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TIME-RELATED SEX DIFFRENCES IN CEREBRAL HYPOPERFUSION-INDUCED BRAIN INJURY

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Abstract – Although the model of cerebral hypoperfusion in rats has been a matter of many investigations over the years, the exact intracellular and biochemical mechanisms that lead to neuron loss and memory decline have not been clearly identified. In the current study, we examined whether cerebral hypoperfusion causes changes in hippocampal protein expression of apoptotic markers in the synaptosomal fraction and neurodegeneration in a time-dependent and sex-specific manner. Adult male and female Wistar rats were divided into two main groups, controls that underwent sham operation, and animals subjected to permanent bilateral occlusion of common carotid arteries. Both male and female rats were killed 3, 7 or 90 days following the insult. The obtained results indicate that the peak of processes that lead to apoptosis occured on postoperative day 7 and that they were more prominent in males, indicating that neuroprotective effects of certain substances (planned for future experiments), should be tested at this time point.

Key words: cerebral hypoperfusion; apoptosis; gender; hippocampus

INTRODUCTION

Sudden and prolonged disturbance of cerebral blood flow (CBF) plays an important role in cognitive impairment and development of various neurodegenerative diseases like vascular dementia and Alzheimer's disease (Farkas et al, 2007). Although the effects of CBF reduction have been matter of investigation over the years, the exact mechanisms that underlie the response to this type of insult, are not completely understood.

Permanent bilateral occlusion of common carotid arteries in rats (2VO) is a well established ischemia model for chronic cerebral hypoperfusion. The time course of CBF changes in this model has three defined phases: acute, chronic and restitution phase. The acute phase begins instantly after the 2VO procedure and lasts 2–3 days. This phase is characterized by sudden and dramatic decrease of CBF and decline of oxygen and glucose levels that compromises proper activity of the brain (Marosi et al., 2006). The chronic phase lasts about 3 months starting from day 3. This phase is featured by long-lasting hypoperfusion, hypoglycemia and oligemia. Since changes that occur during chronic phase of 2VO correspond at the most to the state induced by chronic cerebral hypoperfusion, this phase is used to study its effects (Farkas et al, 2007). The last phase is defined by restitution of CBF and the return of oxygen and glucose to baseline levels.

The literature highlights the significance of apoptotic signaling pathways and proteins involved in response to brain ischemia. Among the many proteins included in apoptotic response, the members of Bcl-2 protein family, anti-apoptotic Bcl-2 and pro-apoptotic Bax, are directly associated with formation of pores in mitochondrial membrane and indirectly with release of cytochrome C (Love, 2003). Its release from mitochondria to cytosol induces the sequence of events that lead to proteolytic processing and activation of caspase 3 (Ouyang et al, 1999) which causes cleavage of large number of proteins including PARP-1 (*poly (ADP ribose) polymerase*-1) whose primary function is maintenance of DNA integrity.

Since various neurodegenerative processes could be instigated in synapses (Mattson and Duan, 1999) and there is possibility of time- and gender-mediated response to cerebral hypoperfusion (Farkas et al, 2007), the current study was carried out to elucidate potential time-dependent and sex-specific mechanisms of apoptotic changes in synaptosomal fractions of the hippocampus. Immunoblot analysis was used to define whether cerebral hypoperfusion causes gender-specific changes in the expression of proteins involved in caspase 3 dependent apoptotic processes in hippocampal synaptosomal fraction at different time points following an ischemic insult. Fluoro-Jade B staining and DNA fragmentation assay were applied to assess the potential outcomes of investigated proteins activities on hippocampal neuronal death rate.

MATERIALS AND METHODS

Animals

The experimental protocol employed in this study was approved by the Ethical Committee for the Use of Laboratory Animals of VINCA Institute of Nuclear Sciences, University of Belgrade, Serbia, according to the guidelines of the EU FELASA-registered Serbian Laboratory Animal Science association (SLASA). All experiments on male (weight 300-350 g) and female (weight 280-300 g) rats, obtained from the local colony and maintained under standard conditions, were conducted in parallel. Three time sets (3, 7, 90 days after surgery) of experiments were carried out. Within each time set, rats were randomly assigned into experimental groups: control, sham-operated animals and 2VO animals that were subjected to permanent bilateral occlusion of the common carotid arteries.

