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Immunohistochemical Characteristics of Age-Related Regressive Changes in the Human Pineal Gland

Características Inmunohistoquímicas de los Cambios Regresivos Relacionados con la Edad en la Glándula Pineal Humana

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SUMMARY: The pineal gland is a small but essential endocrine gland that secretes the hormone melatonin, which regulates the human circadian rhythm. This study aims to analyze regressive changes in human pineal gland tissue by examining the expression of GFAP, vimentin, and CD34. The study included 30 paraffin-embedded blocks of human pineal glands obtained from cadaver autopsies. Samples were stained immunohistochemically with the antibodies GFAP, Vimentin, and CD34. Morphometric data were obtained using the threshold color plugin in Fiji software, and the results were analyzed by age and gender. A statistically significant difference was found in the areal fraction occupied by vimentin between Groups I and II, and Groups I and III. The average areal density of the GFAP marker is highest in the youngest cohort. No statistically significant difference was noted among the analyzed groups in comparing the results. Statistical analysis revealed a statistically significant difference in the density of blood vessels between Group I and Group II. The quantity of pinealocytes and astrocytes diminishes over time, as evidenced by the reduction in the positivity of GFAP-positive cells. Vimentin positivity demonstrates the increase in the proportion of stroma throughout the aging process. Furthermore, the rise in the density of blood vessels, identified by anti-CD34, is relative to their localization within the stroma, which becomes predominant over time.

KEY WORDS: Human; Pineal gland; Vimentin; GFAP; Blood vessels; Aging.

INTRODUCTION

The pineal gland (PG) is a small but essential endocrine gland. People have been familiar with it for 2000 years. René Descartes, a French philosopher, mathematician, and scientist, believed that the pineal gland is where thoughts are formed and the human soul originates (Ilic *et al*., 2005). The pineal gland comprises parenchyma, covered with the connective tissue of the soft meninges (pia mater), which forms its capsule. Connective tissue septa divide the glandular tissue into lobes of different sizes. The parenchyma of the pineal gland itself is made up of two types of cells: pinealocytes, which make up the majority (95 %), and interstitial glial cells (5 %) (Treuting *et al*., 2018). Pinealocytes are round cells with light, basophilic cytoplasm and an irregular euchromatic nucleus, whose role is the production and secretion of the hormone melatonin. A rich network of capillaries surrounds pinealocytes. Glial cells, called interstitial cells, are small, star-shaped supporting cells representing modified astrocytes. Their cytoplasm is darker and localized between pinealocytes (Hyder *et al*., 2011). In addition to cells, calcified structures called brain sand (corpora arenacea), whose function is unknown, can be observed in the pineal gland tissue (Tan *et al*., 2018). It was only in 1958 that the proper function of this gland was discovered (Hyder *et al*., 2011). As a neuroendocrine organ, the pineal gland secretes the hormone melatonin, which regulates the human circadian rhythm—the absence of light conditions and melatonin production. For that reason, the synthesis takes place at night (Bolat *et al*., 2018). The importance of the pineal gland and melatonin in the work of the human organism is manifold. In addition to the circadian rhythm and importance in maintaining sleep, melatonin exhibits anticancer effects through immunomodulatory, anti-inflammatory, antioxidant,

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vasoregulatory, and oncostatic effects (Miller *et al*., 2006; Bonnefont-Rousselot & Collin, 2010; Fernández-Mar *et al*., 2012). As the immunohistochemical characteristics of the human pineal gland tissue have not been determined so far, this study aims to examine the expression of GFAP, vimentin, and CD34 in the human pineal gland tissue, as well as to analyze the changes in their expression during aging.

