**Original article**

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# IMMUNOHISTOCHEMICAL AND MORPHOMETRIC STUDY OF ADENOHYPOPHYSEAL GONADOTROPIC CELLS IN MALE CADAVERS OF DIFFERENT AGES

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The literature data suggest that with advancing age there occurs a functional decline in the gonadotropic cells, while the data concerning structural changes in these cells are rather scarce. The aim of this paper was to detect and quantify the changes in human gonadotropic cells of the anterior pituitary in male cadavers of different ages using immunohistochemical and morphometric methods. The material in this study consisted of adenohypophyseal tissue from 14 male cadavers of different ages, starting from the fourth decade of life. Adenohypophyseal tissue sections were routinely histologically processed and stained with immunohistochemical monoclonal anti-LH antibody to detect gonadotropic LH cells. Digital images of the visual fields of immunohistochemically processed adenohypophyseal sections were then morphometrically analyzed using the Image J system. Statistical analysis was performed using the SPSS statistical software package. The results of the morphometrical analysis showed that volume density of LH cells did not change significantly with advancing age, while their area, perimeter and Feret's diameter increased statistically significantly. Nuclear morphometric parameters did not change significantly, while the nuclear-cytoplasmic ratio of LH cells decreased with ageing, with a statistically significant decline observed in cases aged over 70 years. Based on the obtained results the conclusion may be drawn that the density of LH cells does not change significantly with ageing, but that they undergo hypertrophy in order to maintain normal hormonal secretion. Long-lasting hypertrophy of these cells ultimately leads to their functional decline, which reaches statistical significance after 70 years of age.

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***Key words:*** *luteinizing gonadotropic cells, adenohypophysis, immunohistochemistry, morphometry, ageing*

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## Introduction

Gonadotropic cells, i.e., LH and FSH cells, represent about ten percent of the overall cellular population of the anterior lobe of the pituitary. They are evenly distributed in the distal portion of adenohypophysis and histologically appear basophilic. Gonadotropes are oval cells with round nuclei and well developed organelles. Their cytoplasm contains electron-dense secretory granules which measure 200-250 nm in diameter, and are filled with hormones (1).

The main secretory products of gonadotropes are luteinizing (LH) and follicle stimulating hormone (FSH). Gonadotropin-releasing hormone (GnRH) stimulates the production and secretion of these hormones, while gonadal steroids, inhibin, follistatin and activin, by way of negative feedback loop, inhibit their production and secretion.

In women, LH is involved in ovulation and follicular luteinization, while in men it stimulates Leydig cells to secrete testosterone. FSH in women stimulates follicle maturation, while in men, it stimulates Sertoli cells to secrete androgen-binding protein (2, 3), which binds specifically to testosterone and transports it through the germinative epithelium into the lumen of the tubules. Hereby, it enhances spermatogenesis in the seminiferous tubules of the testis.

LH and FSH represent low molecular weight glycoproteins of about 30 kDa. They exert their effects on target tissues activating the cyclic adenosine monophosphate secondary messenger system,

which further activates specific enzyme systems in appropriate target cells (4).

From the point of view of endocrinology, ageing is predominantly characterized by a decline of function of the systems which support tissue anabolism. In males, it mostly relates to two very important components of the neuroendocrine system: gonadotropic and somatotropic axes. The term gonadopause generally describes a decline in biological availability of testosterone (T), while the term somatopause denotes a decline in biological availability of growth hormone (GH) and/or IGF-1. Nevertheless, these processes may markedly vary between different individuals, and their intensity is influenced as well by environmental factors and life habits.

In men, adenohypophysis begins to secrete gonadotropic hormones with the onset of puberty, and continues with the secretion throughout life following a gradually decreasing pattern. However, in most men, gradually decreasing sexual function becomes evident in late 40s or 50s, and is correlated with decreased testosterone secretion. Testosterone secretion usually drops significantly after 50 years of life, and in 80-year olds it ranges between 20% and 50% of the testosterone amount at the time of its maximal secretion. Despite this, testicular morphology, spermatogenesis and fertility demonstrate only minor changes in older men (4, 5).

Gonadopause (or late hypogonadism) is characterized by a progressive dysregulation of the hypothalamic-pituitary-gonadal axis, most probably under the influence of age-related decline in maximal hypothalamic secretion of GnRH, decline of the maximal and average LH pulse amplitude (with preserved basal frequency of LH pulse secretion), decline of LH-stimulated testosterone secretion, and reduction of testosterone-mediated negative feedback loop (6). The consequence of all of the above is *biochemical* late hypogonadism, with lower serum concentrations of total testosterone, bioavailable testosterone and free testosterone, then with a moderate rise of serum LH and FSH and ultimately with elevated serum levels of globulins which bind sex hormones (7). Late hypogonadism *clinically* manifests with the symptoms similar to those in hypogonadism which affects younger males, including reduced bone and muscle mass, abdominal obesity, loss of pubic hair and beard hair. Moreover, hypogonadism results in a number of non-specific symptoms, including nervousness, irritability, psychological depression, memory loss, weakness, insomnia, hot flashes, periodical sweating, and loss of sexual drive (7).

In their paper, Schwartz et al. (8) have reported that the function of adenohypophysis probably represents the result of integration of multiple input signals, including hypothalamic, peripheral and intrapituitary ones. Intrapituitary factors may exert stimulatory or inhibitory effects on hormone production by the adenohypophysis. The production and secretion of LH is also controlled by a variety of locally produced signalling molecules, which form a complex network and are involved in autocrine/ paracrine control of the function of gonadotropes (9). According to Denef (10), gonadotropes are involved in interactions with other hormonal (mostly lactotropic, somatotropic and corticotropic) and non-hormonal cells, such as folliculostellate (FS) cells.

Considering the fact that hypogonadism which occur with ageing may be the consequence of structural changes at all levels of the gonadotropic axis and that age-related histomorphologic changes, when this regulatory system is concerned, are thoroughly studied only in testis, our aim in this study was to detect and quantify the changes in human gonadotropic cells of the adenohypophysis, as a component part of the gonadotropic axis, in cases of different age, using immunohistochemical and morphometric methods (measurements of volume density, area, perimetar, Feret's diameterand calculation of the nuclear-cytoplasmic ratio).