Surgeries were performed on 5% chloral hydrate (400 mg/kg) anesthetized animals according to Mracsko et al. (2010). After surgery, during postoperative recovery that lasted 3, 7 or 90 days, all rats were closely monitored on their physical health condition on a daily basis.

Tissue and sample preparation

After different times, the animals were killed between 10:00 and 11:00 AM. For DNA fragmentation assay, male and female rats (n=8 per group) were anesthetized with an overdose i.p. injection of 5% chloral-hydrate and perfused transcardially with saline (4°C, 200 ml). Hippocampi from the individual animals were rapidly removed on ice. All samples were immediately frozen in liquid nitrogen and stored at -70°C until use. DNA fragmentation assay was performed as previously stated by Drakulić et al. (2013).

Fluoro-Jade B staining and image analysis of hippocampi obtained from male (n=3 per group) and female (n=3 per group) was performed according to Stanojlović et al. (2014).

Another set of animals (n=8 per group) was quickly decapitated with guillotine and hippocampal brain areas from the same group (4 brains/group/isolation) were quickly taken out for immediate crude synaptosomal fraction isolation as previously described (Horvat et al, 2010). All samples were stored at -80°C until further processing. The modified method of Lowry *was used for determination of* the protein content (Markwell, 1978), using BSA as a standard.

For Western blotting, equal amounts (20 μg) of total protein from each sample was separated on

10% or 12% SDS-PAGE gel, depending on protein molecular mass, and transferred on PVDF membranes (Imobilion-P membrane, Millipore, USA). Membranes were blocked in TBS containing 5% non fat milk and 0.1% Tween 20 for 2 h and incubated overnight at 4°C with the following primary antibodies: anti-Bax (sc-7480, Santa Cruz Biotechnology Inc, USA, 1:1000), anti-Bcl-2 (sc-492, Santa Cruz Biotechnology Inc, USA, 1:1000), anti-procaspase 3 (sc-7148, Santa Cruz Biotechnology Inc, USA, 1:1000), anti-PARP-1 (9542, Cell Signaling Technology, USA, 1:1000). Goat polyclonal anti-β-actin antibody (sc-1615, Santa Cruz Biotechnology Inc, USA, 1:5000) was used for normalization. After washing, the membranes were incubated for 2 h with horseradish-peroxidase-conjugated goat anti-rabbit antibody (sc-2030, Santa Cruz Biotechnology Inc, USA, 1:5000) for Bcl-2, procaspase 3 and PARP; horseradish-peroxidase-conjugated donkey anti-mouse antibody (sc-2318, Santa Cruz Biotechnology Inc, USA, 1:5000) for Bax and with horseradish-peroxidase-conjugated donkey anti-goat antibody (sc-2033, Santa Cruz Biotechnology Inc, USA 1:5000) for β-actin. The antigen-antibody complex was detected using enhanced chemiluminescence (ECL) system (Amersham Bioscience, USA).

Statistical Analysis

The results are presented as percents of the mean of the values in sham-operated males ± SEM, from two independent experiments for each group of animals. The differences among the groups were analyzed by one-way analysis of variance (ANOVA) test followed by the *post-hoc* Tukey test, while gender differences were analyzed by two-way (ANOVA) test followed by the *post-hoc* Tukey test. A p-value of 0.05 or less was considered significant for all statistical analyses.

RESULTS

Cerebral hypoperfusion and apoptosis-related protein expression in the synaptic fraction

In the first set of experiments, we monitored whether cerebral hypoperfusion induced changes in the expression of pro-apoptotic and anti-apoptotic proteins in hippocampal synaptosomal fraction obtained from sham and 2VO male and female rats in time-dependent and sex-specific manner.

