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LIPOFUSCIN ACCUMULATION IN PURKINJE CELLS AS A MARKER OF CEREBELLUM AGING

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Lipofuscin has been known as an "age pigment". The aim of this study was to determine the patterns of lipofuscin accumulation in cerebellum Purkinje cells during the process of aging, as well as the Purkinje cell volume density. The cerebellar tissue samples taken from cadavers were divided into four age groups. Stained tissue samples were analyzed for lipofuscin distribution patterns. Morphometric analysis included the quantification of Purkinje cell volume density. Purkinje cells of neocerebellum predominantly did not contain lipofuscin inclusions, or only discrete diffusely distributed lipofuscin granules were present after the age of 50. Even after 70 years of age, not many cells contained lipofuscin. Besides, no significant changes in the volume density of Purkinje cells were observed. Physiological changes in the elderly associated with cerebellum function impairment may be related to the increased lipofuscinogenesis in Purkinje cells after the age of 50, but especially after the age of 70 years.

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Key words: *cerebellum, lipofuscin, aging, morphometry*

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Introduction

Lipofusicin (LF) has been known as an "age pigment" or a "hallmark of aging" since Knoeff described for the first time in 1886 the relation between LF and the aging process (1, 2). Lipofuscin accumulation in secondary lysosomes is a characteristic of postmitotic cells, such as neurons. Fluorescent yellow-brown electron dense pigment granules are formed out of by cross-linked lipids (20-50%) and protein residues (30-70%). The variability in the proportion of lipid and protein content is due to the fact that LF granules consist of the material that is not degraded and exocytosed by lysosomes, primarily after incomplete mitochondrial degradation (3). It accumulates in cells progressively with aging, even though some authors suggest that there is a possibility of its removal. Besides, various theories about aging are based on different processes that can be related to lipofuscinogenesis (1, 4).

Cerebellum is one of the most sensitive parts of the central nervous system to aging. There is a reduction in white matter mass by 26% (5). A progressive loss of Purkinje cells (PC) (6, 7) is accompanied with the decrease in their mean volume by 33%, but without changes on the nuclear level (5). Nucleus and other cell organel-les may be localized eccentrically. The most prominent changes are in the anterior lobe involved in motor control (5). The presence of amyloid deposits, seen as PAS positive circular laminated glycoprotein corpuscules, were observed for the first time in PC of the cerebellum (3, 6). The accumulation of LF, associated with aging and neurodegenerative diseases, involves the deficiency in ceramide biosynthesis via ceramide synthase 1. As a consequence, along with shortened dendritic arbours, PC death occurs (8). The overload of lysosomes and their decreased hydrolytic capacity lead to LF formation. Even though there may not be a direct relationship between lipofucinognesis and PC ele-ctrophysiology, there is an increase in the number of aberrant slow-firing cells with aging (9).

The aim of this study was to determine the patterns of LF accumulation in cerebellum PC during the process of aging. Additionally, the PC volume density was determined.

Material and methods

This study was approved by the Ethics Committee of the University of Niš, Faculty of Medi-

cine. The study subjects were treated in accordance with the Declaration of Helsinki and for each of them, an informed consent for being included in the study was obtained.

Neocerebellar tissue samples from the inferior side of the left hemisphere posterior lobe, sized 1 cm^2, were taken from cadavers, and routinely autopsied at the Department of Forensic medicine, Faculty of Medicine, University of Niš, Serbia. The sampling of cadaveric tissue was authorized by family members of the deceased and the Ethics Committee of Faculty of Medicine. Forty cadavers aged 17-84 years, were divided into four age groups: Group I (17-29 years), Group II (30-49 years), Group III (50-69 years) and Group IV (over 70 years). None of the human cadavers suffered from brain damage, neurological or psychiatric disease. Not more than 12h passed between the time of death and sampling.

Tissue samples were fixated promptly after being taken, in 10% neutral formalin, for not more than 24h, and then embedded in paraplast. Histological sections were stained with haematoxyllin-eozine (HE), Long Ziehl Neelsen (LZN), periodic acid Schiff (PAS) and silver impregnated by Masson-Fontani (SMF). Each staining method colors LF differently: HE – yellow-brown, LZN and PAS – red, SMF – dark brown-black.

Morphometric analysis was performed on the digital pictures of stained tissue sections, taken using microscope Leica DM2500 and photocamera Leica DFC420 mounted on the third microscope ocular. Each section was divided chessboard-like into fi-elds of vision and every second was photographed under lens magnification of 400x. Morphometric analysis was performed with Image J software after spatial calibration. After analyzing a test sample, we determined a minimum of 20 test fields of vision to be morphometrically analy-zed, in order to obtain satisfactory accuracy, with standard error of less than 5%. Purkinje cell volu-me density (per 1 mm^2) was determined for each sample.

