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Evaluation Effect of Melatonin, Vitamin D3 and VDR expression levels on Histological Structure of Pineal Gland in Male Rats

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Abstract

Background: The pineal gland is an essential neuroendocrine organ that secretes melatonin and regulates biological rhythms. This gland's structure deteriorates with age and is frequently connected to hormonal and nutritional deficits. The purpose of this study was to assess the age-related histological alterations in male rats' pineal glands and investigate their relationship to melatonin levels, blood vitamin D3, and the expression of vitamin D receptors (VDR).

Methods and Procedures: Three age groups of thirty-six male Wistar rats were created: Group I (young, three months), Group II (middle-aged, nine months), and Group III (old, eighteen months). Hematoxylin and Eosin (H&E) and Masson's Trichrome stains were used for the histological analysis. Serum 25(OH)D3, $1,25(OH)_2D3$, and melatonin were evaluated using biochemical methods, and semi-quantitative immunohistochemistry (H-score) was used to measure VDR expression.

Results: Significant progressive shrinkage of the pineal gland, as indicated by a decline in nuclear diameter, pinealocyte count, and gland volume, was linked to aging ($p < 0.01$). Histological results showed widespread calcification (corpora arenacea) and a significant rise in interstitial fibrosis (from 8% in Group I to 42% in Group III). According to biochemical findings, aged rats' serum melatonin and vitamin D3 levels significantly decreased ($p < 0.01$). Additionally, the H-score decreased from 142.4 in young rats to 55.2 in the oldest group, indicating a significant decline in VDR immunoreactivity with age. Vitamin D3 levels and pineal structural integrity and VDR expression were found to be strongly positively correlated by Pearson correlation analysis ($r = 0.889$).

Conclusion: The findings show a strong correlation between decreased melatonin production and the degradation of the Vitamin D3-VDR axis and age-related pineal gland degeneration. These results imply

that vitamin D3 may have a neuroprotective function in preserving the pineal gland's histological vitality and reducing age-related fibrosis and calcification.

Keywords: Pineal Gland, Vitamin D3, VDR, Melatonin, Aging, IHC, Calcification,Rats.

Introduction

The pineal gland is regarded as the central control unit for regulating the body's biological functions. It plays a connecting role between the nervous system and the endocrine system by controlling the secretion of melatonin (Tchekalarova *et al*., 2025). With age, this gland undergoes a series of radical histological changes that affect its functional capacity and overall structure The most prominent histological changes associated with aging include the appearance of corpora arenacea, which are calcifications deposited in the glandular tissue, leading to compression, crowding, and degeneration of the pineal cells (Tan *et al*., 2018). Histologically, a marked increase in the thickness of the fibrous capsule and the intrusion of connective tissue septa into the lobules of the gland are also observed, altering its homogeneous appearance and transforming it into a more fibrous structure with a loss of active cells (Grosshans *et al*., 2016).

Melatonin is a hormone (protein) derived from the amino acid tryptophan. It is the primary secretion of the pineal gland responsible for regulating and supporting biological rhythms and guiding the body's internal clock according to the light-dark cycle (Tan *et al*., 2011). The importance role of this hormone extends beyond its well-known function as a sleep regulator. It is classified as one of the most important powerful natural antioxidants due to its rapid ability to cross the blood-brain barrier and reach to all cellular components. There, it scavenges free radicals and protects DNA and mitochondria from oxidative damage. Related with age, there is a gradual and sharp decline in melatonin production levels due to tissue changes and calcifications affecting the pineal gland (Tan *et al*., 2016). This leads to weakened cellular mechanisms and accelerated tissue aging. Therefore, studying the function of melatonin (especially in conjunction with vitamin D3) leads to pivotal importance in histological research, as it acts as a neuroprotective agent that contributes to reducing degenerative changes, maintaining the cellular density of pineal cells, and preventing age-related tissue fibrosis (Slominski *et al*., 2017).