On postoperative day 3, decreased levels of Bcl-2 protein ($p<0.001$ for males and $p<0.05$ for females, Fig.1A) were detected while Bax was not changed. Further, Bcl-2 protein expression was considerably reduced in 2VO rats in both genders ($p<0.001$ for males and $p<0.05$ for females, Fig. 1A), while the levels of Bax were significantly increased on postoperative day 7 ($p<0.001$ for males and $p<0.01$ for females, Fig. 1B). It should be noted that on postoperative day 7 more pronounced changes in Bax protein expression were observed in hippocampal synaptosomal fraction isolated from male rats when compared to females ($p<0.01$). Alterations in Bcl-2 and Bax protein expression led to decline of Bcl-2/Bax protein ratio ($p<0.001$ for males and $p<0.01$ for females, Fig. 1C).

On postoperative days 3 and 7, cerebral hypoperfusion caused the augmentation of procaspase 3 expression in both genders ($p<0.001$ for males and $p<0.001$ for females, Fig. 2A). As shown in Fig. 2A the expression of this protein was more prominent in females ($p<0.05$). Furthermore, the significant increase of caspase 3 active fragment of 17 kDa was detected 7 days following surgery in both genders ($p<0.001$ for males and $p<0.001$ for females, Fig. 2B). The observed generation of protein fragments resulting from caspase 3 activity was more pronounced in females ($p<0.01$).

The increased expression of 116 kDa full length PARP was detected on day 7 ($p<0.001$ for males and $p<0.001$ for females, Fig. 2C). Consistent with the findings for caspase 3, the quantification of the 89 kDa cleavage product showed considerable augmentation on postoperative day 7 ($p<0.001$ for males and $p<0.001$ for females, Fig. 2D).

