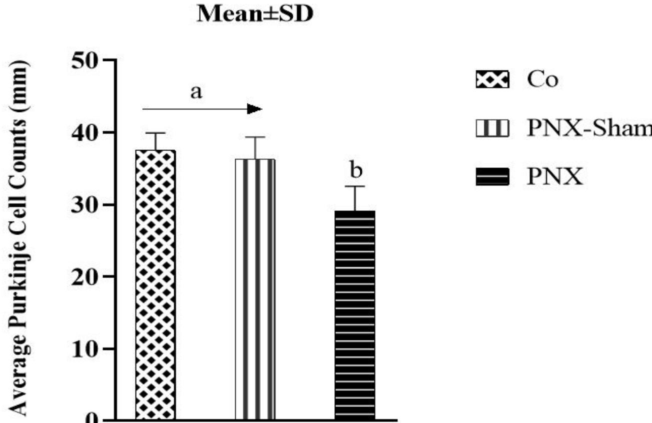

Figure 2. Average Purkinje cell counts

PNX group had a significantly lower number of Purkinje cells than the control and PNX-sham groups (p>0.05). PNX: Pinealectomy surgery group, PNX-sham: Sham-pinealectomy group. a and b presenting the statistical difference between the groups of the study (p<0.05)

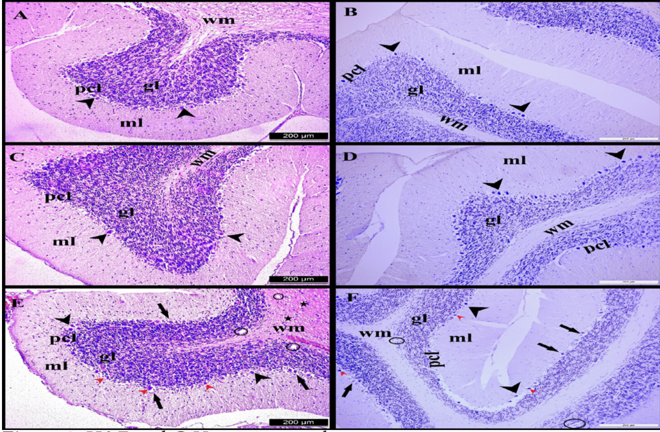

Figure 3. H&E and C-V staining results

ml: Molecular layer, pcl: Purkinje cell layer, gl: Granular layer, wm: White matter, Arrowheads: Purkinje cells, Black circle: Vacuolized areas in the white mater and granular layer, Arrows: Perineuronal vacuolization, Asterisks: Demyelinated areas, Red arrowhead: Necrotic Purkinje cells and pyknotic nuclei, A: H&E stain of the control group, C: H&E stain of the PNX-sham group, E: H&E stain of the PNX- group, B: C-V stain of the control group, D: C-V stain of the PNX-sham group, F: C-V stain of the PNX group, H&E: Hematoxylin-eosin, C-V: Cresyl-violet, PNX: Pinealectomy surgery group

The GFAP Immunohistochemical Staining Results

Immunohistochemical staining with GFAP antibody revealed that the immune response and staining intensity were comparable in the control and PNX-sham groups; however, the PNX group exhibited a significant presence of GFAP (+) astrocytes, characterized by intense staining in the granular layer of the cerebellar cortex and the area of substantia alba. The cytoplasmic extensions of astrocytes in the PNX group had a pronounced trajectory in the molecular layer. Cytoplasmic extensions of astrocytes were shown to be interwoven. Histoscore (H-score) was calculated by a semi-quantitative assessment of both the intensity of staining (graded as: 0, non-staining; 1, weak; 2, median; or 3, strong using adjacent normal cells as the median) and the percentage of positive cells. The H-score for GFAP immunoreaction is given in Figure 4, 5.