In this regard, vitamin D3 plays a pivotal role not only as a nutrient but also as a tissue regulator that protects and maintains nerve and glandular cells. Studies indicate that the deficiency of vitamin D3 levels coincides with accelerated calcification and fibrosis within the pineal gland, reducing its appility to produce melatonin (Holick *et al*., 2011; Eyles *et al*., 2005). The association between vitamin D deficiency and low hormone levels leads to a deterioration of the gland's histological architecture, with pineal cells becoming smaller and fewer in number, and collagen fibers accumulating around blood vessels. Understanding the relationship between these hormonal variables (vitamin D3 and melatonin) and the histological deterioration of the gland across different life stages is crucial. Histological changes are not merely a marker of aging; they directly reflect the body's biochemical state (Spiro & Buttriss, 2014; Tripkovic *et al*., 2012) . Therefore, this study aims to evaluate changes in the histological structure of the pineal gland in male rats, focusing on manifestations of fibrosis, calcification, and cellular degeneration, and to correlate these manifestations with vitamin D3 and melatonin levels to determine their impact on maintaining the vitality of this important tissue.

Material and Methods

2.1. Experimental Animals: Thirty-six male Wistar rats of varying ages were used in this experiment. The rats were obtained from Kut Technical Institute/Middle technical University, Iraq in March, 2023, the rats were placed in clean polypropylene cages and kept for ten days according to standard guidelines for the care and use of laboratory animals, including a temperature between 22 and 25°C and a relative humidity between 50 and 70%. They were continuously fed appropriate laboratory feed. A 12-hour light/dark cycle was maintained (lighting from 7:00 AM), with standard rodent food and unlimited drinking water provided. Based on their age at sacrifice, the animals were divided into three groups of twelve rats each.

Group I: included young rats males (3 months) with weight mean 211 ± 14.2 g.

Group II: included middle-aged adult rats males (9 months) with weight. Mean 319 ± 23.7 g.

Group III: included old rats males (18 months) with weight 381 ± 27.9 g.

2.2. Samples Collection: After a gentle 12-hour fast, the animals were carefully placed under deep anesthesia with chloroform. The next step involved collecting blood samples through a puncture in a cardiac vein, ensuring a sterile environment with heparin-free tubes. Once collected, the serum was separated with care using centrifugation set at 4000 rpm for ten minutes. To maintain the integrity of the samples, automatic pipettes were employed for transferring the serum into sterile tubes, where it was lovingly stored at -20°C until it was ready for analysis. This conscientious process reflects a deep respect for the well-being of the animals, ensuring that every step is handled with the utmost care and attention.

2.3. Biochemical Analysis: ELISA of serum 25 (OH)D3 and 1,25(OH) 2D3 was done (Immunodiagnostic Systems Ltd., UK). Melatonin (in serum) was quantified on the basis of the melatonin ELISA kit (ALPCO Diagnostics, USA).

2.4. Histological Preparation: In the careful embrace of standard histological practices, we prepared pineal gland tissues designated for histological examination with attention and care. These precious samples were gently fixed in a 10% neutral-buffered formalin solution and then embedded in paraffin. Once encased, the paraffin was expertly cut into thin sections, each just 4 µm thick, using a microtome, which allowed us to preserve the integrity of the tissues. Later, these delicate sections were stained with a harmonious mixture of hematoxylin and eosin (H&E) solution, highlighting the intricate details within. After the staining process, each slide was thoughtfully mounted and covered with a suitable medium, ensuring that the samples remained in pristine condition. Finally, we turned to our microscope from Olympus, based in Tokyo, Japan, to explore and examine the rich tapestry of cellular structures within the tissues. For a thorough investigation, we selected three sections from each group, allowing us to gather meaningful insights with compassion and diligence (Masar & Kazaal, 2024).

2.5. Immunohistochemical Analysis of VDR: Each case's hematoxylin and eosin-stained slides from paraffin-embedded and formalin-fixed blocks were examined before being chosen for VDR immunohistochemistry staining. Following dewaxing, rehydration, and endogenous peroxidase quenching with 3% v/v H 2O 2 in methanol for 15 minutes; antigen retrieval in 0.01 M citrate buffer at 98°C water bath for 40 minutes; application and incubation of primary antibody (VDR mouse monoclonal D-6, sc-

13133, Santa Cruz Biotechnology, Texas, USA) at 1:200 dilution for 1 hour at room temperature; incubation with peroxidase-based EnVision+/Horseradish peroxidase (Dako A/S, Glostrup, Denmark) for 3 minutes. After that, the sections were dried, mounted, and counterstained with Mayer's hematoxylin. VDR expression was assessed on the entire section's pineal gland cells using nuclear and cytoplasmic labeling. VDR expression levels in pineal gland cells were assessed semi-quantitatively using nuclear and cytoplasmic staining after immunolabeled slices were examined using a Nikon Eclipse 80i light microscope at 10× magnification. Degree of expression calculated according to rate of cells scoring as detected in previous study (Juhász *et al*., 2022).