Evaluation of cell death by Fluoro-Jade B staining and nuclear DNA fragmentation

In the second set of experiments, Fluoro-Jade B

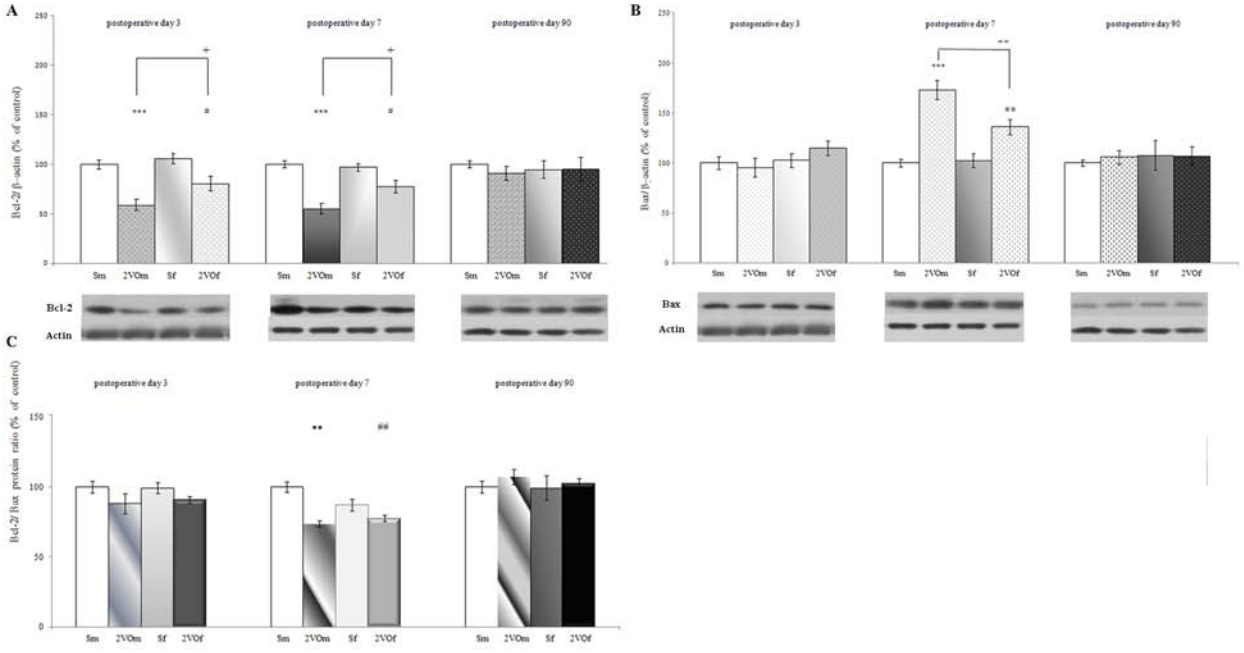

Fig. 1. The effect of cerebral hypoperfusion on Bcl-2 and Bax protein abundance and the Bcl-2/Bax protein ratio in male and female rat hippocampal synaptosomal fractions at different time points following the insult. **Panel A**. Representative Western blots and graphics showing the relative abundance of Bcl-2 in the hippocampal synaptosomal fraction of sham (S) and bilaterally occluded (2VO) male (m) and female (f) rats, quantified by densitometry, normalized against β-actin in the same sample and expressed as percentages relative to matching controls. **Panel B**. Representative Western blots and graphics showing the relative abundance of Bax protein in the hippocampal synaptosomal fraction of sham (S) and bilaterally occluded (2VO) male (m) and female (f) rats, quantified by densitometry, normalized against β-actin in the same sample and expressed as percentages relative to matching controls. **Panel C.** The Bcl-2/Bax protein ratio in hippocampal synaptosomal fractions of sham (S) and bilaterally occluded (2VO) male (m) and female (f) rats expressed as percentages relative to matching controls. The results are expressed as percentages of the means of values obtained in sham-operated males±SEM. The differences between the groups (marked as * for males and # for females) were analyzed by one-way analysis of variance (ANOVA), followed by the *post-hoc* Tukey test; gender differences (marked as +) were analyzed by two-way (ANOVA), followed by the *post-hoc* Tukey test. A p-value of 0.05 or less was considered to be significant.

staining and DNA fragmentation assay were used to estimate whether cerebral hypoperfusion induced time-dependent and sex-specific neurodegeneration and apoptosis-specific DNA fragmentation in 2VO rats. Fig. 3A, B shows an increase in the number of Fluoro-Jade B labeled cells in the hippocampus of hypoperfused rats on postoperative day 7 in both male and female rats. Furthermore, the number of cells contained in foci of compact, Hoechst-staining chromatin, was significantly increased in 7th day following surgery, which indicated that neurons were undergoing apoptosis (Fig. 3A, B). A quantitative analysis of Fluoro-Jade B staining of hippocampi of hypoperfused rats (Fig. 3C) shows a significant increase in the number of Fluoro-Jade B labeled cells only on postoperative day 7 in both male and female rats ($p<0.001$ for males and $p<0.001$ for females). Their number peaked in males ($p<0.05$, Fig. 3C).

As shown in Fig. 3D, in response to cerebral hypoperfusion, only on postoperative day 7, content of apoptosis-specific DNA fragments was considerably increased in both male and female animals ($p<0.01$ for males and $p<0.01$ for females). Moreover, the significant difference in amount of apoptosis-specific DNA fragments between males and females was observed ($p< 0.05$, Fig. 3D).

