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Changes of Neurons and Blood Vessels of Human Substantia Nigra in Aging- Morphometric Study

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ABSTRACT

Introduction: With aging, populations of dopaminergic neurons in the central nervous system show prominent pathological changes compared to other brain regions. Previous studies on substantia nigra were performed in cases of Parkinson's disease and in old age.

Aim of the Study: Since Parkinson's disease is a disorder associated with age, it is important to examine how the relationship between neurons and blood vessels is associated with normal aging.

Patients and Methods: Ten brainstems were sliced into three strata. Each stratum was sliced in semiserial sections and stained by Mallory method. Studied phases were neurons and blood vessels of substantia nigra. The analysis was conducted by camera "Leica EC3" under the 40x magnification of light microscope „Leica" DM 1000, using ImageJ software (version 1.42 e). Determined morphometric parameters of neurons and blood vessels were: volume and surface density, and absolute numbers per visual field. Statistical analysis was performed using SPSS software, version 16.0, using Student's t-test and Pearson correlation coefficient.

Results: Volume and surface density, and total number of neurons per visual field of substantia nigra significantly decreased with age, while the volume and surface density and absolute number of blood vessels per visual field significantly increased ($p < 0.05$).

Conclusion: Decrease in size and number of neurons occurs with aging, which is compensated by the increase of vascular network. This affects the supply of nutrients from the blood to neurons, as well as the availability of blood cells or toxic substances, but also the susceptibility to neuronal diseases.

Key words: Aging; substantia nigra; humans.

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Introduction

With aging, populations of dopaminergic neurons in the central nervous system show more prominent pathological changes compared to other regions of the brain. This is especially pronounced with the pars compacta of the substantia nigra. Buchman and associates have showed that one-third of elderly persons without clinically diagnosed Parkinson's disease (PD) have a moderate to significant loss of neurons in this part of the substantia nigra (SN).[1] Between the older and younger groups of patients, Rudow and associates found a difference in the number of neurons of 28.3%, or a loss of 4.35% for a period of 10 years.[2] This loss is lower compared to a loss of 48% till 60 years of age, found by McGeer and associates and 35% loss till 90 years, as reported by Mann and associates.[3,4]

In a stereological study of SN pars compacta, Ma and co-workers found a significant age-dependent reduction in the total number of pigmented neurons.[5] The study of Vaillancourt and associates provided the first in vivo evidence that the microstructural integrity of the dorsal part of SN depended on age, while this was not true for ventral SN.[6] In their study, Fearnley and Lees took a step further and found that the ventral and lateral part of the SN pars compacta during the aging process was relatively spared (a loss of 2.1% at ten years) compared to the dorsal part (6.9% per decade).[7] The authors concluded that pigmented nigral cells in the ventral part of the SN pars compacta were most affected by PD, while aging affected pigmented cells in the dorsal SN.

In terms of arteries, SN is supplied (going rostrally) by anteromedial and anterolateral perforant and penetrating branches of superior cerebellar artery, posterior cerebral artery, collicular artery, posterior medial choroid artery, posterior communicant artery, and anterior choroidal artery.[8-11] Indeed, anteromedial arteries are typical perforating, interpeduncular (thalamoperforating) arteries,[11] and only their lateral arteries supply the most medial part of SN. Anterolateral arteries, also known as peduncular arteries, supply the largest part of SN.[11] Perfusion branches of posterior cerebral arteries also supply SN.

Vascular lesions of SN are extremely rare because of a large number of neurons, but also because of the large number of arteries that supply SN from various sources. Barcia and colleagues used the stereological methods and noticed an increase in the number of neurons with positive expression of vascular endothelial growth factor (VEGF), and an increase in the number of blood vessels and their volume in the SN pars compacta in monkeys.[12] These changes do not only occur by the increase of blood vessels, but by neuromicroangiogenesis, and they can affect the supply of nutrients to neurons from the blood, as well as on the availability of blood cells or toxic substances and the susceptibility of neurons to PD.