MATERIAL AND METHOD

The research was performed at the Department of Histology and Embryology, Faculty of Medicine, University of Novi Sad. The study includes 30 formalin-fixed, paraffin-embedded (FFPE) blocks of human pineal glands obtained from the autopsy of cadavers. According to age, samples were divided into three groups: Group I (n=10) includes samples of the pineal gland of subjects aged 20 to 30; Group II (n=10) includes samples of persons aged 31 to 64; Group III (n=10) includes samples of individuals older than 64. Nineteen samples belonged to men and 11 to women. In the material used in the research, the youngest respondent was 20, and the oldest was 83 years old. The average age was 53.4 ± 18.42 for males and 50.9 ± 20.94 for females. In the youngest age group, the average age was 27.3 ± 3.3; in the second group, 57.7 ± 5.0; in the oldest, it was 76.2 ± 4.7.

Histological technique. Pineal glands were fixed in buffered formalin (24 hours at +4 °C) and decalcified with 14 % ethylenediaminetetraacetic acid (EDTA). They were then dehydrated in rising isopropanol concentrations, embedded in paraffin (Histowax, The Netherlands), and cut on a rotary microtome (Leica, Germany) at 5 μm. The histology slides were stained with a standard hematoxylin-eosin technique.

Immunohistochemical staining was performed using the following primary antibodies: GFAP (mouse monoclonal, 1:100 dilution, Thermo Fisher Scientific, Waltham, MA, USA), Vimentine (rabbit monoclonal, 1:500 dilution, Abcam, Boston, MA, USA) and CD34 (rabbit monoclonal; 1:3000 dilution, Abcam, Boston, MA, USA) detected with the UltraVision LP Detection System using HRP Polymer & DAB Chromogen (Thermo Fisher Scientific, Waltham, MA, USA). Before incubation of both antibodies (30 min. at room temperature), antigen retrieval was performed using a citrate buffer (pH 6.0) in a microwave oven at 850 W for 20 min. Mayer's hematoxylin was used as a counterstain for immunohistochemistry before mounting and coverslipping the slides (Bio-Optica, Milan, Italy).

The slides were analyzed using a Leica DMLB microscope (Leica, Germany) and photographed with a Leica MC 190 HD camera (Leica, Germany) at a magnification of 40x using the Leica Application Suite (LAS).

Surface fraction assessment. Ten randomly selected microscopic fields of view were photographed at 40x magnification. Afterward, using Fiji morphometry software and a Color Deconvolution plugin, DAB-positive labels for the analyzed markers were isolated from the microphotographs and transferred to a black-and-white binary format using the threshold function. Based on the contrast, the surface fraction (area fraction) was analyzed using the Analyze Particles function for black-and-white photos, i.e., the percentage occupied by DAB-positive areas to the entire photograph surface (Fig. 1A).

Statistical analysis. Statistical analysis was performed using IBM SPSS statistical software, version 23.0 (IBM Corp., Armonk, NY, USA). Data were reported as the mean ± standard deviation (SD). One-way analysis of variance (ANOVA) or Kruskal–Wallis test and Student's t-test were employed to compare experimental groups. A statistically significant difference between groups was considered for a P value of less than 0.05 ($P < 0.05$).

RESULTS

The expression of vimentin and glial fibrillary acidic protein (GFAP) within the pineal gland. Immunoexpression of both examined markers was observed in the pineal gland tissue samples. By analyzing the samples stained with the GFAP antibody, the immunoreactivity of the parenchyma within the gland's lobes is noted across all age groups. Immunopositive cytoplasmic extensions of astrocytes encircle the bodies of pinealocytes, creating a dense network (Fig. 1B). On the slides stained with an antibody to vimentin, the immunopositivity of astrocytes is evident in the peripheral regions of the lobes. Alongside astrocytes, endothelial cells of blood vessels and fibrocytes within the connective septa also demonstrate an immunopositive reaction to vimentin (Fig. 1C). The average surface density of vimentin in group I is recorded at 19.37 ± 6.36 %; in group II, it is reported at 23.8 ± 7.39 %, and in group III, it is noted as 24.31 ± 5.94 % (Fig. 1D). A highly statistically significant difference in the area occupied by vimentin was found between groups I and II, as well as between groups I and III. However, no significant difference was observed when comparing groups II and III. The average surface density of GFAP markers in the youngest group (group I) is 25.07 ± 3.36 %; in Group II, it is recorded at 24.26 ± 6.86 %, while in the oldest group (Group III), this percentage is 23.17 ± 6.57 % (Fig. 1E). When comparing the results obtained, no statistically significant difference was noted among the analyzed groups ($p \geq 0.05$).