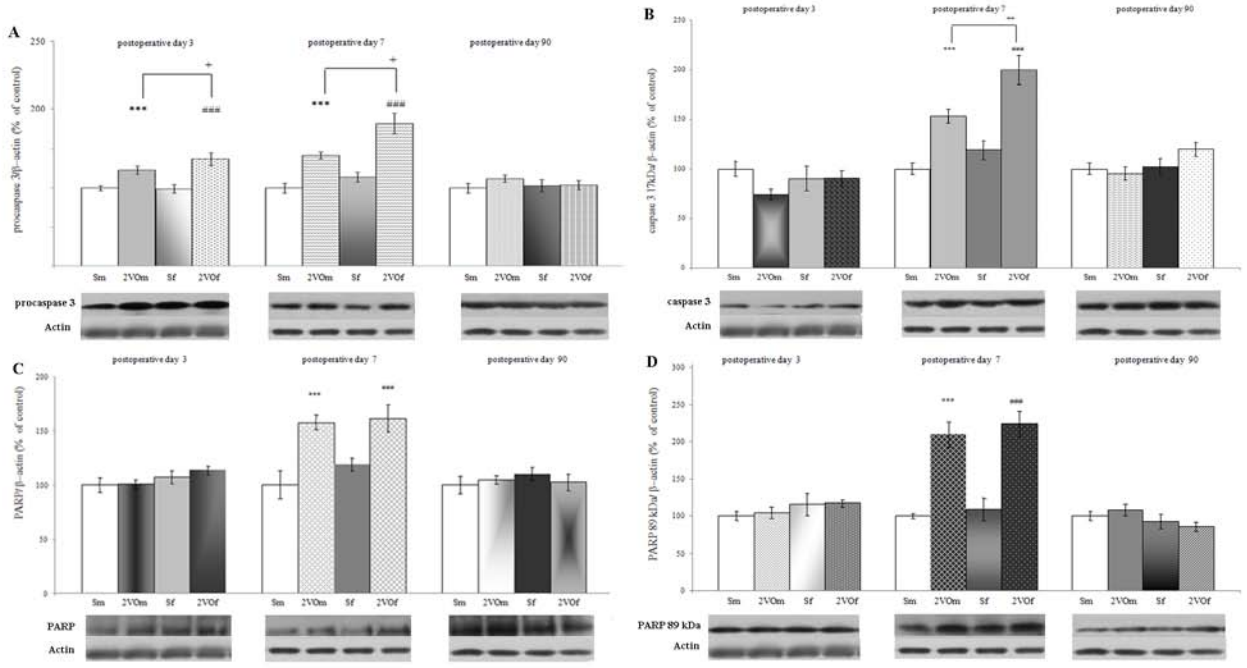

Fig. 2. The effect of cerebral hypoperfusion on procaspase 3, cleaved caspase 3, full length PARP-1 and cleaved PARP-1 protein abundance in male and female rat hippocampal synaptosomal fractions at different time points following the insult. **Panel A**. Representative Western blots and graphics showing the relative abundance of procaspase 3 in hippocampal synaptosomal fractions of sham (S) and bilaterally occluded (2VO) male (m) and female (f) rats, quantified by densitometry, normalized against β-actin in the same sample and expressed as percentages relative to matching controls. **Panel B**. Representative Western blots and graphics showing the relative abundance of cleaved caspase 3 in hippocampal synaptosomal fractions of sham (S) and bilaterally occluded (2VO) male (m) and female (f) rats, quantified by densitometry, normalized against β-actin in the same sample and expressed as percentages relative to matching control. **Panel C**. Representative Western blots and graphics showing the relative abundance of full length PARP-1 protein in hippocampal synaptosomal fractions of sham (S) and bilaterally occluded (2VO) male (m) and female (f) rats, quantified by densitometry, normalized against β-actin in the same sample and expressed as percentages relative to matching controls. **Panel D.** Representative Western blots and graphics showing the relative abundance of cleaved PARP-1 protein in hippocampal synaptosomal fractions of sham (S) and bilaterally occluded (2VO) male (m) and female (f) rats, quantified by densitometry, normalized against β-actin in the same sample and expressed as percentages relative to matching controls. The results are expressed as percentages of the mean values obtained in sham-operated males ± SEM. Differences between the groups (marked as * for males and # for females) were analyzed by one-way analysis of variance (ANOVA), followed by the *post-hoc* Tukey test; gender differences (marked as +) were analyzed by two-way (ANOVA), followed by the *post-hoc* Tukey test. A p-value of 0.05 or less was considered to be significant.

DISCUSSION

The understanding of the hippocampal gender-specific response and time course of the expression of Bcl-2 family members as well as the expression profiles of other proteins such as procaspase 3, caspase 3 and PARP-1 in apoptosis induced by cerebral hypoperfusion is limited. Thus, the current study was undertaken to obtain data on the dynamics and possible contributions of the aforementioned proteins in cell death following cerebral hypoperfusion. Our results point to several interesting findings.