## Materials and methods

The study took place at the Institute of Anatomy, University of Niš Faculty of Medicine, Center for Forensic Medicine in Niš, and Center for Pathology, Clinical Center Niš, all of which are the teaching bases of this Faculty. Immunohistochemical processing of histological sections of adenohypophysis was done at the *Siniša Stanković* Institute for Biological Research in Belgrade.

### *Material*

The study material consisted of hypophyseal tissue taken from 14 male cadavers, aged 41 to 87 years. The tissue samples were taken during routine autopsies performed at the Center for Forensic Medicine in Niš, after a post-mortem period which did not exceed 24 hours, abiding by the ethical norms regulating the use of cadaveric material for biomedical research purposes (decision by the Ethics Committee of the University of Niš Faculty of Medicine, № 12-2307-2/8 of March 10, 2016). The cadavers used in this study were without any diagnosed neurological, psychiatric or endocrine disorders during life. There was no visible damage to the brain or hypophysis on autopsy in any of the cases. Further, histopathological evaluation of the brain and hypophysis excluded the presence of a possible hidden or misdiagnosed disease. Cadavers were classified into three age groups: first (I), with cases aged 30-49 years; second (II), with cases aged 50-69 years; and third (III), with cases aged 70 years and older.

The methodology of the study involved an adequate dissection procedure for sampling of the hypophysis, followed by histological preparation of the samples and their morphometrical and statistical analysis.

### Dissection procedure

The sellar diaphragm (*diaphragma sellae*) was the first to be removed by dissection. After that, the pituitary stalk (*infundibulum*) was sectioned, and the hypophysis was then carefully detached from the

surrounding osseous structures of the sella turcica and removed *en bloc*.

**Histological preparation**

Our histological analysis, in the sense of identification of possible changes in the structure of gonadotropic cells of the adenohypophysis with ageing, was based on the light microscopy evaluation of their properties.

The removed hypophyses were fixed for 24 hours in 10% buffered formalin and then embedded in paraffin. The resulting paraffin molds were used to obtain up to 5 μm thick hypophyseal tissue sections using the Leica 2235 microtome, which were then routinely stained with hematoxylin-eosin (H&E) and immunohistochemically processed. The presence of cells with a positive reaction to the applied immunohistochemical marker was established by immunohistochemical analysis.

*Immunohistochemical staining of LH gonadotropic cells of the adenohypophysis*

The peroxidase-antiperoxidase (PAP) method was used for immunohistochemical staining of LH gonadotropic cells (11). Immunocytochemical methods, in particular the specific reaction between the primary antibody and antigen enables differential staining of cells carrying the specific antigen, in this case a hormone. After deparaffinization and rehydration of the tissue sections, first the activity of endogenous peroxidase was blocked by incubation of tissue sections in 0.3% $H_2O_2$ in methanol for 15 minutes. The sections were then washed in 0.01M phosphate buffer (Phosphate Buffer Saline PBS; pH 7.6; 2 x 5 min.), and the reduction of non-specific staining was accomplished by incubation of the sections in normal swine serum (Normal swine serum, Dako Dakopatts, Denmark; dilution 1:10 in PBS) for 1 hour.

Primary anti-LH (1:100) anti-rat antibodies (a donation from Dr Parlow; NIH, Bethesda, Md., USA) diluted in PBS were used to detect LH gonadotropic cells (12). The incubation lasted for 24 hours at room temperature. After the incubation with the primary antibody, the sections were washed in PBS (2 x 5 minutes) and then incubated for 1 hour with secondary conjugated antibody (Policlonal swine anti rabbit IgG (HRP), Dako Dakopatts, Denmark; dilution 1:200 in PBS). After washing in PBS, visualization was made possible by using DAB (Dako). Contrast enhancement was performed using Mayer's hematoxylin solution (Merck-Alkaloid, Alkaloid, Skopje, Republic of North Macedonia), dehydration with increasing ethanol series, and mounting by using DPX.

**Morphometric analysis**

Morphometric analysis was performed using the digital image taken with 1.3 megapixel digital camera. Twenty visual fields were obtained from each of the adenohypophyseal lateral wings and 20 from the middle portion in each analyzed case, with 60 visual fields in total per each analyzed case. Image analysis was accomplished using the ImageJ system (https://imagej.nih.gov/ij/).

*Astereological analysis*

Our astereological analysis of gonadotropic LH cells included the measurement of their area (ALH), perimeter (BLH) and Feret's diameter (DfLH). Additionally, for each of the selected somatotropic LH cells, we measured the parameters of area (ANLH), perimeter (BNLH) and Feret's diameter (DfnLH) of the nucleus. The nuclear-cytoplasmic ratio, as the parameter of metabolic activity and functional status of somatotropic LH cells ((N/C)LH), was calculated as the ratio of nuclear area and cytoplasmic area, with the cytoplasmic area obtained as the difference of the area of these cells and area of their nuclei. The measurement of 60 gonadotropic LH cells was performed for each of the analyzed cases.

*Stereological analysis*

Stereological analysis was performed using the multipurpose test system M168 (d = 17.88 μm, a = 15.49 $\mu m^2$, AT = 2601.54 $\mu m^2$, LT = 1501.92 μm), placed over the analyzed digital image of histological sections. Volume density of gonadotropic LH cells (VVLH) was obtained as the ratio of the number of dots in the test system which hit immunopositive cells (PF) and the total number of dots in the system (PT = 168) (13), per each analyzed field.

The values of area, perimeter and Feret's diameter, then of nuclear area, perimeter and Feret's diameter, nuclear-cytoplasmic ratio and volume density of LH cells per each analyzed case were obtained as the average of values for all measured visual fields.

**Statistical analysis**

Statistical analysis was performed using the SPSS statistical software package (version 16). The correlation of age with the measured morphometric parameters was evaluated by the calculation of linear correlation and linear regression.

A more precise dynamics of the values of morphometric parameters for the age groups was analyzed using One Way ANOVA and Tukey-Kramer *post hoc* test.

The t-test was used to establish statistical significance of the differences between two dependent samples.

**Results**

*Histological analysis*

Histological analysis of adenohypophyseal gonadotropic LH cells during ageing involved the analysis of immunohistochemically stained tissue sections of the gland.

In younger cases, gonadotropic LH cells were dispersed in the lateral wings of adenohypophysis, while in the mucoid, wedge-shaped portion of the gland these cells were localized within the acinar structures, the number of which varied between cases. Gonadotropic LH cells were in such cases oval or polygonal and with centrally or (more often) eccentrically positioned immunonegative blue-stained euchromatic nucleus. Their cytoplasm was immunopositive, brown-stained, with prominent granular appearance due to the presence of numerous secretory granules (Figure 1).