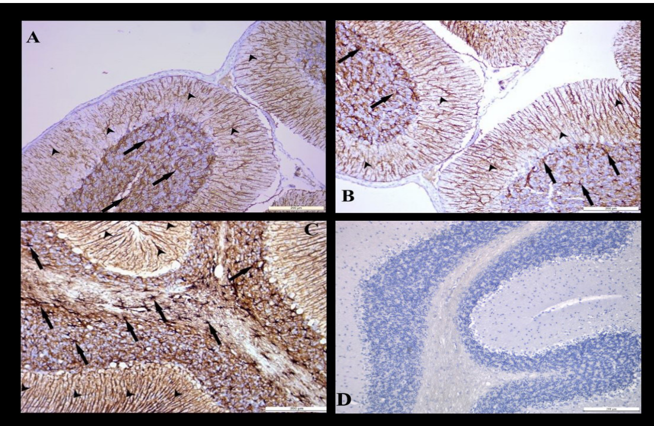

Figure 4. The immunostaining results for GFAP

Arrowheads: The immunoreaction in the cytoplasmic processes of the astrocytes located in the molecular layer, Arrows: Immunoreactive astrocytes located in the granular layer and white matter, A: GFAP immunohistochemistry stain of the control group, B: GFAP immunohistochemistry stain of the PNX-sham group, C: GFAP immunohistochemistry stain of the PNX group, D: Negative control for GFAP immunstain, GFAP: Glial fibrillary acidic protein, PNX: Pinealectomy surgery group

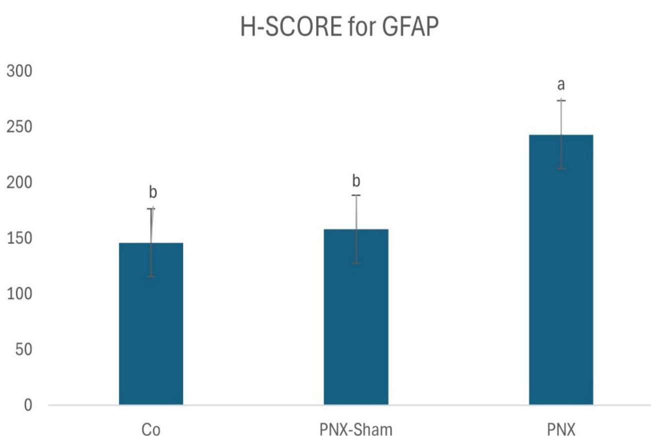

Figure 5. H-score for GFAP immunostaining

PNX group had a significantly higher number of immunoreactive cells than the control and PNX-sham groups (p>0.05). H-score: Histoscore, GFAP: Glial fibrillary acidic protein, PNX: Pinealectomy surgery group, PNX-sham: Sham-pinealectomy group. a and b presenting the statistical difference between the groups of the study (p<0.05)

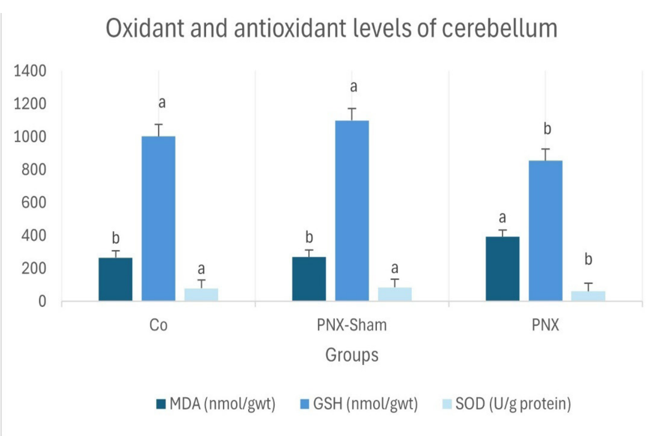

Figure 6. The oxidative stress parameters in the groups

Data are expressed as mean±standard deviation (n=8). MDA: Malondialdehyde, GSH: Reduced glutathione, gwt: Gram wet tissue, SOD: Superoxide dismutase. Superscripts represent the statistically significant difference MDA; ^{a}p<0.05 vs Co and PNX-sham, ^{b}p>0.05 Co vs PNX-sham; GSH and SOD; ^{a}p>0.05 Co vs PNX-Sham, ^{b}p<0.05 vs Co and PNX-sham

Biochemical Results

Biochemical analyses of cerebellar tissue revealed that pinealectomy resulted in elevated MDA levels and reduced GSH and SOD levels. The elevation of MDA levels in the pinealectomy group was statistically significant when compared to the Co and PNX-sham groups (p>0.05), although no significant difference was observed between the Co and PNX-sham groups (p<0.05). The reduction in GSH and SOD levels was statistically significant when compared to the Co and PNX-sham groups (p>0.05), although no significant change was observed between the Co and PNX-sham groups (p<0.05). The biochemical results are summarized in Figure 6.