Neovascularization occurs in the brain after various insults cause the neuron loss.[13] Neuropathological analysis showed that PD patients had an increased number of nuclei of endothelial cells,[14] which could be related to the increased number and density of blood vessels or changes in the thickness of the wall of blood vessels. Morphometric studies on animal tissues, in which the PD was induced, showed an increase in the area occupied by blood vessels by 25% in SN pars compacta.[15,16] Increased vascularization in Parkinsonism seems to be induced by loss of dopaminergic cells. Angiogenesis in the affected parenchyma could also be related to the high demands for metabolites by surviving neurons.

Aim of the Study

Previous morphometric studies of SN were performed in cases of PD and in old age. Since this disease is related to age, it is important to examine how the relationship between neurons and blood vessels is associated with normal aging.

Patients and Methods

The research was done with the permission of the Ethics Committee of the University Clinical Center of the Republic of Srpska, on 10 brains of adults without diagnosed neurological diseases. Using an ordinary autopsy technique, brains were extracted from the cranial cavity, and then immersed in a 10% formalin solution for fixation. In order to reach SN samples, brainstems were separated from forebrain, by cutting the brain mass at the level of the posterior edge of mammillary bodies and from the cerebellum, by cutting cerebellar pedicles. After fixation, brainstems were cut in 3 mm thick strata in the transverse plane (stratified sampling), going caudally from the level of: middle of the superior mesencephalic colliculus (5 samples) and the caudal border of the superior mesencephalic colliculus (5 samples). Obtained strata were used to make semiserials sections (5, 10, 15, 20), 4 μm thick, which were stained by the Mallory method. Control of proper verification of blood vessels was carried out by the immunohistochemical method of antigen factor VIII.

In objects with a complex structure, at the beginning of morphometric analysis, the place and significance of their individual components should be determined, as well as their mutual relations. This will create a hierarchical model of the object.[17] For this research, we built the hierarchical model of SN. Reference space in all cases was

SN. Studied phases were nerve cells and blood vessels of SN. Images of objects of the research were taken with the camera "Leica EC3" in RGB format, 24-bit resolution of 2048 x 1536 pixels, under a 40x magnification of light microscope „Leica" DM 1000. For the analysis, resulting images were processed in the Adobe Photoshop 7.0 using "Auto Color" image adjustments and "unsharp mask" filter. For morphometric analysis, the program for the analysis and processing of digital images ImageJ (version 1.42 e) was used. In sample selection procedure, we picked up every second field, and the sample sizes, i.e. the required number of measurements for each variable and for each group was determined according to the formula:[18]

$$n = (200/ y \times s / x)^2$$

n– number of visual fields that should be analyzed, x – mean of the orientation sample, s– standard deviation of the orientation sample, y– allowed mean tolerance. Calculated number n represents the number of tests fields which should be morphometrically analyzed with a 95 % confidence interval.

Prior to analysis, the spatial calibration was done, using images of object micrometer taken at the same magnification as samples in this research. On a 24-bit image of object micrometer for a given magnification (400x), we measured the distance between the two notches on the object micrometer (10 μm), using the "straight line" selection. Option "set scale" in the software menu was used to convert the values from pixels to microns. By selecting the option "global", obtained calibration was applied to all images analyzed in one occasion.

After calibration, the parameters of the test system A100 were determined. Based on those parameters and software option"grid" we formed the grid of test system A100. Basic parameters of this system were: the total number of test points 100, the length of a line of test system was 0.020386 mm, and the surface of the test area was 0.04156 mm².