In older cases, except for the interstitial fibrosis of variable degree and consequential reduced presence of blood vessels, the distribution and presence of LH cells did not differ significantly from that in younger cases. Gonadotropic cells were larger, more often round, and with eccentrically positioned, smaller, hyperchromatic, immunonegative nuclei compared to younger cases. Immunopositivity reaction was similar to that of the same gonadotropes in younger cases (Figure 2).

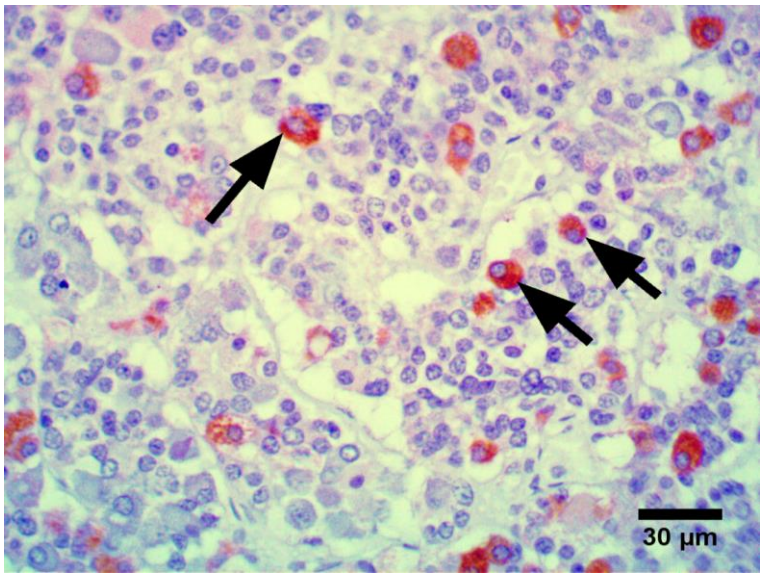



**Figure 1.** Tissue section from a 41-year old man; immunopositive LH cells with eccentric or centrally positioned euchromatic nucleus (arrows); anti-LH antibody; PAP, x40

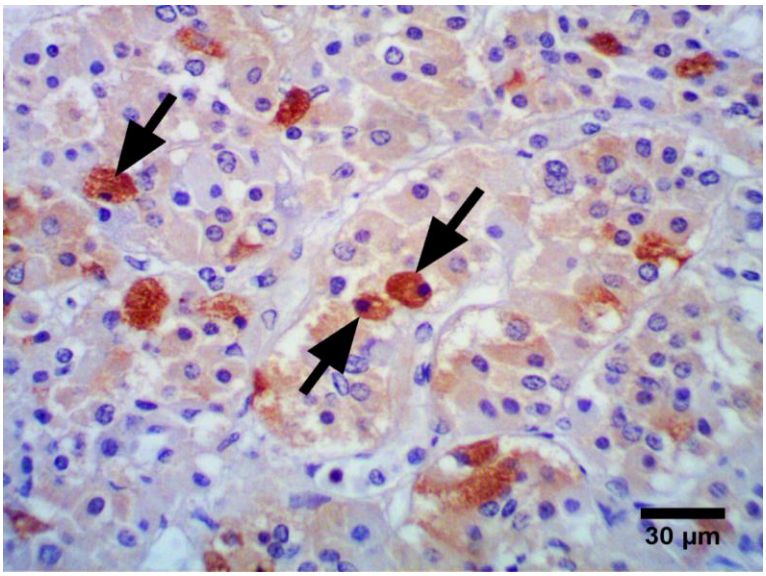



**Figure 2.** Tissue section of an 87-year old man; immunopositive LH cells of the adenohypophysis; large immunopositive LH cells with small, eccentrically positioned hyperchromatic nuclei (arrows); anti-LH antibody; PAP, x40

*Morphometric analysis*

The results of morphometric analysis of adenohypophyseal LH immunoreactive cells are presented in Table 1.

The case number 1 was excluded from further morphometric analysis due to a significant age difference compared to the other group 1 cases.

*Correlation analysis* indicated the presence of a statistically significant correlation between age and astereological parameters (average area, perimeter and Feret's diameter) and nuclear-cytoplasmic ratio of LH immunoreactive cells of the adenohypophysis in analyzed cases (Table 2).

**Table 1.** Results of morphometric analysis of adenohypophyseal LH immunoreactive cells

| Case | Age | Group | $A_{LH}$ ($\mu m^2$) | $B_{LH}$ ($\mu m$) | $D_{FLH}$ ($\mu m$) | $A_{NLH}$ ($\mu m^2$) | $B_{NLH}$ ($\mu m$) | $D_{FNLH}$ ($\mu m$) | $(N/C)_{LH}$ | $V_{VLH}$ (%) | $V_H$ ($cm^3$) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 22 | I | 123.26 | 41.16 | 14.94 | 20.35 | 16.62 | 5.97 | 0.20 | 2.90 | 0.7 |
| 2 | 41 | I | 107.60 | 38.57 | 13.84 | 22.16 | 17.56 | 6.29 | 0.26 | 3.12 | 0.5 |
| 3 | 45 | I | 136.72 | 43.83 | 15.63 | 24.37 | 17.99 | 6.32 | 0.22 | 3.31 | 0.4 |
| 4 | 48 | I | 109.52 | 38.92 | 13.83 | 20.60 | 16.82 | 6.09 | 0.23 | 3.45 | 0.7 |
| 5 | 48 | I | 142.16 | 44.87 | 16.18 | 28.55 | 19.67 | 7.17 | 0.25 | 3.61 | 0.7 |
| 6 | 57 | II | 131.29 | 42.64 | 15.50 | 23.54 | 17.89 | 6.45 | 0.22 | 4.13 | 0.25 |
| 7 | 61 | II | 121.01 | 40.54 | 14.57 | 24.18 | 17.92 | 6.28 | 0.25 | 8.04 | 0.3 |
| 8 | 65 | II | 147.83 | 45.66 | 16.37 | 27.37 | 19.28 | 6.96 | 0.23 | 4.35 | 0.4 |
| 9 | 65 | II | 138.10 | 43.29 | 15.50 | 20.58 | 16.68 | 5.94 | 0.18 | 3.00 | 0.4 |
| 10 | 66 | II | 156.08 | 45.90 | 16.55 | 30.12 | 20.10 | 7.23 | 0.24 | 8.23 | 0.5 |
| 11 | 76 | III | 165.11 | 50.00 | 18.51 | 26.25 | 19.12 | 6.93 | 0.19 | 7.70 | 0.8 |
| 12 | 76 | III | 123.46 | 42.41 | 15.82 | 19.97 | 16.73 | 6.21 | 0.19 | 2.54 | 0.4 |
| 13 | 77 | III | 161.76 | 48.14 | 17.76 | 27.55 | 19.64 | 7.26 | 0.21 | 5.54 | 0.6 |
| 14 | 78 | III | 129.99 | 43.13 | 15.65 | 23.46 | 17.82 | 6.42 | 0.22 | 1.92 | 0.4 |
| 15 | 87 | III | 168.33 | 49.03 | 17.73 | 21.55 | 17.41 | 6.32 | 0.15 | 4.82 | 0.4 |