2.6. Statistical analysis: To analyze the data, we turned to the tools of Statistical Package for the Social Sciences (SPSS) version 19 and Excel 2010. With care, we employed ANOVA to compare the results from each of the three groups against the control group. In addition, we used the t-test to uncover any significant differences in the serological and biochemical indicators between the two groups. We defined a probability value of less than 0.05 as an indicator of statistical significance, allowing us to draw meaningful conclusions from our findings. Thank you for joining us on this journey of exploration and understanding.

Results

The histological sections with H&E staining of young rats males (group 1) also showed compact parenchyma densely filled with large, rounded pinealocytes and the sparse connective tissue stroma (figure 1).(middle-aged rats males (group II) depicted moderate cellular reduction, with augmented fibrous interstitium, and scattered tiny foci of calcification (Figure 2). Old rats males in Group III had severe atrophic alterations that were characterized by severe depletion in pinealocyte count, thick collagenous stroma as verified by Massons trichrome, and large corpora arenacea of different sizes (figure 3).

The histological results in (figure 4) comparing the three groups, show a clear gradient in the degree of fibrosis and collagen deposition within the gland tissue. In Group I, minimal fibrosis (8%) was observed, with the tissue appearing largely intact in its normal structure and limited collagen fiber deposition, indicating a near-normal state or minimal impact from the studied agent. In Group II, moderate fibrosis (22%) was present, characterized by a marked increase in collagen fiber deposition (stained blue) and a decline in normal tissue organization, suggesting a gradual progression in the severity of tissue damage. Group III exhibited extensive damage with extensive collagen deposition (49%) exhibiting a dense and irregular distribution of collagen fibers and significant structural damage, reflecting an advanced stage of fibrosis. These results indicate a progressive relationship between the studied agent and the severity of fibrosis, supporting the hypothesis that continuous or intense exposure exacerbates tissue changes and increases collagen deposition.

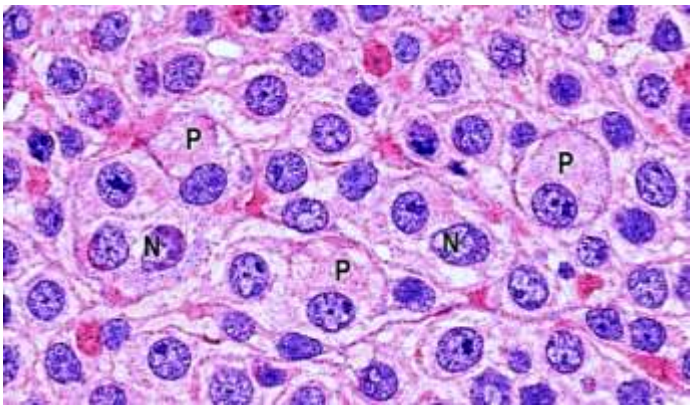


Fig. 1. Group I (3 months). H&E × 400. Dense parenchyma with large pinealocytes (P) having prominent vesicular nuclei (N) and scant fibrous stroma. Corpora arenacea are entirely absent. Scale bar = 50 µm.