First, the time-specific down-regulation of Bcl-2 accompanied with increased levels of procaspase 3 and no changes in the expression of Bax, cleaved caspase 3 and PARP emerged in synaptosomal fraction on postoperative day 3. Given that Bcl-2 is an estrogen-responsive gene in brain tissue, its expression is elevated in estrous and estradiol-treated rats

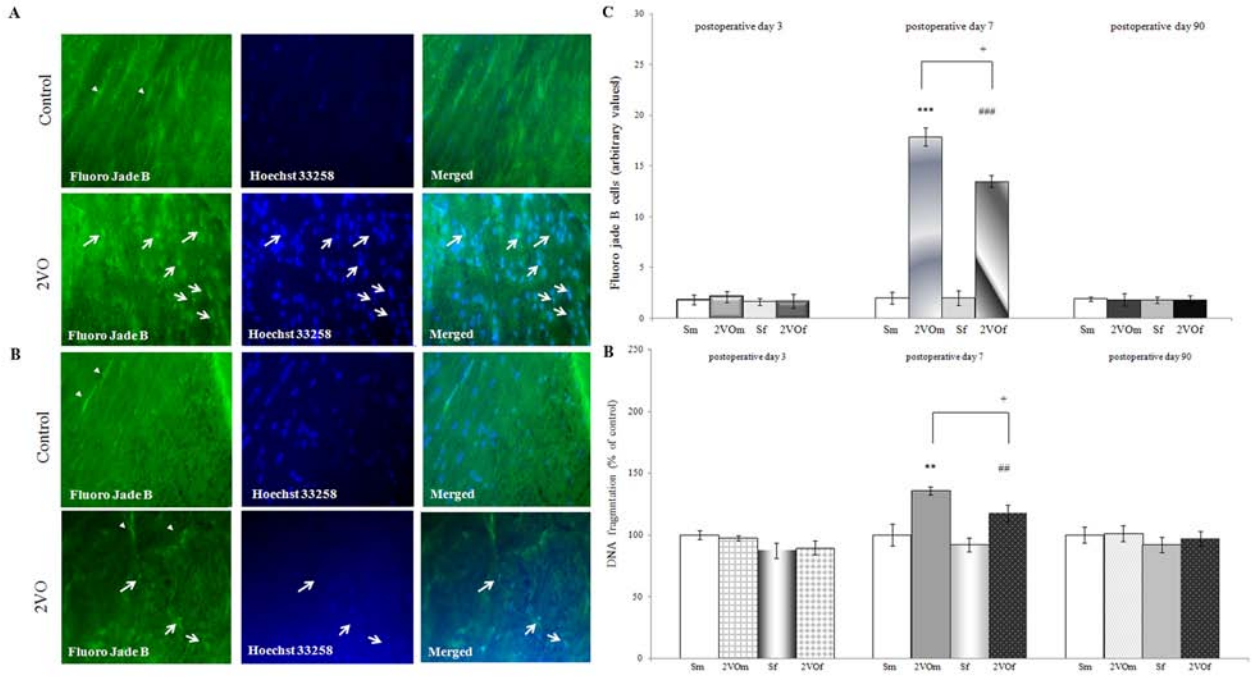

Fig. 3. Staining of adult male (**Panel A**) and female (**Panel B**) rat brains with Fluoro-Jade B and Hoechst 33258 at different time points following the insult (magnification X 40). Degenerating neurons are marked with arrows; arrowheads point to blood vessels. **Panel C**. Quantitative analysis of Fluoro-Jade B staining of adult male and female hippocampi. The results are expressed as means±SEM. Differences between the groups (marked as * for males and # for females) were analyzed by one-way analysis of variance (ANOVA), followed by the *post-hoc* Tukey test; gender differences (marked as +) were analyzed by two-way (ANOVA), followed by the *post-hoc* Tukey test. A p-value of 0.05 or less was considered to be significant. **Panel D**. Measurement of DNA fragments with the diphenylamine (DPA) colorimetric assay. Results are presented as the percentage of control fragmentation, according to the formula: % of fragments = ($OD_{600\ nm}$ T/$OD_{600\ nm}$ (T+B)) × 100; T = fragmented DNA; B = intact DNA obtained from male (m) and female (f) rats subjected to sham (C) and bilateral occlusion (2VO) 3, 7 and 90 days following the insult. The results are expressed as percentages of the mean values relative to the values obtained in sham-operated males±SEM. Differences between the groups (marked as * for males and # for females) were analyzed by one-way analysis of variance (ANOVA), followed by the *post-hoc* Tukey test; gender differences (marked as +) were analyzed by two-way (ANOVA), followed by the *post-hoc* Tukey test. A p-value of 0.05 or less was considered to be significant.

(Garcia-Segura et al., 1998) and injury-induced changes of this proteins are prevented by estradiol (Won et al., 2005), we are tempted to speculate that female gonadal hormones (estradiol and progesterone) might vary the levels of this molecule in ischemic conditions and also alleviate the injury-induced downregulation of Bcl-2 observed in females when compared to males. The absence of neurodegeneration indicates that the investigated proteins probably do not play the same role during early hypoperfusion-induced injury as during chronic reduction of CBF. Moreover, previous studies point to the involvement of caspase 3 in cytoskeleton remodeling and axon guidance (Villapol et al, 2008; McLaughlin, 2004), thus we assume that during acute phase of cerebral hypoperfusion, this and other examined proteins contribute mostly to morphological changes that could be associated with cell death in later investigated time point.