**Table 2.** Correlation matrix of age and values of morphometric parameters of LH immunoreactive cells of the adenohypophysis in analyzed cases

| Parameter | | $A_{LH}$ | $B_{LH}$ | $D_{FLH}$ | $A_{NLH}$ | $B_{NLH}$ | $D_{FNLH}$ | $(N/C)_{LH}$ | $V_{VLH}$ |
|---|---|---|---|---|---|---|---|---|---|
| Age | R | 0.92 | 0.92 | 0.92 | 0.20 | 0.27 | 0.35 | -0.86 | 0.25 |
| | p | < 0.001 | < 0.001 | < 0.001 | 0.49 | 0.36 | 0.22 | < 0.001 | 0.38 |
| | N | 14 | 14 | 14 | 14 | 14 | 14 | 14 | 14 |

Linear regression analysis was aimed at evaluation of the association between astereological parameters (average area, perimeter and Feret's diameter) of adenohypophyseal gonadotropic cells and age of the analyzed cases (Table 3). The results demonstrated that the age of analyzed cases was a statistically significant predictor of area ($(F(1,12) = 68.59$, $p < 0.001$), perimeter ($F(1,12) = 63.37$, $p < 0.001$) and Feret's diameter ($F(1,12) = 62.67$, $p < 0.001$), which can be represented by the following three models: ALH = 28.83 + Age x 1.79, BLH = 24.03 + Age x 0.32 and DFLH = 8.04 + Age x 0.13. The average area, perimeter and Feret's diameter of gonadotropic cells of the adenohypophysis statistically significantly increased during the process of ageing, and age could be held accountable for 85% of total variance of area ($R^2 = 0.85$) (Graph 1), 84% of total variance of perimeter ($R^2 = 0.84$) and 84% of variance of Feret's diameter ($R^2 = 0.84$) (Graph 2), and in all three cases represents a large magnitude effect.

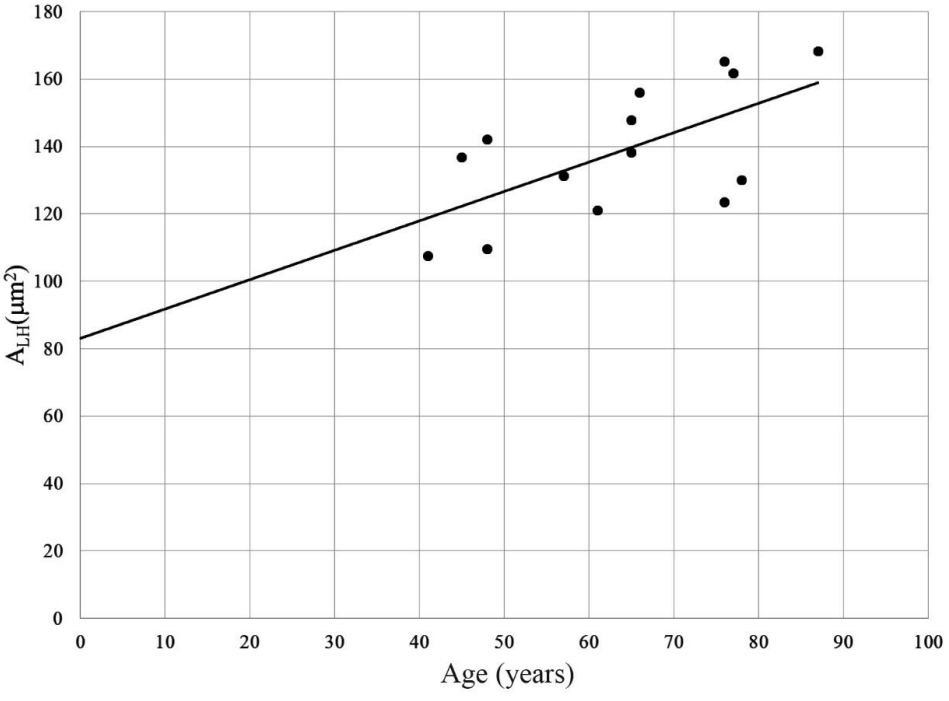
In contrast to the above, age was not a statistically significant predictor when astereological parameters of the nuclei of gonadotropic adenohypophyseal cells were concerned (area, perimeter and Feret's diameter) in the examined cases ($p > 0.05$) (Table 2).

Age represented a statistically significant predictor of average nuclear-cytoplasmic ratio of adenohypophyseal gonadotropic cells ($F(1,12) = 32.53$, $p < 0.001$) (Table 3). This ratio could be identified using the following model: $(N/C)LH = 0.384 - Age \times 0.003$, which meant that the values of the nuclear-cytoplasmic ratio statistically significantly declined during ageing (Graph 3). Age was accountable for 73% of total variance of this parameter ($R^2 = 0.73$), which was a large magnitude effect.