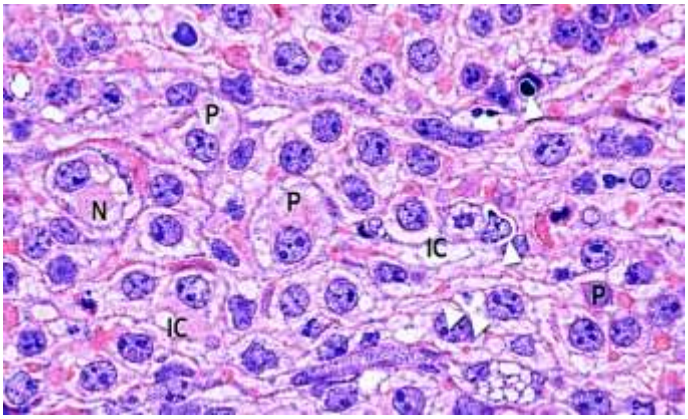


Fig. 2. Group II (9 months). H&E × 400. Moderate reduction in pinealocyte density, increasing fibrous interstitium (F), and small early calcification foci (arrow). Scale bar = 50 µm.

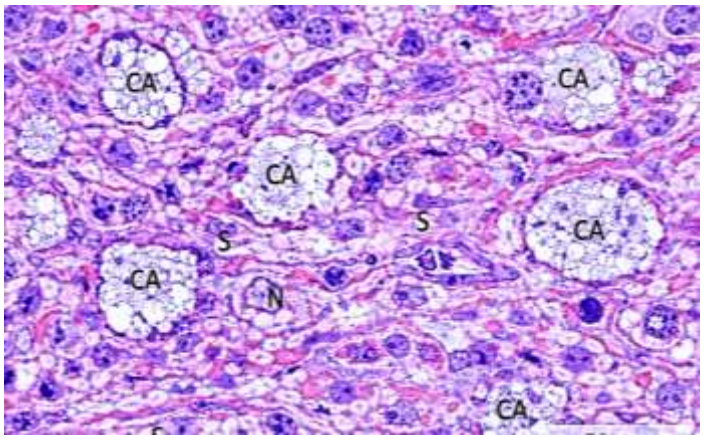
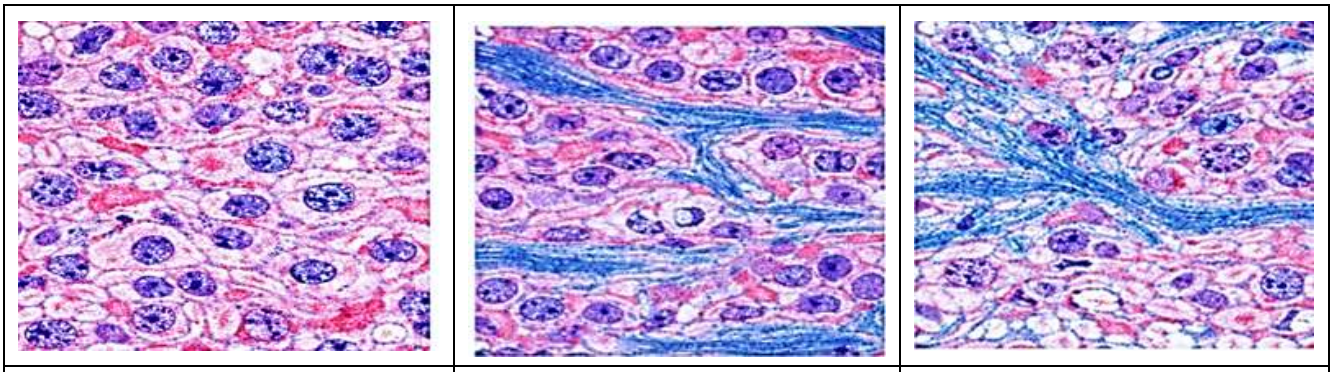


Fig. 3. Group III (18 months). H&E × 400. Marked atrophy of glandular parenchyma with severe reduction in pinealocyte count, extensive fibrous stroma (F), and multiple large corpora arenacea (CA). Scale bar = 50 µm.



Group I	Group II	Group III

Fig. 4. Masson's Trichrome × 200. Blue color represents collagenous stroma. Group I shows minimal fibrosis (8%), Group II moderate fibrosis (22%), and Group III extensive collagen deposition (42%), consistent with progressive stromal expansion.