All data analyses were performed by Statistical Package for Social Sciences (SPSS 16.0; Chicago, IL, USA). Quantitative data were presented as mean ± standard deviation. The significance of differences between age groups was tested by ANOVA with Tukey post-hoc test. A p-value <0.05 was considered to be a measure of statistical significance.

Results

Patterns of lipofuscin distribution

The presence of LF was analyzed in PC of the cerebellar cortex after four methods of staining for LF identification. In the pictures taken at small magnification of 10x (Figure 1) stained with HE, PC were observed between molecular and granular layer. They were oval or pyriform cells, sized 35-65 μm, with eosinophilic cytoplasm rich in Nissl substance. The nucleus was large and strictly demarcated, with discrete chromatine fibrils. The hyperchromic nucleolus was located centrally.

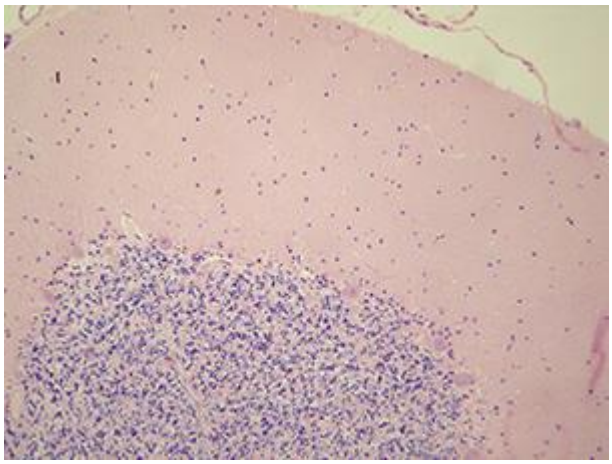

Figure 1. Cerebellar cortex with Purkinje cells (HE, 10x)

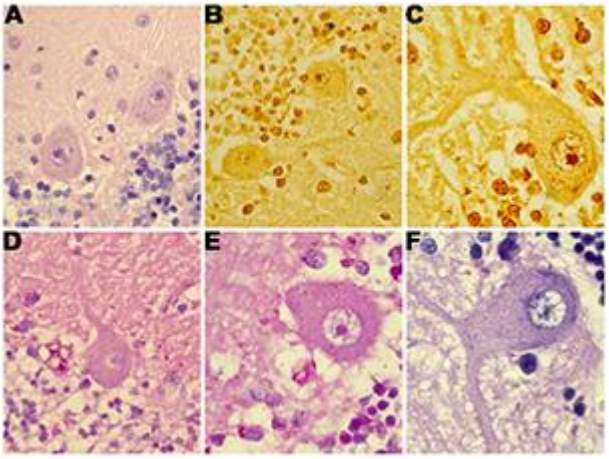

Figure 2. Purkinje cells without lipofusin inclusions in the age group I (A. HE, 40x; B. SMF, 40x; C. SMF, 100x; D. PAS, 40x; E. PAS, 100x; F. LZN, 100x)

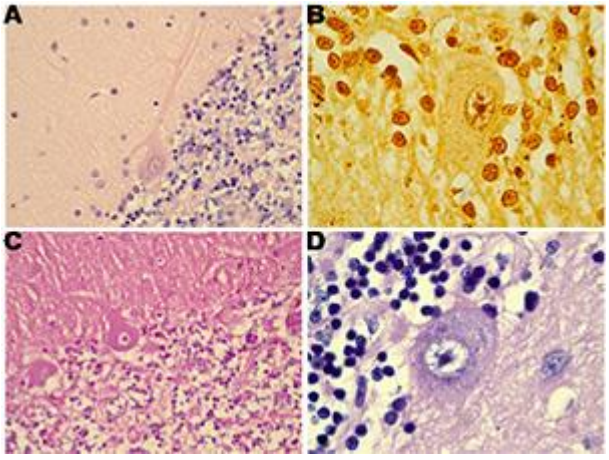

Figure 3. Purkinje cells without lipofusin inclusions in the age group II (A. HE, 40x; B. SMF, 100x; C. PAS, 40x; D. LZN, 100x)

Figure 2 represents the cerebellar tissue of

the first age group. Up to four dendrites could be seen on the apical pole of PC, pointing towards the surface of cerebellum and ending in the molecular layer. At the 100x magnification (Figure 2c), the dichotomous branching was observed. Neurites, with wide basis on the basal cell pole, were the single projections from the cerebellar cortex reaching ce-rebellar nuclei, transiting granular layer. As seen in Figures 2 and 3, no LF accumulation was seen in the first two age groups, i.e., until the age of 50.