Vimentin in males has an average areal density of 23.08 ± 7.067 % of the surface. In contrast, in females, this

marker occupies an average fraction of 22.91 ± 6.946 % of the surface of the PJ (Fig. 1F). Based on the results, no statistically significant difference was found between males and females ($p \geq 0.05$). The average area occupied by the GFAP marker in men is 24.1 ± 6.69 %, whereas in women, this value is 24.27 ± 6.77 % (Fig. 1G). No statistically significant difference was found when comparing the values obtained for both sexes ($p \geq 0.05$).

No statistically significant difference was observed when comparing the percentage of the vimentin marker to that of the GFAP marker across all three age groups. Statistical analysis revealed no significant difference in sex regarding the areal density of vimentin compared to the areal density of GFAP. In males, vimentin accounts for an average areal fraction of 23.08 ± 7.07 % of the gland's surface, while the average value for the GFAP marker is 24.1 ± 6.49 %. In females, vimentin constitutes 22.91 ± 6.95 % of the surface, while GFAP covers 24.27 ± 6.77 % of the gland's surface (Fig. 1H).

Density of blood vessels in the pineal gland. In specimens stained with the CD34 antibody, the density of blood vessels was calculated using Fiji software. Blood vessel density was 1.78 ± 1.38 % for Group I, while 2.42 ± 1.84 % for Group II; samples in the oldest group (Group III) had a value of 2.01 ± 1.21 %. Statistical analysis indicated a statistically significant difference between Group I and Group II ($p \leq$