Table (1) showed histological changes in the pineal gland across the three studied age groups and demonstrated progressive age-related degenerative changes. A significant decrease in gland size and dimensions (width and length) was observed from the first to the third group ($p<0.05$ and $p<0.01$), indicating progressive atrophy. Both the diameter and number of pineal cells per microscopic field decreased, as did the diameter of the nuclei, reflecting a decline in the functional activity of the cells responsible for melatonin secretion. Conversely, our results showed a significant increase in the proportion of interstitial tissue, indicating the replacement of functional cells with supporting fibrous tissue. Furthermore, the area of calcifications increased significantly with age, a hallmark of pineal gland aging. Overall, these findings reflect clear functional and structural changes in the pineal gland with age, including cellular atrophy, increased fibrosis, and calcification, which may negatively impact its hormone production efficiency.

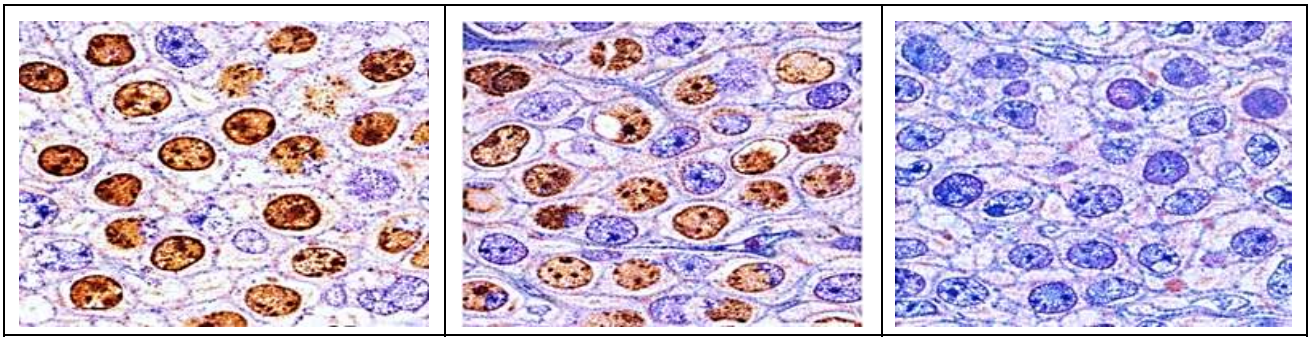
Table 1. Histological properties of pineal gland across age groups

Tissue properties	Group I (n=12) Mean ± Standard Deviation	Group II (n = 12) Mean ± Standard Deviation	Group III (n = 12) Mean ± Standard Deviation
Gland Volume (mm^3)	2.13 ± 0.15	1.87 ± 0.22*	1.43 ± 0.19**
Gland Length (µm)	1827 ± 86	1640 ± 104*	1396 ± 81**
Gland Width (µm)	986 ± 57	865 ± 64*	733 ± 49**
Pinealocyte Diameter (µm)	13.4 ± 1.09	10.7± 1.5*	8.9 ± 1.3**
Pinealocyte Count/HPF	27.9 ± 3.4	24.2 ± 2.5*	19.1 ± 4.4**
Nuclear Diameter (µm)	6.1 ± 0.5	5.7 ± 0.2*	4.7 ± 0.5**
Interstitial Tissue (%)	19.2 ± 2.7	26.7 ± 4.02*	39.5 ± 4.3**
Calcification Area ($µm^2$)	5.1 ± 0.2	139 ± 34*	895 ± 120**

**p<0.05; **p<0.01 vs. Group I.*

The results of this study showed a clear difference in the level of vitamin D receptor (VDR) immunoreactivity and histological changes among the three groups as discussed in figure (5). Histological images showed that Group I exhibited strong nuclear VDR expression stained with DAB (brown) with mild fibrosis (8%), while expression intensity in Group II decreased to a moderate level with increased fibrosis (22%). Group III showed the lowest levels of expression with weak or absent staining and significant collagen deposition, indicating clear histological deterioration. These observations are supported by the immunohistochemical analysis, which showed a mean H-score for VDR expression in Group I (142.4 ± 12.3) compared to a decrease in Group II (98.6 ± 11.7) and Group III (55.2 ± 9.8), with statistically significant differences ($p<0.05$ and $p<0.01$, respectively). The percentage of VDR-positive cells also gradually decreased from 74.3% in the first group to 52.1% and then to 31.6% in the third group, with a rise in weak expression (42%) and a decrease in strong expression (17%) in the latter group, reflecting a gradual decline in receptor function (table 2) Concurrently, serum results showed a significant decrease in vitamin D3 levels (both 25(OH)D3 and 1,25(OH)2D3) as well as melatonin. The first group recorded the highest values (38.6, 52.3, and 88.4, respectively), followed by the second group, and then the lowest values in the third group (19.4, 24.7, and 34.8 pg/ml), with clear statistical significance as described in table 3 .