In the third age group (50-69 years old specimens), the cells without LF still predominant (Figures 4a-c). Yellow-brown LF granules (HE), red (LZN and PAS) or dark brown-black (SMF) were observed in a small number of cells (Figures 4d-f). It was distributed diffusely in the cytoplasm. No other pattern of distribution was present, and there were no cells congested with LF inclusions.

After the age of 70 (IV age group), there was still a predomination of cells without LF pigment (Figures 5a-d). Sporadically, cells with diffuse LF granules were seen (Figures 5e-h). Besides, less fre-quently, LF was located solely on one of the PC poles, at the base of dendrites or neurites (Figure 6). In

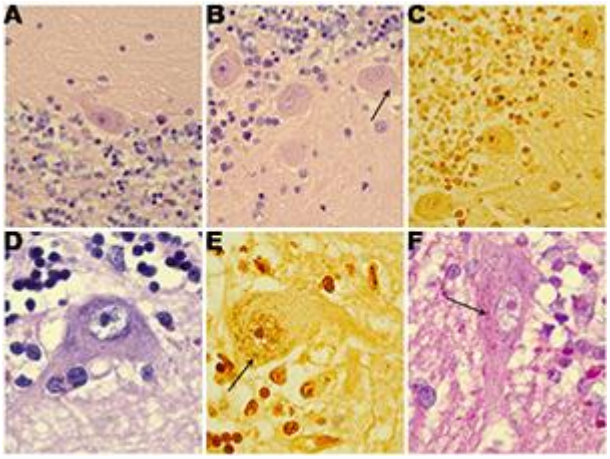

Figure 4. Purkinje cells in the age group III, lipofuscin inclusions are marked with arrows (A. HE, 40x; B. HE, 40x; C. SMF, 40x; D. LZN, 100x; E. SMF, 100x; F. PAS, 100x)

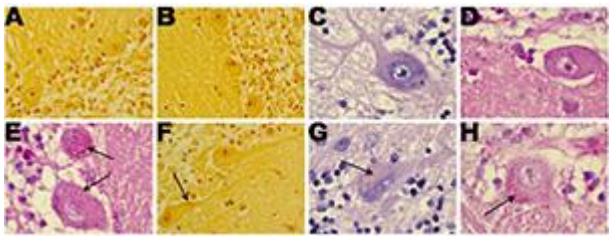

Figure 5. Purkinje cells in the age group IV, lipofuscin inclusions are marked with arrows (A. SMF, 40x; B. SMF, 40x; C. LZN, 100x; D. PAS, 100x; E. PAS, 100x; F. SMF, 40x; G. LZN, 100x; H. PAS, 100x)

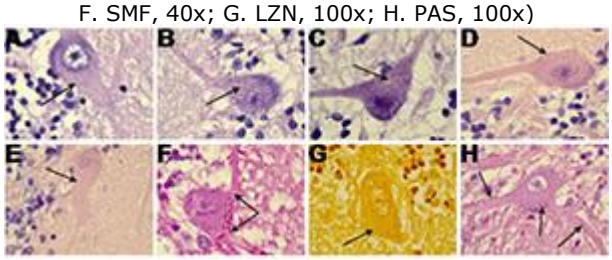

Figure 6. Lipofuscin in Purkinje cells extending into dendrites and neurites in the age group IV (A. LZN, 100x; B. LZN, 100x; C. LZN, 100x; D. HE, 100x; E. HE, 100x; F. PAS, 100x; G. SMF, 100x; H. HE, 100x)

the rest of the cytoplasm, it was either absent or only discrete diffuse granules were observed. Lipofuscin inclusions were detected along the dendrites and neurites, as well.

Morphometric analysis of lipofuscin distribution

Due to a low frequency of PC filled with LF in all four age groups, the variation in the cell proportion and volume density, of those with and without lipofuscin, could not be analyzed. Therefore, the morphometric analysis included solely PC volume density as the function of age (Figure 7). A statistically significant decrease in PC volume density was noted between age groups II and III ($p < 0.05$). Since other differences were not significant, this result may be due to chance.