After grid superimposing, such image was analyzed with the cell counting tool ("cell counter"). For the analysis of the blood vessels SN we used the following morphometric variables: the volume density, surface density and an absolute number of blood vessels per field. On the same samples, the volume density, surface density and an absolute number of nerve cells per field were determined. For these tests, we used the conventional morphometric procedures.[19-21]

For calculation of volume density (Vv), the following formula was used: Vv = Pf / Pt (mm0), where Pf was the number of points of the test system falling on the studied phase, and Pt – total number of points within the test system A100.[18]

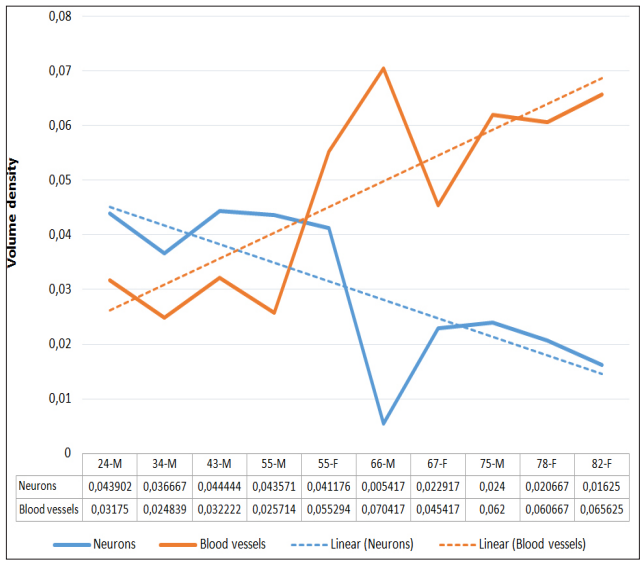
Surface density (Sv) was determined by the formula: Sv = 2×If / Lt (mm^{-1}), where If stood for the number of intersections of test system lines with studies phase, and Lt represented the total length of test lines.[17]

Statistical analysis of the results was performed using SPSS software, version 16.0, using Student's t-test and Pearson's correlation coefficient. Statistical significance was tested for the level of statistical significance of 5%.

Results

Morphometric measurements were carried out on 10 human brains, aged from 24 to 82 years. Volume and surface density and an absolute number per visual field of neurons and blood vessels of adult SN were determined in samples without diagnosed neurological diseases. Figure 1 shows values of volume density, Vv (mm0) of SN neurons and blood vessels in relation to age.

Figure 1. Age-related changes of volume density, Vv (mm^0) of neurons and blood vessels of the substantia nigra.



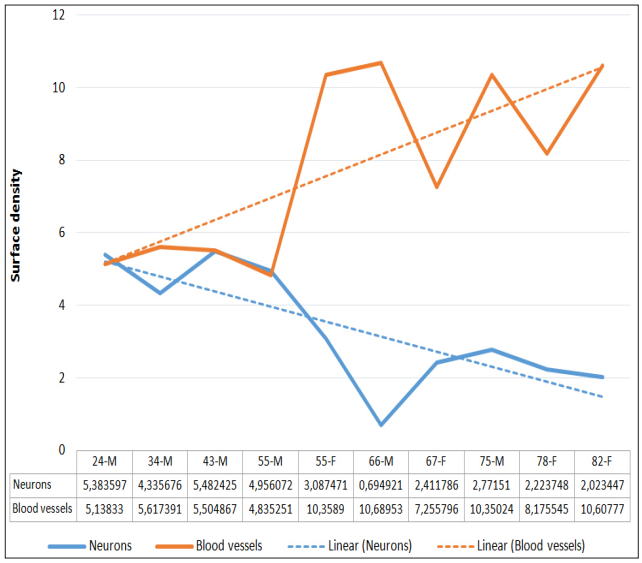
Pu Solid lines represent individual values, while dashed lines represent trendline of values of volume densities. Legend: M- male, F- female (afore values represent age)

By observing the linear trend of growth, it is obvious that the volume density of SN neurons decreased while SN blood vessels volume density increased with aging, as shown by Pearson's correlation coefficient of r = -0.82713. Using the t-test comparisons for different variances, we

obtained a statistical significance of p = 0.024.

Figure 2. shows the change of values of the surface density, Sv (mm^{-1}) of the blood vessels and neurons of SN according to age.