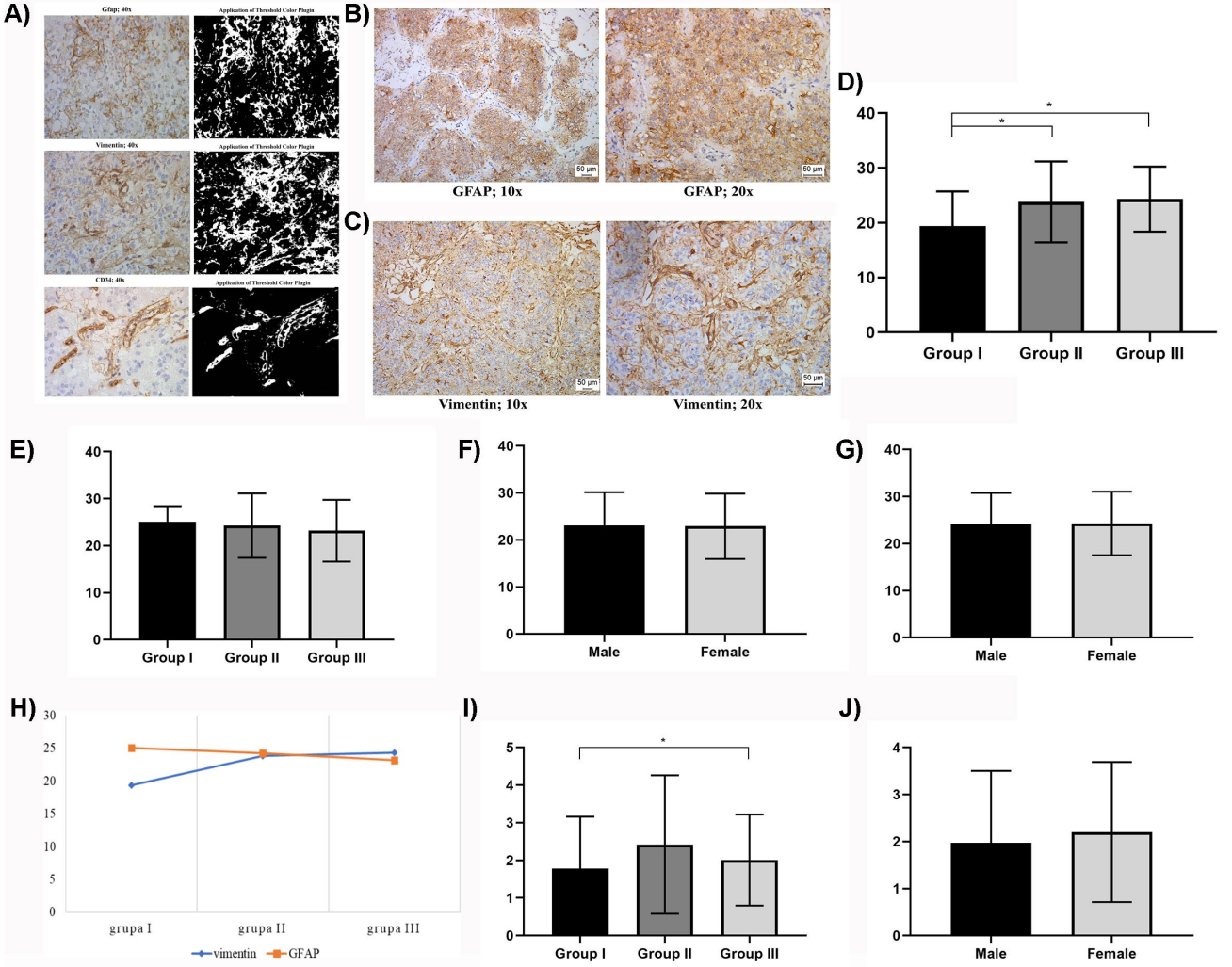

Fig. 1. A) Results of applying the threshold color plugin in Fiji software to immunohistochemically stained samples of the pineal gland; B) Immunoexpression of GFAP in the pineal gland; C) Immunoexpression of Vimentin in the pineal gland; D) Graphical representation of the average surface density of Vimentin in the pineal gland across all three age groups; E) Graphical representation of the average surface density of GFAP in the pineal gland across all three age groups; F) Graphical representation of the average surface density of Vimentin in the pineal gland by sex; G) Graphical representation of the average surface density of GFAP in the pineal gland by sex; H) Comparison of Vimentin and GFAP surface densities in the pineal gland by age; I) Average density of blood vessels in the pineal gland across all age groups; and J) Representation of the average density of blood vessels in the pineal gland by sex.

0.05). In contrast, the differences in blood vessel density between Groups I and III, as well as between Groups II and III, were not statistically significant ($p \geq 0.05$) (Fig. 1I). Additionally, the results showed that the mean density of blood vessels near the pineal glands in females was 2.20 ± 1.49 %, compared to 1.97 ± 1.53 % in males (Fig. 1J). Statistical analysis revealed no statistically significant difference between these two groups ($p \geq 0.05$).

DISCUSSION

Glial fibrillary acidic protein and vimentin are intermediate filaments, about 10 nm in size, located in the cytoplasm of glial cells. During the maturation of astrocytes, the expression of intermediate filaments changes. In the initial development of astrocytes, the expression of vimentin is more pronounced than the expression of GFAP, while in mature cells, the representation of these two proteins is reversed (López-Muñoz *et al*., 1992). Today, GFAP is considered a characteristic constituent of mature cells, and the presence of vimentin in cells is a sign of immaturity (Eng, 1985). A study by Fabrizio Busolini *et al.* (2017) involved examining the spatial distribution of vimentin, GFAP, and protein S-100 in the interstitial cells of the pineal gland in developing chinchillas compared to adults. GFAP was predominantly expressed in stellate cells with long finger-like extensions surrounding individual pinealocytes or groups of pinealocytes. At the same time, vimentin was observed in cytoplasmic extensions of astrocytes, as well as in endothelial cells and perivascular spaces. By qualitative analysis of the preparation, we could see that the distribution of GFAP and vimentin followed a similar ratio as in Fabrizio's study (Busolini *et al*., 2017). The parts of the parenchyma labeled with the GFAP marker were located in the lobules themselves. GFAP is found in glial cells within a lobule, forming a visible network of filaments surrounding the pinealocytes. Contrary to GFAP, vimentin expression was detectable predominantly at the periphery of the lobules, in perivascular spaces and endothelial cells, similar to the results of the study above. Research by Papasozomenos (1983) conducted with samples of the human pineal gland showed that immunopositivity for GFAP is observed as early as in the 24th week of gestation, as well as that with aging, the interstitial network of astrocyte extensions becomes more pronounced. Using immunomarker S100b, earlier studies showed that the number of glial cells increases with aging (Popovic´ *et al*., 2016). Our research showed that GFAP is found in the cytoplasm of mature astrocytes and is almost equally represented in cells of all age groups, confirmed by statistical analysis. Vimentin was initially considered a marker of mesenchymal cells and radial glia, and it shows strong immunoexpression in the cytoplasm of immature astrocytes (Schnitzer *et al*., 1981). The literature states that the expression of vimentin is significant in the early stage of cell development but that as the cell matures, this filament is replaced by another (Fernández-Mar *et al*., 2012).