DISCUSSION

In this study, the cerebellar oxidant and antioxidant status were revealed using MDA, SOD, and CAT, and the GFAP immunoreaction in cerebellar tissue was shown in control, Pinealectomy and sham-pinealectomy performed rat groups. The histomorphometric measurements for granular, molecular, and total cortex thickness were also employed as well as the Purkinje cell counts. The findings indicated that the lack of melatonin resulted in elevated MDA levels in the cerebellum and a statistically significant reduction in GSH and SOD levels relative to the control and PNX-sham groups (p<0.05). A statistically significant increase in GFAP immunoreactivity in the cerebellum coincided with a statistically significant reduction in Purkinje cell count, as well as in the thickness of the molecular, granular, and total cortex (p<0.05).

In animals, including humans, the circadian system is structured hierarchically, with a central pacemaker located in the SCN.[21] The SCN coordinates circadian physiological and behavioral rhythms, such as sleep and wakefulness, temperature, eating, neuroendocrine, and autonomic functions, with a 24-hour cycle to align with the ambient light-dark cycle, thus establishing an optimum internal temporal order. Light serves as the principal stimulus for synchronizing the SCN rhythm period and phase with the external environment.[22] In humans and other diurnal organisms, melatonin functions in the SCN to diminish the wake-promoting signals of the circadian clock.[23] Although melatonin was discovered over fifty years ago and several papers addressing its neuroprotective properties,[24] shockingly little is understood about the effects of melatonin on the cerebellum. The majority of research investigating the consequences of melatonin deficiency has predominantly focused on specific tissues.[25] The cerebellum performs numerous essential activities, including motor coordination, learning, motivational processes, regulation of feeding and food anticipation, language, attention, and working memory.[26] Many of these tasks rely on a functional cellular clock and a stable circadian structure.[27] Even the cerebellum was found to have specific binding areas for melatonin,[19] yet the effect of the pinealectomy surgery on the cerebellar tissue has not been well-documented. Antioxidant and anti-inflammatory properties of melatonin have gained prominence in recent years,[28] whereas melatonin has a multifaceted impact by modulating factors in oxidative and inflammatory pathways, including the regulation of fundamental physiological and biological activities.[29] Moreover, melatonin metabolites significantly mitigate oxidative stress and inhibit inflammatory responses.[30] Melatonin is an effective free radical scavenger, and besides models of neurodegenerative illnesses, CNS

trauma, and ischemia-reperfusion injury, melatonin safeguards the brain from hyperoxia, excitotoxicity, ionizing radiation, and DNA damage.[31] Melatonin administration was shown to affect levels of MDA, GSH, SOD, and GPx of cerebellar tissue compared to the control groups, on behalf of the antioxidants.[32] The optimal operation of the endogenous molecular clock relies on cellular redox equilibrium and an antioxidant milieu.[33] The equilibrium between oxidants and antioxidants in healthy tissues is sustained by a preponderance of antioxidants. Diverse factors that may cause tissue damage disturb the oxidant/antioxidant equilibrium, favoring oxidants.[34] One contributing factor is the reduction in circulating hormone levels. In the literature, some studies explain how oxidative stress levels of the cerebellum are related to hormone levels.[35,36] Nevertheless, while the therapeutic or protective effects of melatonin on the cerebellum against various substances have been examined, no research has specifically addressed the harm resulting from melatonin shortage. The existence of hormone-specific receptors in an organ indicates that its function will be regulated by that hormone,[37] such as in the case of cerebellum and melatonin. While not explicitly detailed, it is evident that melatonin contributes to the maintenance of cerebellar function.[38] Although data suggest that exogenous melatonin, leveraging its antioxidant characteristics, mitigates oxidative damage in cerebellar tissue,[29,32,39] the deficiency of melatonin and its recognized antioxidant properties on cerebellar tissue merit thorough investigation. In this study, the lack of melatonin in the PNX group caused an increase in the levels of MDA which was statistically significant ($p<0.05$) compared to the control and PNX-sham groups, but a decrease in the levels of GSH and SOD which was statistically significant ($p<0.05$) compared to the control and PNX-sham groups. These results are attributed to the anti-oxidant property of melatonin. We believe that even sham pinealectomy operation caused little difference in the oxidative levels of the cerebellum, it did not affect the situation statistically so all the changes seen in the PNX group are related to the lack of melatonin synthise from the pineal gland.