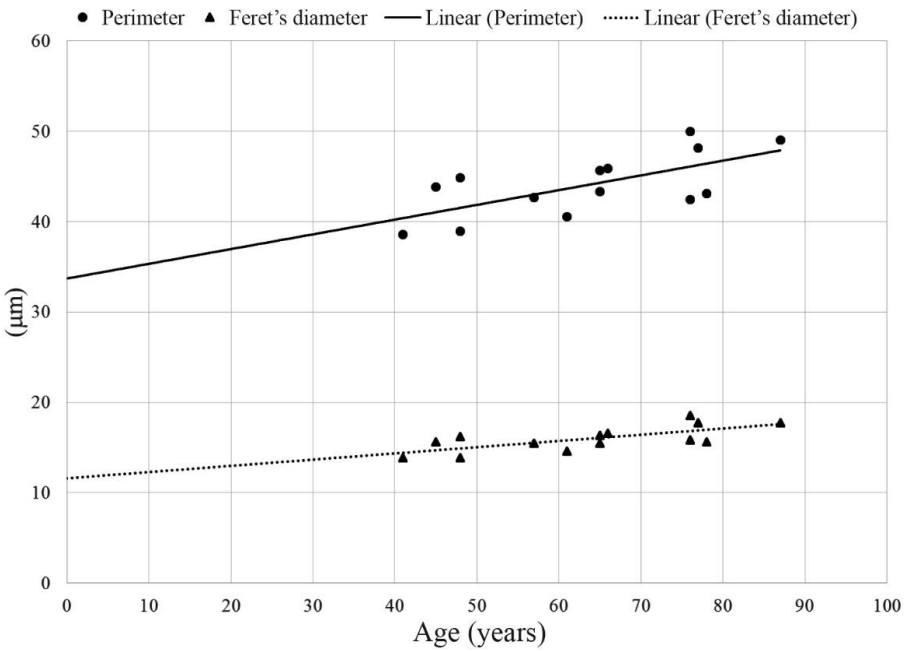
Age did not represent a statistically significant predictor of volume density of adenohypophyseal gonadotropic cells in the same cases ($p > 0.05$) (Table 2).

**Table 3.** Results of bivariate linear regression analysis of age as an independent, and analyzed morphometric parameters of adenohypophyseal LH immunoreactive cells as the dependent variables

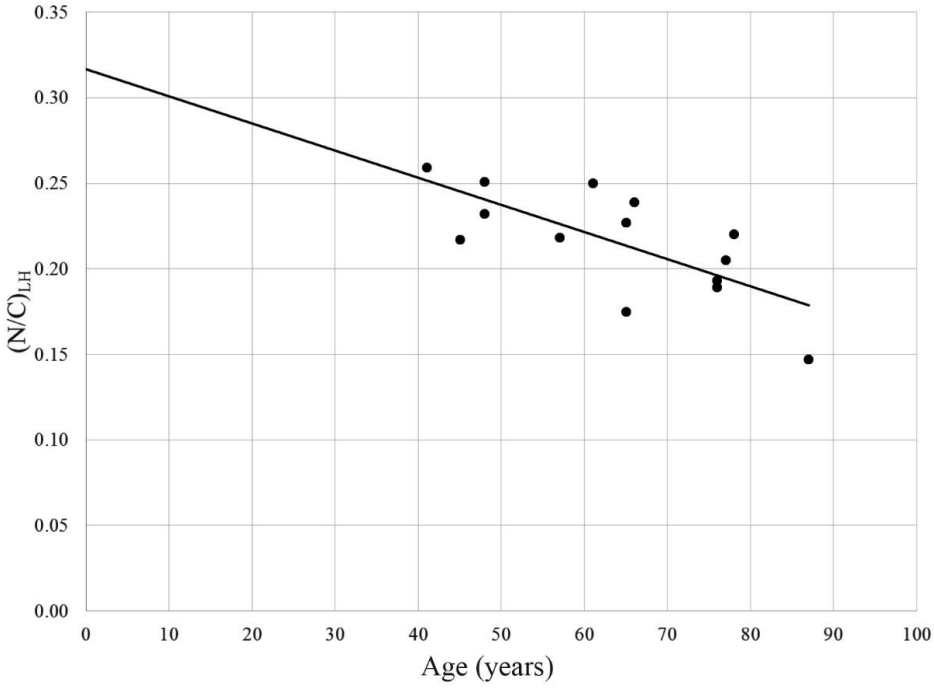
| $A_{LH}$ | | | | | |
|---|---|---|---|---|---|
| Variable | B | SEB | β | t | p |
| Constant | 28.83 | 14.03 | | 2.05 | 0.062 |
| Age | 1.79 | 0.22 | 0.92 | 8.28 | < 0.001 |
| $R^2 = 0.85$; $F(1,12) = 68.59$, $p < 0.001$; Model: $A_{LH} = 28.83 + Age \times 1.79$ | | | | | |
| $B_{LH}$ | | | | | |
| Variable | B | SEB | β | t | p |
| Constant | 24.03 | 2.64 | | 9.09 | < 0.001 |
| Age | 0.32 | 0.04 | 0.92 | 7.96 | < 0.001 |
| $R^2 = 0.84$; $F(1,12) = 63.37$, $p < 0.001$; Model: $B_{LH} = 24.03 + Age \times 0.32$ | | | | | |
| $D_{FLH}$ | | | | | |
| Variable | B | SEB | β | t | p |
| Constant | 8.04 | 1.05 | | 7.67 | < 0.001 |
| Age | 0.13 | 0.02 | 0.92 | 7.92 | < 0.001 |
| $R^2 = 0.84$; $F(1,12) = 62.67$, $p < 0.001$; Model: $D_{FLH} = 8.04 + Age \times 0.13$ | | | | | |
| $(N/C)_{LH}$ | | | | | |
| Variable | B | SEB | β | t | p |
| Constant | 0.384 | 0.031 | | 12.511 | < 0.001 |
| Age | -0.003 | 0.0005 | -0.8547 | -5.7036 | < 0.001 |
| $R^2 = 0.73$; $F(1,12) = 32.53$, $p < 0.001$; Model: $(N/C)_{LH} = 0.384 - Age \times 0.003$ | | | | | |




**Graph 1.** Correlation between age and area of adenohypophyseal LH immunoreactive cells in the examined cases




**Graph 2.** Correlation between age and perimeter, i.e. Feret's diameter, of adenohypophyseal LH immunoreactive cells in the examined cases