Second, time- and gender-specific damage consistent with the occurrence of neurodegeneration was detected in hippocampus 7 days after 2VO insult. Our data provide strong evidence that chronic cerebral hypoperfusion induces apoptosis through activation of intrinsic apoptotic pathway characterized by changes in the expression of Bcl-2 family members, caspase activation and proteolysis of

PARP. Although observed apoptotic cell death in females could be contributed to activation of caspase-dependent pathways as previously showed by Du et al. (2004), the elevated number of Fluoro-Jade B positive cells along with doubled stained Fluoro-Jade B- Hoechst cells and increased apoptosis-related DNA fragmentation detected in males could not be explained just by the activation of this apoptotic pathway alone but also by inclusion of additional pro-apoptotic signaling pathways.

Third, according to our findings, the activation of apoptosis-related signaling pathways and neurodegeneration could be reduced as time elapses following the injury. Namely, on postoperative day 90, although, we have not measured the CBF and diameter of the arteries, we assume that in current experimental setup glucose and oxygen levels, essential for cell homeostasis and survival, are normalized through the activation of adaptive and compensatory mechanisms and restoration of CBF as previously showed in several other studies (Smidt-Kastner et al, 2005; Choy et al, 2006).

CONCLUSION

Our findings indicate that the hippocampus exhibits time- and sex-specific tissue responses to persistent reduction of CBF associated with modulation of expression and activation of investigated proteins in the synaptosomal fraction. The activation of a caspase-dependent pathway that leads to neurodegeneration was more prominent in females whereas additional apoptotic pathways are most likely involved in males. The activation of those signaling pathways needs to be investigated furter. The time-window and sex-specific cellular and molecular mechanisms that underlie the pathophysiological processes in cerebral hypoperfusion are essential for establishing the neurorestorative and neuroprotective therapies that would promote the recovery of cognitive functions observed after this type of brain injury.

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Authors' contribution

Miloš Stanojlović participated in performing the experiments, literature search and drafting the article. Ivana Guševac took the part in performance of experiments. Ivana Grković and Nataša Mitrović contributed in critical revision of the manuscript. Anica Horvat designed the subject and aims of the research and provided critical revision of the manuscript. Dunja Drakulić contributed substantially to the study conception and design, performance of all experiments as well as data acquisition, analysis and interpretation, literature search, and writing of the manuscript.

Conflict of interest

All authors gave their final approval for publication and declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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