Combined data (n=32) Pearson correlation analysis showed that 25(OH)D3 and structural integrity parameters had strong positive correlations, and calcification and fibrosis had strong negative correlations. The most significant correlation was between 25(OH)D3 and VDR H-score (r=0.889) and between 25(OH)D3 and calcification area (r= -0.876) as shown in table 4.



Group I	Group II	Group III

Fig. 6. VDR immunoreactivity (brown, DAB) in pinealocytes counterstained with hematoxylin. Left: Group I — strong nuclear positivity (VDR⁺ ≈ 74%). Centre: Group II — moderate positivity (VDR⁺ ≈ 52%). Right: Group III — weak to absent staining (VDR⁺ ≈ 32%). Scale bar = 25 µm.

Table 2. Immunohistochemistry analysis of VDR Expression

IHC Results	Group I (n=12)	Group II (n = 12)	Group III (n = 12)
	Mean ± Standard Deviation	Mean ± Standard Deviation	Mean ± Standard Deviation

VDR Expression (H-score)	142.4 ± 12.3	98.6 ± 11.7*	55.2 ± 9.8**
VDR Positive Cells (%)	74.3 ± 6.8	52.1 ± 5.9*	31.6 ± 5.4**
Degree of expression	N (%)	N (%)	N (%)
Weak	2 (17%)	3 (25%)*	5 (42%)**
Moderate	2 (17%)	4 (33%)*	3 (25%)
Strong	8 (66%)	6 (50%)*	2 (17%)**

*n = 10 per group. *p<0.05; **p<0.01 vs. Group I.*

Table 3. Serum concentration of vitamin D3 and Melatonin

Markers (pg/ml)	**Group I (n=12)**	**Group II (n = 12)**	**Group III (n = 12)**
	Mean ± Standard Deviation	**Mean ± Standard Deviation**	**Mean ± Standard Deviation**
Serum 25(OH)D3	38.6 ± 4.2	28.1 ± 3.8*	19.4 ± 3.1**
Serum 1,25$(OH)_2$D3	52.3 ± 5.1	38.9 ± 4.6*	24.7 ± 4.0**
Melatonin	88.4 ± 9.2	61.3 ± 8.4*	34.8 ± 6.7**

*n = 10 per group. *p<0.05; **p<0.01 vs. Group I.*

Table 4. Pearson Correlation Coefficients Between histological and Biochemical Parameters

Histomorphometric Parameter	**25(OH)D3 r / p**	**VDR Score r / p**	**Melatonin r / p**
Gland Volume	0.847 / <0.001	0.812 / <0.001	0.796 / <0.001
Pinealocyte Count	0.823 / <0.001	0.778 / <0.001	0.814 / <0.001
Pinealocyte Diameter	0.791 / <0.001	0.745 / <0.001	0.768 / <0.001
Calcification Area	−0.876 / <0.001	−0.831 / <0.001	−0.854 / <0.001
Interstitial Tissue	−0.834 / <0.001	−0.801 / <0.001	−0.819 / <0.001
VDR Expression	0.889 / <0.001	—	0.801 / <0.001

r = Pearson correlation coefficient. All correlations on pooled data (n=36). |r| > 0.8 indicates strong correlation. p < 0.001 for all listed correlations. (—) = same variable, not computed.

4. Discussion

The detailed results of this research revealed a progressive, age-related functional and histological disturbance in the pineal gland of male rats. Microscopic examination showed a fundamental transformation in the gland's structure from a dense, homogeneous tissue composed of pinealocytes at 3 months of age to atrophic tissue characterized by significant fibrosis and a notable accumulation of collagen fibers, reaching 42% by 18 months of age. This aligns with the findings of Tan *et al*. (2018) regarding the role of radical histological changes in reducing the gland's functional efficiency with age . The dense appearance of corpora arenacea in aged rats reflects a histological degeneration mechanism that leads to compression of secretory cells and a significant decrease in their number and diameter. These

findings are supported by the study of Humbert & Pévet (1991), which linked calcification to a loss of cellular vitality .