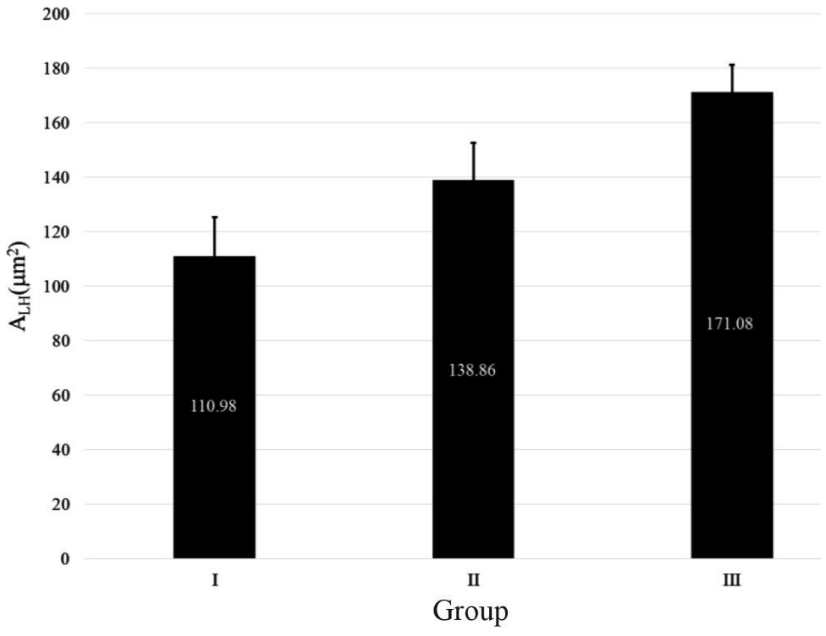

**Graph 3.** Correlation between age and nuclear-cytoplasmic ratio of adenohypophyseal LH immunoreactive cells in the examined cases

A more detailed dynamics of age-related changes of the average values of morphometric parameters of adenohypophyseal gonadotropic cells was evaluated using the One Way ANOVA test (Table 4).

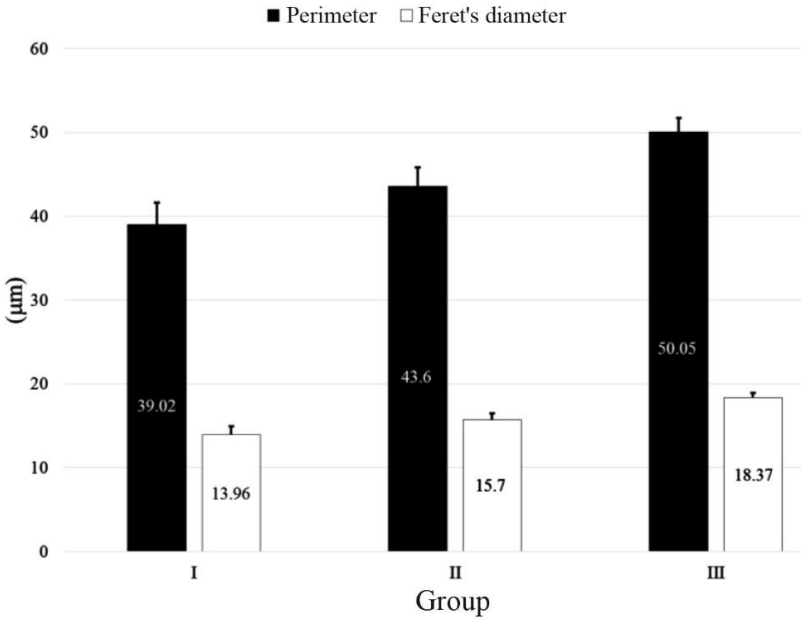
Average area ($F(2,11) = 24.88$, $p < 0.001$), then average perimeter ($F(2,11) = 29.82$, $p < 0.001$) and average Feret's diameter ($F(2,11) = 37.61$, $p < 0.001$) of adenohypophyseal gonadotropic cells statistically significantly increased during the process of ageing (Graphs 4 and 5).

**Table 4.** Results of univariate ANOVA test involving the average values of morphometric parameters of adenohypophyseal LH immunoreactive cells in the analyzed age groups