Figure 2. Age-related changes of surface density, Sv (mm^{-1}) of neurons and blood vessels of the substantia nigra.



	24-M	34-M	43-M	55-M	55-F	66-M	67-F	75-M	78-F	82-F
Neurons	5,383597	4,335676	5,482425	4,956072	3,087471	0,694921	2,411786	2,77151	2,223748	2,023447
Blood vessels	5,13833	5,617391	5,504867	4,835251	10,3589	10,68953	7,255796	10,35024	8,175545	10,60777

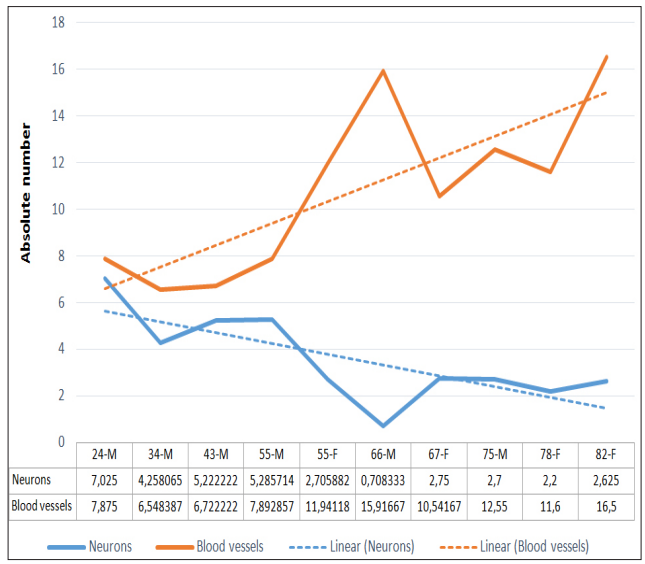
Solid lines represent individual values, while dashed lines represent trendline of values of surface densities. Legend: same as Figure 1.

By observing the linear trend of growth, it is obvious that the surface density of SN neurons decreased, while the surface density of SN blood vessels increased with aging, as shown by Pearson's correlation coefficient of r = -0.8468. Using the t-test comparisons for different variances, we obtained a significant statistical significance of p = 0.00022.

Figure 3.shows changes in the absolute value of the number of blood vessels and neurons of SN with aging.

Linear growth trend showed that the absolute number of SN neurons decreased, while SN blood vessels increased with aging, as shown by Pearson's correlation coefficient of r = -0.8025. Using the t-test comparisons for different variances, we obtained a significant statistical significance of p = 0.000056.

Figure 3. Age-related changes of absolute number, N of neurons and blood vessels per visual field of the substantia nigra.



	24-M	34-M	43-M	55-M	55-F	66-M	67-F	75-M	78-F	82-F
Neurons	7,025	4,258065	5,222222	5,285714	2,705882	0,708333	2,75	2,7	2,2	2,625
Blood vessels	7,875	6,548387	6,722222	7,892857	11,94118	15,91667	10,54167	12,55	11,6	16,5

Solid lines represent individual values, while dashed lines represent trendline of values of an absolute number of neurons and blood vessels. Legend: same as Figure 1.

Discussion

Detailed knowledge of SN microanatomy is necessary to explain the changes that occur during aging and in pathological conditions.[22-26] In the available literature, only a small number of data could be found, which were obtained by morphometric studies of neurons during the aging process, while the changes that occur in blood vessels are rarely documented. Available data are mainly related to the dimensions of the investigated nuclei and cells that are building them,[27] while changes in quantitative indicators with aging cannot be found. This paper presents data derived from the morphometric study of SN neurons and blood vessels of people who are classified into groups by age of both sexes without the diagnosis of a neurological disease. By processing the obtained results, we observed and described quantitative changes that occur in the neurons and blood vessels with the tissue aging.