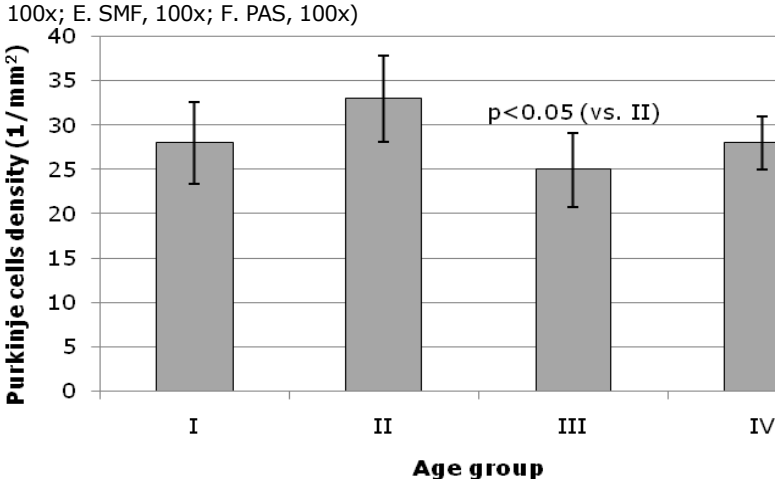

Figure 7. Purkinje cell volume density in function of age

Discussion

Lipofuscinogenesis is slower in the cerebellar cortex compared to other parts of the brain, such as the cerebral cortex and hippocampus (10). It has been shown to be more intense in the phylogenetically older structures of the central nervous sys-tem. Besides, the higher the activity of the brain structure, the more intense is lipofuscinogenesis (11). Therefore, as previously described, PC of neocerebellum predominantly did not contain LF inclusions, or only discrete diffusely distributed LF granules were present after the age of 50. Even after 70 years of age, not many cells contained LF. Besides, no significant changes in the volume den-sity of PC were observed.

In rats, at about the age of 20-30 months, the accumulation of LF starts, predominantly at the apical pole of soma. It is shown to be age-dependent (12, 13). Granules become larger and more concentrated around nucleus and at the basis of apical dendrite (13). Purkinje cells are more susceptible to age-associated LF accumulation and cell death than other cells in the cerebellar cortex. Initial diffuse presence of LF is replaced with large inclusions in the older specimens with polar localization in the dendritic region (14). Similar results have been found in other species (7). Lipofuscin deposits may be even found in basket or stellate cells (11). In humans, at the young age (2-6 years) no or few deposits of LF are observed. On the contra-ry, at the age beyond 80, LF granules are detected in PC, as well as large ectracellular deposits (15). In accordance with the literature data, our results show that more prominent accumulation of LF is seen after the age of 70, but without large depo-sitions. It was mainly distributed diffusely in the cytoplasm, or concentrated at the base or along the neuron projections. Lipofuscin granules are electron-dense, finely granular, sometimes with amorphous central material and surrounding membrane fragments. Compared to the other parts of the brain, they are much smaller in PC (11).

A significant PC loss is observed in rats 20 months old (12). In contrast to the previous studies, we did not find significant changes in the volume density of PC. This may be the result of both the decrease in the absolute cell number (6, 7) and the involution of cerebellar mass (5). After the age of 40-50, PC become more rounded, linear density decreases, as well as the quantity of Nissl substance. After 60 years, their diameter decreases in contrast to the increase in the diameter until the middle age (16, 17).

Age-associated changes in the cells of cerebellar cortex are associated with impaired motor function in the elderly. Age-related changes in PC may be related in higher degree to the cognitive dysfunction (17). Nevertheless, it is still not clear weather LF overload leads to functional impairment, or it is just the consequence of intense cell functioning (14). There is no clear relationship between RNA content and LF content in PC (15).

Conclusion

Based on the previously described results of our study, we may conclude that physiological changes in the elderly, associated with impaired cerebellum function, may be related to the increased lipofuscinogenesis in Purkinje cells after the age of 50, but especially after the age of 70 years.

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AKUMULACIJA LIPOFUSCINA U PURKINJEOVIM ĆELIJAMA KAO MARKER STARENJA MALOG MOZGA

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Lipofuscin je poznat kao "pigment starenja". Cilj ovog istraživanja bio je da se utvrde obrasci akumulacije lipofuscina u Purkinjeovim ćelijama malog mozga tokom procesa starenja, kao i da se odredi zapreminska gustina Purkinjeovih ćelija. Uzorci tkiva malog mozga uzeti sa kadavera podeljeni su u četiri starosne grupe. Nakon bojenja, analizirani su obrasci akumulacije lipofuscina. Morfometrijska analiza je podrazumevala određivanje zapreminske gustine Purkinjeovih ćelija. Purkinjeove ćelije neocerebeluma uglavnom nisu sadržale inkluzije lipofuscina, ili su bile prisutne diskretne, difuzno raspoređene granule lipofuscina u uzorcima starosti preko 50 godina. Čak ni nakon 70. godine života, u malom broju ćelija bile su prisutne akumulacije lipofuscina. Osim toga, nije bilo značajnih razlika u zapreminskoj gustini Purkinjeovih ćelija. Fiziološke promene kod starih osoba, udružene sa poremećenom funkcijom malog mozga, mogu biti posledica povećanog stvaranja lipofuscina u Purkinjeovim ćelijama nakon 50, a naročito nakon 70. godine života.

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Ključne reči: *mali mozak, lipofuscin, starenje, morfometrija*