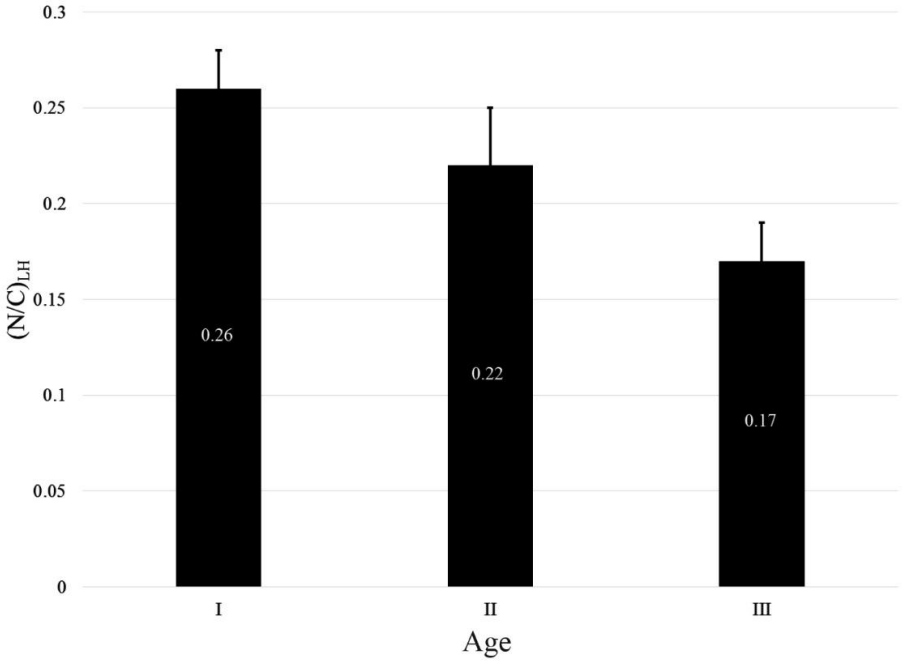
| Parameter | Group | N | Average | SD | SE | 95% CI | | Tukey post hoc test |
|---|---|---|---|---|---|---|---|---|
| | | | | | | LB | UB | |
| $A_{LH}$ (μm²) | I | 4 | 110.98 | 14.41 | 7.20 | 88.06 | 133.9 | a, b |
| | II | 5 | 138.86 | 13.72 | 6.14 | 121.82 | 155.9 | a, c |
| | III | 5 | 171.08 | 10.25 | 4.58 | 158.36 | 183.81 | b, c |
| ANOVA | F (2,11) =24.88, p < 0.001 | | | | | | | |
| $B_{LH}$ (μm) | I | 4 | 39.02 | 2.62 | 1.31 | 34.85 | 43.19 | a, b |
| | II | 5 | 43.60 | 2.23 | 1.00 | 40.83 | 46.38 | a, c |
| | III | 5 | 50.05 | 1.64 | 0.73 | 48.01 | 52.08 | b, c |
| ANOVA | F (2,11) = 29.82, p < 0.001 | | | | | | | |
| $D_{FLH}$ (μm) | I | 4 | 13.96 | 0.95 | 0.48 | 12.45 | 15.48 | a, b |
| | II | 5 | 15.70 | 0.80 | 0.36 | 14.71 | 16.69 | a, c |
| | III | 5 | 18.37 | 0.57 | 0.25 | 17.66 | 19.08 | b, c |
| ANOVA | F (2,11) = 37.61, p < 0.001 | | | | | | | |
| $A_{NLH}$ (μm²) | I | 4 | 22.87 | 3.67 | 1.83 | 17.04 | 28.71 | / |
| | II | 5 | 25.16 | 3.68 | 1.64 | 20.60 | 29.72 | / |
| | III | 5 | 24.37 | 2.89 | 1.29 | 20.78 | 27.96 | / |
| ANOVA | F (2,11) = 0.51, p = 0.62 | | | | | | | |
| $B_{NLH}$ (μm) | I | 4 | 17.50 | 1.22 | 0.61 | 15.55 | 19.45 | / |
| | II | 5 | 18.37 | 1.33 | 0.60 | 16.72 | 20.03 | / |
| | III | 5 | 18.27 | 1.27 | 0.57 | 16.69 | 19.84 | / |
| ANOVA | F (2,11) = 0.60, p = 0.57 | | | | | | | |
| $D_{FNLH}$ (μm) | I | 4 | 6.25 | 0.45 | 0.22 | 5.54 | 6.96 | / |
| | II | 5 | 6.57 | 0.52 | 0.23 | 5.93 | 7.22 | / |
| | III | 5 | 6.65 | 0.50 | 0.23 | 6.02 | 7.27 | / |
| ANOVA | F (2,11) = 0.78, p = 0.48 | | | | | | | |
| $(N/C)_{LH}$ | I | 4 | 0.26 | 0.02 | 0.01 | 0.23 | 0.29 | b |
| | II | 5 | 0.22 | 0.03 | 0.01 | 0.19 | 0.26 | c |
| | III | 5 | 0.17 | 0.02 | 0.01 | 0.14 | 0.20 | b, c |
| ANOVA | F (2,11) = 16.26, p = 0.001 | | | | | | | |
| $V_{VLH}$ (%) | I | 4 | 3.45 | 0.23 | 0.12 | 3.07 | 3.82 | / |
| | II | 5 | 5.55 | 2.42 | 1.08 | 2.55 | 8.55 | / |
| | III | 5 | 4.73 | 2.06 | 0.92 | 2.17 | 7.29 | / |
| ANOVA | F (2,11) = 1.33, p = 0.30 | | | | | | | |
| a - I : II, p < 0.05; b - I : III, p < 0.05; c - II : III, p < 0.05 | | | | | | | | |




**Graph 4.** Average area of adenohypophyseal LH immunoreactive cells in the analyzed age groups




**Graph 5.** Average perimeter and average Feret's diameter of adenohypophyseal LH immunoreactive cells in the analyzed age groups




**Graph 6.** Average nuclear-cytoplasmic ratio of adenohypophyseal LH immunoreactive cells in the analyzed age groups

The *post hoc* Tukey-Kramer test indicated that the above parameters showed an identical trend during ageing, with the values in the III age group being statistically significantly greater compared to the II and I age group ($p < 0.05$). Average values of these parameters in the II age group were also statistically significantly greater compared to the I age group ($p < 0.05$) (Table 4).

Average nuclear-cytoplasmic ratio of adenohypophyseal gonadotropic cells was statistically significantly different between I and III and between II and III analyzed age group, while the difference between groups I and II did not reach statistical significance ($F(2,11) = 16.26$, $p = 0.001$). Nuclear-cytoplasmic ratio decreased from I to III age group, but the decrease was statistically significant in age

group III, suggesting a functional decline of gonadotropic cells with ageing, which was most conspicuous after 70 years of life (Table 4, Graph 6).

## Discussion

In recent decades, more and more authors have considered the endocrine, i.e. neuroendocrine system, responsible for numerous changes occurring in the body with growing age. In particular, in the endocrine, similar to other tissues in the body, some structural changes occur with ageing, which have as a consequence respective functional abnormalities. These are reflected in a disturbed synthesis and secretion of hormones created in particular glands. The pattern of hormonal secretion at different levels (hypothalamus and peripheral gland tissues) varies considerably. With ageing, the levels of some hormones increase, the levels of others decrease, while the levels of some of them do not change significantly.

Ageing of the hypophysis, from the functional point of view, manifests with a decline of its secretory activity, above all by decreasing levels of growth hormone, gonadotropic hormones, prolactin and thyroid-stimulating hormone in the blood. These changes lead to so called ageing diseases, which predominantly affect the target-organs of these hormones (14-16). The above adenophyseal changes are most commonly only a link in the chain of changes which involve the hypothalamic-adenohypophyseal axes (hypothalamic-somatotropic (HSO or GH), hypothalamic-pituitary-gonadal (HPG) axis, hypothalamic-pituitary-adrenal (HPA) axis, and hypothalamic-pituitary-thyroid axis). These functional changes stem from the structural changes at different levels of these axes.

Although adenohypophysis has an important role in the maintenance of overall homeostasis in the organism and is characterized by progressive functional decline with ageing, the knowledge of the accompanying structural changes is not sufficient, especially regarding the quantity and dynamics of these changes.

In the available literature on the subject older studies tend to prevail; these have been performed mostly on experimental animals and utilizing semi-quantitative methodologies (17-20).

The performed quantitative immunohistochemical and electron-microscopy studies used as their material mostly the hypophyses obtained from experimental animals, especially rats (21-26).