The specificity of the SN is that this structure shows more pathologic changes with aging in comparison to other regions of the brain. The study on more than 750 older individuals without a defined PD revealed that in nearly one- third of the samples there was a serious loss of neurons in the SN, and 10% showed pathological Lewy bodies.1 It has been estimated that the loss of neurons in the SN per decade was 4.7 %,7 while older stereological techniques showed loss of up to 9.8%.[28]

Rudow and colleagues examined the relationship between the loss of neurons in the SN in normal aging and in PD.[2] They measured the total number and volume of the body of neuromelanin- containing pigmented neurons in the SN. The research was performed on younger control subjects (n = 7, average age: 19.9 years), middle- aged (n = 9, mean age: 50.1 years) and elderly control subjects from the Baltimore Longitudinal Study of Aging (n = 7, the average age: 87.6), as well as in patients with PD (n = 8, mean age: 74.8). On randomly, systematically selected paraffin sections stained by the Nissl method,they used an optical fractionator for estimation of the total number of neurons in SN on one side. Using nucleator, they measured the volume of these neurons. In younger and older control subjects, they also assessed the total number and volume of tyrosine hydroxylase-positive (TH +) nigral neurons. They observed a significant loss of pigmented (-28.3%, p< 0.01) and TH + (-36.2%, p< 0.001) neurons in older control subjects compared to the younger. The analysis of the distribution by size of pigmented and TH + neurons showed a significant degree of hypertrophy in elderly control subjects compared to the younger (p< 0.01). In contrast, a substantial atrophy of pigmented neurons in relation to all control groups was noticed in PD (p< 0.01). These data indicate that hypertrophy of neurons represents a compensatory mechanism within individual neurons in the SN, which allows normal motor function despite the loss of neurons during normal aging. It is assumed that in PD, this compensatory mechanism is out of order or it is in the shadow of pathological events caused by this disease, which leads to the appearance of the characteristic motoric disturbances.

There is no consensus on a change in the volume of neurons in the SN during normal aging. Nonstereological studies provided different results. Although Ma and colleagues have noted the existence of finer pigmented neurons in the SN in elderly subjects,[28,29] Cabello and colleagues, using the rotator method, have found that there is an increased volume of these neurons.[30] Measures, made with nucleator,2 the different kind of stereological test compared to the one used by Cabello and colleagues, agree with the estimates in the aforementioned study.[30] Our observations also showed an increased volume of neurons in the SN in older subjects and that, in addition to the fact that comparison of mean values of the volume of neurons in younger compared to older control subjects did not reach the level of statistical relevance, the analysis of frequency of certain volumes of neurons showed a significant difference between the younger and older subjects. Also it is important that the examination of the histogram of frequencies of distribution showed that an increase in the average volume of SN in elderly subjects was not the result of a selective loss of small size neurons, but a real hypertrophy of pigmented nerve cells bodies. Morphological study of TH + neurons in the human SN has not been able to establish the correlation between cellular body size and age.[31] Although in stereological studies of SN the volume of TH + neurons in normal aging has not been studied, there is evidence that pigmented and TH + neurons behave similarly.[32] Accordingly, the hypertrophy of TH + neurons in normal aging would be expected. This is exactly what the studies have found - TH + neurons with normal aging become larger, a change similar to the one observed in pigmented neurons. The mechanism behind this hypertrophy of neurons is not directly examined in this study, but in addition, some theoretical possibilities could be considered. Progressive accumulation of neuromelanin and other pigments may be the reason for neuronal enlargement. However, this does not explain why the hypertrophy of neurons is present in elderly subjects, but there was no difference when the younger group and middle-aged subjects were compared. Another possibility is that the enlargement of neurons is a result of damage.[33] The third option, which seems most likely, is that since with aging there is a loss of neurons in the SN, the remaining neurons take over and re-innervate deafferented target zones, especially in the striatum. This hypothesis of the compensation, proposed by Cabello and co-workers,[30] is supported by the observation that the total volume (multiplication of volume and the number of neurons) of pigmented neurons in the SN is constant for all age groups. A similar mechanism is also mentioned when it comes to the hypertrophy of cortical nerve cells in asymptomatic dementia. Hypertrophy associated with aging was observed for the pigmented neurons in the locus coeruleus in humans, in a study conducted by Iwanaga and associates[34], attributing the spread of cytoplasm of neuronal bodies to the embrace of the synaptic terminals.