Martin Oudega and Enrico Marani examined the expression of vimentin and GFAP in developing rat spinal cord cells. Their study showed that during the first two weeks of life, vimentin is replaced by GFAP and that their expression had a similar pattern (Oudega & Marani, 1991). Examining the pineal glands of cats and dogs, Boya & Calvo (1993) observed that in both types of animals, the pineal cells show vimentin immunopositivity regardless of the animal's age. Namely, cells stained with vimentin had a similar immunohistochemical profile as those stained with GFAP. This phenomenon is explained in the literature as a delay in the maturation of astrocytes in the pineal glands compared to astrocytes of the central nervous system. This is supported by findings that in certain parts, such as the cerebellum and large myelinated pathways, astrocytes retain immunopositivity for vimentin and GFAP (Schnitzer *et al*., 1981; Boya & Calvo, 1993). Our examination of the distribution of vimentin markers showed that the highest expression of vimentin was observed in the oldest group, while it was the lowest in the youngest group. A statistically significant difference exists between Groups I and II and between Groups I and III. No statistically significant differences were demonstrated when comparing the surface density of GFAP and vimentin markers between age groups. This phenomenon can be explained by the fact that, in addition to astrocytes, vimentin is also expressed in blood vessel cells and fibrocytes of connective septa within the pineal tissue, which resulted in a similar surface density with the GFAP marker (positive immunoexpression is considered evidence of the astrocytic nature of the cells). In the available literature, we could not find any papers that examined these parameters on a human sample and compared them by sex. Today, it is known that astrocytes undergo the process of reactive gliosis due to various pathological changes in the central nervous system. During this process, there is not only an increase in the number of astrocytes (proliferation) but also an increase in cellular processes. In addition, increased production of intermediate filaments, both glial fibrillary acidic protein, and nestin and vimentin (filaments characteristic of immature astrocytes) were observed (Eng *et al*., 2000; Pekny, 2001). Glial cells do not show immunopositivity for the examined markers, only in pineal glands with normal histology, but also in tumor changes of the pineal parenchyma; it was observed that glial cells show immunopositivity for S100, GFAP, as well as vimentin (Yamane *et al*., 2002).

Vasculogenesis refers to the process of formation of blood vessels during embryonic development. In contrast, angiogenesis is the physiological process of forming new blood vessels by multiplication (branching) from existing ones (Cabral *et al*., 2017). Angiogenesis occurs both prenatally and postnatally in the usual developmental processes of the organism but also in some pathological processes such as injuries, inflammatory and vascular diseases, and tumors (Cabral *et al*., 2017). In tumors, angiogenesis is an unfavorable factor that leads to metastases, first described by Grinblatt and Shubik in 1968 as a cause of tumor expansion (Greenblatt & Shubik, 1968). The factors that lead to angiogenesis are a drop in arterial blood pressure for an extended period, increased metabolic needs of the body, and the tissue's need for oxygen. The density of blood vessels is a measure of angiogenesis in an organ.