Danilova et al. (27) have analyzed ultrastructural changes in all types of adenohypophyseal endocrine cells in rats during ageing and identified changes in almost all cellular organelles: nuclei, Golgi apparatus, endoplasmatic reticulum, mitochondria and secretory granules, reporting also the presence of numerous lysosomal bodies and fat vacuoles. Older animals had also a greater number of atrophic cells, which indicated their progressive degeneration with growing age. In apparently morphologically normal cells of these animals the signs of ultrastructural changes were seen, which indicated their increased functional activity. These changes were aimed at preservation of the levels of hormonal secretion, but in the long term they resulted in a depletion of the functional reserves of adenohypophyseal endocrine cells in older animals. It was clear that these changes in an ageing hypophysis could be viewed as an attempt of compensatory structural reorganization which would preserve normal gland functioning and help in the maintenance of basic homeostatic mechanisms in the ageing organism.

Analyzing rat hypophyses with quantitative immunohistochemistry methods, Console et al. (28) reported a significant decrease in the number, volume and surface density, area and perimeter of somatotropic cells with ageing. They also noticed a reduced number of other adenohypophyseal cell types, such as gonadotropic cells (FSH and LH), but the reduction was not associated with a corresponding decline of LH and FSH levels.

In general, morphometric studies about the cellular composition of adenohypophysis are relatively rare, especially those dealing with human adenohypophysis in the process of ageing.

Morphometric studies of age-related changes of gonadotropes are also rare. In contrast to other hormonal cells of the adenohypophysis which store a single hormone within special cell types, numerous gonadotropes show immunoreactivity to both LH and FSH. This additionally complicates their quantification in the process of ageing, since in theses cells, FSH and LH expression may vary significantly in different phases of postnatal life.

Meeran et al. (29) evaluated the changes in gonadotrope subtypes in puberty and adolescence of rhesus monkeys and concluded that the number of gonadotropes increased in adults compared to juvenile rhesus monkeys, mostly due to increased numbers of LH and bihormonal cells.

Console et al. (22) performed a morphometric analysis of gonadotropes from young, old and very old rats and established a progressive age-associated reduction of cellular density, volume density and surface density of LH cells. On the other hand, area and perimeter of gonadotropes increased in both young and old animals, but were drastically reduced in oldest animals. Basal levels of serum LH and FSH showed a tendency identical to that established for surface and perimeter of gonadotropes.

Kurosumi et al. (30) classified rat gonadotropes according to the size of their secretory granules into two types: type I, which contained both small and large secretory granules and expressed both FSH and LH; and type II, which contained only small granules, immunopositive to LH. They found that in young adult rats type I gonadotropes are more common compared to type II ones. In middle-aged rats, type I gonadotropes were predominant, but the expression of FSH in most of them was rather weak. In older rats, the ratio of type I and II was reversed compared to young and middle-aged rats, i.e. type II gonadotropes were more common. Kurosumi et al. finally concluded that with advancing age LH cells became predominant in the hypophysis of male rats, which was associated with a considerable decline in FSH and a slow depletion of LH contents.

In our study, the density of LH cells did not change significantly with ageing. However, in contrast to the findings of Console et al. (22), the area of LH cells increased significantly even after 70 years of age. In contrast to the factor of area, nuclear-cytoplasmic ratio gradually decreased with advancing age, and the decrease became significant after 70 years of life. Therefore, our findings of relatively stable density of LH cells and their larger size with advancing age, together with age-related increased production of LH reported by some authors (31), led us to the conclusion that in men these cells probably developed hypertrophy (32) with ageing. This may have indicated their exposure to increased functional stress with time, probably due to disturbed testosterone feedback loop or excessive stimulation by some extra- or intrapituitary factors. Gradual decline of nuclear-cytoplasmic ratio of LH cells represents the sign of their functional decline, which becomes significant after 70 years of life, most probably due to their exhaustion caused by long-lasting hypertrophy. This agrees with the results obtained by Kurosumi et al. (30), who noticed gradual depletion of LH contents with ageing.

However, with the exception of a greater irregularity and reduced amplitude of the LH pulse, as we have described, the prevailing opinion in the scientific community is that LH secretion does not only change insignificantly, but that in men it even slightly increases with advancing age (33-35). This is inconsistent with the findings of reduced GnRH secretion and the assumption that disturbed testosterone feedback loop at the level of hypothesis and hypothalamus releases LH secretion during ageing (36).

## Conclusion

Based on the investigations performed so far, a conclusion may be drawn that there is a relatively stable density and increased size of adenohypophyseal gonadotropic LH cells during ageing in men, which probably reflects their hypertrophy in order to maintain normal hormonal secretion, with the consequence of their functional decline.

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# IMUNOHISTOHEMIJSKO I MORFOMETRIJSKO PROUČAVANJE GONADOTROPNIH ĆELIJA ADENOHIPOFIZE KADAVERA MUŠKOG POLA RAZLIČITE STAROSTI

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Podaci iz literature ukazuju na opadanje funkcije gonadotropnih ćelija sa starenjem, dok su podaci koji se odnose na strukturne promene istih oskudni. Cilj ovog rada bio je da se primenom imunohistohemijske i morfometrijske metode, detektuju i kvantifikuju promene kod humanih gonadotropnih ćelija adenohipofize kadavera muškog pola različitog uzrasta. Materijal je predstavljalo tkivo adenohipofize 14 kadavera muškog pola različite životne dobi, počev od četvrte decenije. Tkivni preseci adenohipofize standardno su histološki obrađivani i bojeni imunohistohemijski monoklonalnim anti–LH antitelom za detekciju gonadotropnih LH ćelija. Digitalne slike vidnih polja imunohistohemijski obrađenih preseka adenohipofize, zatim su morfometrijski analizirane pomoću Image J sistema. Statistička analiza vršena je pomoću SPSS statističkog paketa. Rezultati morfometrijske analize pokazali su to da se zapreminska gustina LH ćelija nije značajno menjala sa godinama, dok su njihova area, perimetar i Feretov dijametar statistički značajno rasli. Nuklearni morfometrijski parametri nisu se značajno menjali, a nukleocitoplazmatski odnos LH ćelija opadao je sa starenjem, pri čemu je taj pad bio statistički značajan kod slučajeva starijih od 70 godina. Na osnovu dobijenih rezultata, može se zaključiti da se tokom starenja gustina LH ćelija ne menja značajno, već da one hipertrofiraju, u cilju održavanja normalne sekrecije hormona. Dugotrajna hipertrofija ovih ćelija na kraju dovodi do njihovog funkcionalnog pada, koji postaje značajan nakon 70. godine života.

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***Ključne reči:*** *luteinizirajuće gonadotropne ćelije, adenohipofiza, imunohistohemija, morfometrija, starenje*