Neurons have traditionally been seen as the fundamental functional units of the central nervous system, whereas glial cells were perceived only as supportive components. This idea has recently undergone rapid transformation; it has been claimed that the proper functioning of the neuron-microglia-astrocyte "trio" is essential to the functional organization of the central nervous system (CNS).[40,41] Astrocytes, a kind of glial cell, are recognized for their critical functional role in the development and maturation of the CNS. They participate in the regulation of brain extracellular ionic homeostasis, the migration and maturation of neurons, the synthesis and reuptake of specific neurotransmitters, and possess the capability to store energy as glycogen.[42] Astrocytes possess two varieties of cytoskeletal intermediate filaments: glial fibrillary acidic protein (GFAP) which is a type II intermediary filament and vimentin.[43] GFAP, mostly located in fibrous astrocytes and to a lesser degree in protoplasmic astrocytes serves as a dependable and extensively utilized marker for astroglia.[44] GFAP is also regarded as a key immunohistochemistry marker for astrocytes.[45] The body of work about GFAP transcription is extensive, as nearly any alteration or disruption of homeostasis in the CNS results in modifications to GFAP expression.[46] GFAP expression elevates in reaction to oxidative damage, aging, and environmental toxin exposure.[47] On the other hand, melatonin, the primary secretion of the pineal gland during the dark phase of the photoperiod, can influence the organization of microfilaments, microtubules, and intermediate filaments by functioning as a cytoskeletal modulator.[48] According to our results, an elevation of GFAP immunoreactivity in the pinealectomized rat group was seen. We speculate that this can be attributed to the loss of melatonin's cytoskeletal modulatory function for the organism. Moreover, we plan to further examine the potential that the lobes of the cerebellum, recognized for their distinct functions, may likewise serve varying roles for GFAP. We hypothesized that increased astrocyte activity serves as a compensatory response to neuronal damage and correlated the increase in astrocyte activity with increased GFAP expression.

Pinealectomy triggers apoptosis in Purkinje cells.[49] According to the results of our study, there was a significant decrease ($p>0.05$) in the number of Purkinje cells in the cerebellum. In our study even if the cascade of apoptosis was not explained, the Purkinje cell loss might be related to the apoptosis caused by the lack of melatonin. There is not a lot of literature planned to observe the effects of pinealectomy on the cerebellum, nonetheless, it is stated that pineal melatonin did not promote the survival of Purkinje cells during the developmental phase.[50] We believe in adult rats the contribution of melatonin for the survival of the Purkinje cells is more essential since the loss of the Purkinje cells in the PNX group is statistically significant compared to other groups of the study.

In the literature, some studies focused on the effects of pinealectomy on the Purkinje cells, but all these studies put forward the importance of melatonin for the normal development of cerebellar layers. However, there are no studies that focused on the effect of melatonin on the cerebellum in adult rats. Conversely, certain studies in the literature indicate the beneficial effects of specific antioxidant compounds on the overall structure of the cerebellum. In our study, a lack of melatonin not only resulted in the loss of Purkinje cells, but also revealed a substantial reduction in molecular, granular, and total cerebellar cortex thicknesses. These results underscore the necessity for comprehensive study, including volume calculation of the cerebellum in the pinealectomy model.

Limitations

This work elucidates the levels of oxidants and antioxidants, GFAP immunoreactivity, Purkinje cell counts, and cerebellar layer thickness in the cerebellum of a pinealectomized rat model for the first time; nonetheless, it possesses several limitations. The primary issue is that the impact of exogenous melatonin on the cerebellum could not be assessed with the existing parameters. This is attributable to financial limitations. It may be advantageous to design new studies addressing this problem.

CONCLUSION

This study has, for the first time, elucidated the amounts of oxidants and antioxidants, GFAP immunoreactivity, Purkinje cell counts, and cerebellar layer thickness in the cerebellum of a pinealectomized rat model. This study is the inaugural investigation to elucidate the association between melatonin and the cerebellum, a topic hitherto overlooked in the literature, thereby establishing a significant foundation. The contribution of more studies on the topic is essential.

ETHICAL DECLARATIONS

Ethics Committee Approval

The study was carried out with the permission of the Karabük University Rectorate Animal Experiments Local Ethics Committee (Date: 26.12.2024, Decision No: 2024/12/32).

Informed Consent

Since this study was conducted with experimental animals, a written consent form was not obtained.

Referee Evaluation Process

Externally peer-reviewed.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

Financial Disclosure

The authors declared that this study has received no financial support.

Author Contributions

All of the authors declare that they have all participated in the design, execution, and analysis of the paper, and that they have approved the final version.

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