Although the pineal gland has been known to man since ancient times, we have not found any information about the density of blood vessels in the pineal gland in the literature. In this study, an analysis of the surface (areal) density of blood vessels of the pineal gland was performed. Data for the density of blood vessels was obtained with the help of the Fiji software and Plugin called Threshold-Colour, and the anti-CD34 antibody was used as an immunohistochemical marker. The analyzed data shows a statistically significant difference between age Groups I and II. The value of blood vessel density obtained by this method is the highest for Group II and amounts to 2.42 % of the examined area. By comparing the obtained results, the average value of the density of blood vessels in persons of female and male sex is approximate, and there is no statistically significant difference between them. Viacava *et al*. (2003) conducted research similar to ours. In their research, we came across data on the density of blood vessels in the healthy and tumor-altered pituitary gland. The findings from their study show that healthy pituitary tissue is significantly more densely vascularized compared to tumor-altered tissue (adenoma). Within the adenomas themselves, there is no statistically significant difference in the density of blood vessels compared to the histological type of tumor, tumor size, age, and patient sex (Auer, 1994). Thanks to the introduction of anti-angiogenic therapy, the density of blood vessels is an important prognostic and therapeutic factor in today’s medicine. Much attention is paid to the density of blood vessels in malignancies of the breast, colon, uterus, head, and neck (Sharma *et al*., 2005). A semi-automated method involves computer software that facilitates sample analysis (Auer, 1994; Romero *et al*., 2000).

Applied software solutions and digital technologies will be able to remove the subjectivity of researchers and offer a more accurate evaluation of immunohistochemical markers in the future, thus ensuring a more precise result. Studies of this type would significantly help create reference values that could be used to compare the vascularization of healthy and pathologically altered tissues. This study identified regressive changes in the pineal gland, reflected in the gradual disappearance of the pineal gland's parenchyma and the stroma's compensatory proliferation. The number of pinealocytes and astrocytes attached to pinealocytes decreases over time, which could be seen through the decrease in the positivity of GFAP-positive cells. The increase in the proportion of stroma during aging is clearly shown through vimentin positivity. Although obvious, the increase in the density of blood vessels detected by the anti-CD34 marker can be attributed to relative values considering their localization in the stroma, which becomes dominant over time.

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RESUMEN: La glándula pineal es una glándula endocrina pequeña pero esencial que secreta la hormona melatonina, la cual regula el ritmo circadiano humano. Este estudio tiene como objetivo analizar los cambios regresivos en el tejido de la glándula pineal humana mediante el examen de la expresión de GFAP, vimentina y CD34. El estudio incluyó 30 bloques de glándula pineal humana incluidos en parafina, obtenidos de autopsias de cadáveres. Las muestras se tiñeron inmunohistoquímicamente con los anticuerpos GFAP, vimentina y CD34. Los datos morfométricos se obtuvieron mediante el plugin de color de umbral del software Fiji, y los resultados se analizaron por edad y sexo. Se encontró una diferencia estadísticamente significativa en la fracción de área ocupada por vimentina entre los Grupos I y II, y entre los Grupos I y III. La densidad de área promedio del marcador GFAP es mayor en la cohorte más joven. No se observaron diferencias estadísticamente significativas entre los grupos analizados al comparar los resultados. El análisis estadístico reveló una diferencia estadísticamente significativa en la densidad de vasos sanguíneos entre el Grupo I y el Grupo II. La cantidad de pinealocitos y astrocitos disminuye con el tiempo, como lo demuestra la reducción en la positividad de las células GFAP-positivas. La positividad para vimentina demuestra el aumento en la proporción de estroma a lo largo del proceso de envejecimiento. Además, el aumento en la densidad de vasos sanguíneos, identificado por anti-CD34, es relativo a su localización dentro del estroma, que se vuelve predominante con el tiempo.

PALABRAS CLAVE: Humano; Glándula pineal; Vimentina; GFAP; Vasos sanguíneos; Envejecimiento.

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