The presence of striated muscle fibers in the pineal glands of several mammals, including humans, cattle, pigs, bats, and rats, has been previously reported (Tomonari *et al*., 2012). In our study, striated muscle fibers were rare, with an age-related increase as in previous reports, although their incidence varied among the reports. Interestingly, these fibers were found in male rats in our study; however, there are some reports indicating their presence in females. Quay reported finding striated muscle fibers in the pineal glands of two males and one ovariectomized female rats in a series of approximately 1200 rats (Tomonari *et al*., 2012), (Allen *et al*., 1982). Dale also reported the distribution of striated muscle in the pineal glands of one female and another female who had undergone biadrenal resection (Dill, 1963). Other studies, however, have reported only male mice, except for those whose sex was not determined. The histological characteristics of the striated muscle fibers in our study were similar to those reported previously, with the fibers appearing in the connective tissue near the pineal gland stalk, particularly in the presence of fibrosis (Diehl, 1978).Immunohistochemical staining in our study showed positive for lipase and negative for myogenin, indicating fiber maturation. After examining the fibers and considering the arguments of several other researchers, the origin of the fibers remains unknown (Moridi *et al*., 2020).

Vitamin D3 and melatonin supplementation help preserve the histological structure of the rat pineal gland, particularly mitigating age-related fibrosis and mineralization, while enhancing antioxidant defenses against degeneration. Melatonin acts as a potent free radical scavenger, supporting pineal integrity, whereas Vitamin D3 acts to prevent degenerative damage, with effects often dependent on the age and metabolic state of the rats (Moridi *et al*., 2020). From a biochemical and immunological perspective, this study revealed a very strong positive correlation between decreased serum vitamin D3 levels and reduced expression of its receptor (VDR) within the pineal gland, with the H-score decreasing from 142.4 to 55.2. This suggests that the deterioration of the vitamin D3-VDR axis plays a pivotal role in accelerating fibrosis and calcification processes, consistent with the hypotheses of Holick (2011) and Eyles et al. (2005) regarding the regulatory and protective role of this axis in neural and glandular tissues.

This structural deterioration necessarily led to a severe secretory deficit, manifested in a decrease in melatonin levels from 88.4 pg/ml to 34.8 pg/ml. This supports the study by Reiter et al. (1981) that pineal gland aging is associated with the loss of the antioxidant protection provided by melatonin, thus entering the cell into a vicious cycle of oxidative damage and tissue fibrosis. Based on the strong statistical correlation ($r = 0.889$) between vitamin D3 and pineal gland health, this study demonstrates that maintaining adequate levels of vitamin D3 and its receptors is vital for protecting the pineal gland from age-related degenerative changes (Reiter et al., 1981; Tchekalarova et al., 2025). These findings are consistent with those of Barrenetxe et al. (2004) regarding the metabolic functions of melatonin and its interaction with fat-soluble vitamins, suggesting that the complementarity between vitamin D3 and melatonin forms a protective shield for maintaining the integrity of the skeletal gland, and that the absence of this complementarity in advanced age is responsible for the observed fibrotic and calcifying transformations (Barrenetxe *et al*., 2004).

Conclusion

The results of this study showed that the age-related decline in the pineal gland of male rats is associated with significant functional and structural deterioration, characterized by a decrease in the number, size, and diameter of pineal cells, along with a significant increase in colloidal fibrin deposition and the expansion of calcification zones (pine sand). Biochemical and immunological analysis revealed a sharp decrease in serum melatonin and vitamin D3 levels, accompanied by a decline in VDR expression within the gland tissue. Statistical correlations demonstrated a strong positive relationship between vitamin D3 bioavailability and the maintenance of the gland's structural integrity and secretory capacity, indicating that the vitamin D3-VDR axis plays a vital protective role in mitigating the fibrotic and calcific degenerative changes associated with aging.

CONFLICT OF INTEREST

The author declares no conflict of interest.

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