Vaillancourt and colleagues examined the effects of aging on the ventral and dorsal part of SN by diffuse tensor imaging (DTI).[35] Measurements collected by DTI images of 16 young adults (19- 27 years) and 15 older adults (55-71 years) have shown that in the dorsal SN fractional anisotropy is decreased, and radial diffusivity increased with age. In the ventral SN and the red nucleus measurements, using DTI did not show differences depending on age. DTI represents a non-invasive technique that accurately reflects the established pattern of cell loss caused by the aging of the dorsal and ventral part of the SN, which indicates the great potential in the use of DTI to describe degeneration of nigrostriatal pathway in healthy and diseased persons. Studies showed that the loss of neurons with age occurred in the dorsal, while in extrapyramidal disorders, the loss is in the ventral part of the SN.

Some studies have measured the loss of neurons with

aging in other regions of the brain that have not shown a similar degree of neuronal loss as in SN. It turned out that the number of neurons remains relatively stable over the life in the hippocampus, the putamen, the medial mammillary body and Meynert's nucleus, while neocortical neurons have a loss of 10% through the lifetime. But in other dopaminergic populations involved in the ventral tegmental area and retrorubral area loss is up to 50%. These studies show that the dopaminergic neuron populations are increasingly vulnerable to age-dependent loss compared to other brain regions.[36]

It is known that age induces changes in angiogenesis in the brain and other tissues, and that the vascular endothelial growth factor (VEGF) is a powerful regulator of angiogenesis and is thought to be involved in age-related changes of angiogenesis.[37] It is noted that physical exercise improves the effect of age-induced angiogenesis in many tissues.[37,38] Also, studies have shown that VEGF is also a neuroprotective molecule for dopaminergic neurons.[39]

In a study on experimental mice, divided into three age groups and two subgroups,[40] one group was assigned to physical activity on the treadmill and the other one was a control group. The results showed that age was likely related to chronic ischemia and therefore induced the reduction in the density of blood vessels and VEGF levels in the SN, which may have increased the vulnerability of dopaminergic neurons to additional injury.

Uchida and colleagues examined how transient focal brain ischemia could lead to neuronal damage in remote areas, including the thalamus and SN on the affected side, as well as in the ischemic core.[41] These researchers studied the long-term changes in rats SN that occurred between the first and twentieth week on the same side affected by 90-minute attack of transient focal brain ischemia, through immune marking using TH, protein NeuN, Iba-1, glial fibrillary acidic protein (GFAP) and brain-derived neurotrophic factor (BDNF). The results showed that transient focal cerebral ischemia in rats could cause serious and lasting damage to neurons in the striatum of the affected hemisphere. Also, the results obtained on the basis of TH immunolabeling and NeuN showed that atrophy of SN hemisphere affected by transient focal ischemia of the brain was not static, but had a progressive character. In addition, the double-immunohistochemical labeling indicated that the NFMP, released by GFKP-positive astrocytes, could play a key role in the preservation of dopaminergic neurons in the SN during the chronic phase on the same hemisphere affected by transient focal ischemia of the brain, although the surface of SN on that side was progressively reduced after ischemia. Thus, this study provided additional information on the pathogenesis of neuronal damage after transient focal cerebral ischemia.

Do the changes in vascularization have positive or negative effects during aging, or is it a combination of both? Manipulation by vascularization on experimental models can help finding the answer to this question and possibly identify new targets for treating diseases of SN. The formation of blood vessels may, in fact, come through a variety of processes and inflammation stimulates angiogenesis in various diseases. Numerous mechanisms can be the basis of such kind of neovascularization. There are several types of angiogenic factors which are released from the cells, which stimulate neovascularization. One of these mechanisms might be associated with the loss of neurons in the SN pars compacta since in parkinsonism, the existence of cytokines that promote an inflammatory process and the infiltrating cells of blood origin has been described.[12]

It was observed that with age- dependent decline of degrees of nigral vascularization and nigral VEGF, both degrees have increased after the implementation of locomotor exercises.[40] One study showed a beneficial effect that exercise had on neuroprotection in cerebral ischemic damage,[38] while other study suggested that exercises of the locomotor system induced an increase in VEGF expression, probably as a compensatory mechanism leading to increased capillary surface in response to the increased demand for oxygen and energy.[40]

Our previous studies of neurons in the magnocellular part of the red nucleus have shown the reduction of the quantitative parameters with age, but the decrease did not reach statistical significance.[42] We mention this because of the proximity of red nucleus and SN at the same midbrain level, and there is a joint functional activity in the extrapyramidal system. This is confirmed by Lambert and associates, who showed the reduction in the number of neurons, gliosis and increased levels of iron with aging in SN and red nucleus.[43] The increase in iron in vivo results in an increase neuromelanin, the substance that is then released by dying neurons and causes new neuronal damage.

One of the limitations observed in our study is that we do not have information whether the analyzed human tissue belonged to individuals whose lifestyle in the older age included intense physical activity and exercise, which could be accounted for increased angiogenesis in the elderly. This fact opens the door for further research on whether the increased activity of the musculoskeletal system in old age significantly affects the maintenance activity of the nervous system.

Conclusion

With aging, there is a statistically significant increase in the values of morphometric parameters of blood vessels and reduction of values of morphometric parameters of SN neurons. Taking into account that we have studied healthy individuals at different ages, this relationship explains that, with aging, there is a decline of neurons which is compensated by the increase of vascular network of SN.

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Promjene neurona i krvnih sudova substantiae nigrae kod čovjeka tokom starenja - morfometrijsko istraživanje

SAŽETAK

Uvod: Populacije dopaminergičkih neurona u centralnom nervnom sistemu sa starenjem pokazuju izraženije patološke promjene u poređenju sa drugim dijelovima mozga. Dosadašnja ispitivanja substantiae nigrae su rađena kod Parkinsonove bolesti i u starosti.

Cilj rada: S obzirom na to da je Parkinsonova bolest poremećaj povezan sa starosnom dobi, važno je ispitati na koji način je odnos neurona i krvnih sudova povezan sa normalnim starenjem.

Ispitanici i metode: Deset moždanih stabala je rezano u tri stratuma, od kojih su pravljeni semiserijski rezovi bojeni Mallory metodom. Proučavane faze su neuroni i krvni sudovi substantiae nigrae. Analiza je rađena kamerom "Leica EC3", pri povećanju objektiva 40x svjetlosnog mikroskopa "Leica" DM 1000, korišćenjem programa ImageJ (verzija 1.42 e). Određivani morfometrijski parametri neurona i krvnih sudova su bili: volumenska i površinska gustina, i apsolutni broj po vidnom polju. Statistička analiza je urađena pomoću softvera SPSS, verzija 16.0, upotrebom Studentovog t-testa i Pearson-ovog koeficijenta korelacije.

Rezultati: V Volumenska i površinska gustina neurona, te apsolutni broj neurona po vidnom polju substantiae nigrae su se statistički značajno smanjivali sa godinama života, dok su se volumenska i površinska gustina krvnih sudova i apsolutni broj krvnih sudova po vidnom polju statistički značajno povećavali ($p < 0,05$).

Zaključak: Starenjem dolazi do smanjenja dimenzija i broja neurona koji kompenzuje porast vaskularnog korita jedra i utiču na snabdijevanje neurona nutrijentima iz krvi, kao i na dostupnost krvnih ćelija ili toksičnih supstanci, ali i na podložnost neurona bolesti.

Ključne riječi: Starenje, substantia nigra, čovjek