Original Article

Time Course of Cerebral Hypoperfusion-Induced Neurodegenerative Changes in the Cortex of Male and Female Rats

(neurodegeneration / apoptosis / cerebral hypoperfusion / cerebral cortex / rat)

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Abstract. To study time-dependent and gender-specific intracellular and biochemical mechanisms that lead to neurodegeneration due to moderate but persistent reduction of cerebral blood flow, 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. Animals were sacrificed 3, 7 or 90 days following the insult. Expression of several apoptotic proteins in synaptic fractions along with Fluoro-Jade B staining and DNA fragmentation assay were used to estimate the apoptotic processes and potential neurodegeneration in cerebral cortex. Data suggest a time-specific increase of Bax as well as time- and gender-associated down-regulation in protein expression of Bcl-2, up-regulation of procaspase 3, accompanied with increased cleavage of procaspase 3 and PARP in synaptic terminals. Furthermore, time- but not gender-specific neurodegeneration was observed. Our findings support the concept of time- and gender-associated response to permanent bilateral occlusion of common carotid arteries, which would enable better understanding of the mechanisms underlying cerebral hypoperfusion.

Introduction

Cerebrovascular occlusive diseases and cerebral hypoperfusion due to carotid insufficiency and other ischemic states associated with aging have been matters of extensive investigation over the years. To study the neuronal damage and cognitive decline that arises as a consequence of moderate but persistent reduction of cerebral blood flow in humans, an experimental model of common carotid artery ligation or two-vessel occlusion (2VO) in rodents is widely exploited. This model has been predominantly used to investigate the neurodegenerative processes in the hippocampus. However, it was shown that other brain regions, including cerebral cortex, are sensitive to this type of ischemic insult (Farkas et al., 2007). The importance of studying the effects of cerebral hypoperfusion on cerebral cortex lies in the fact that this brain area exerts a pivotal role in memory formation, sustaining attention, perception of the environment, linking different aspects of behaviour, etc.

A large body of evidence indicates that much of the biochemical machinery involved in apoptosis can be activated in synaptic terminals, where it can alter the synaptic function and promote localized degeneration of synapses and neurites, and finally lead to nuclear DNA condensation and fragmentation (Mattson and Duan, 1999). Although the exact molecular mechanism leading to biochemical and morphological changes as well as neuronal loss following cerebral hypoperfusion is still matter of controversy, several studies point to time- and gender-associated response to this type of brain injury (Farkas et al., 2007). Given that a number of various disorders initiate neurodegenerative processes in synapses and there is the possibility of time- and gender-mediated effects of cerebral hypoperfusion, the current

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Abbreviations: 2VO – two-vessel occlusion, ANOVA – one-way analysis of variance, ECL – enhanced chemiluminescence, EDTA – ethylenediamine tetraacetic acid, NFM – non-fat milk, PARP – poly (ADP-ribose) polymerase, PCA – perchloric acid, PFA – paraformaldehyde, PVDF – polyvinylidene difluoride.

study was undertaken to obtain insight into the prospective time-dependent and gender-specific mechanisms of apoptotic changes in synaptosomal fractions of the cerebral cortex. To test our hypothesis, the levels of a few apoptosis-related molecules such as Bcl-2 family members (Bcl-2 and Bax) and procaspase 3 together with the proteolytic degradation of caspase 3 and its specific substrate PARP in adult male and female rats on postoperative days 3, 7 and 90 were determined by Western blot. Additionally, Fluoro-Jade B staining and DNA fragmentation assay were used to estimate the potential neurodegeneration and apoptosis-specific DNA degradation in the rat cerebral cortex after 2VO insult.

Material and Methods

Animals

The Ethics Committee for the Use of Laboratory Animals of VINCA Institute of Nuclear Sciences, University of Belgrade, Belgrade, Serbia approved the experimental protocol employed in this study, according to the guidelines of the EU FELASA-registered Serbian Laboratory Animal Science Association (SLASA).

Adult Wistar rats (VINCA Institute of Nuclear Sciences, University of Belgrade, Serbia) (males weighing 300–350 g and oestrous females weighing 280–300 g) were maintained under the standard conditions. Both female and male rats were randomly assigned into two main experimental groups: I) control, sham-operated animals and II) 2VO, animals subjected to permanent bilateral occlusion of the common carotid arteries. Three time sets (3, 7, 90 days after surgery) of experiments were carried out, using the third of the rats of each group at each time point.

Surgical procedure

Surgeries were performed on 5% chloral hydrate (400 mg/kg) anesthetized animals. A neck ventral midline incision was made to expose both carotid arteries. The arteries were carefully separated from the carotid sheath, cervical sympathetic and vagus nerve. In 2VO surgery, each carotid artery was double-ligated with 5-0 silk suture. In sham operations, both arteries were exposed but not ligated. The time points studied were 3, 7 and 90 days following the surgical procedures.

Tissue and sample preparation

After different survival periods, animals were sacrificed between 10:00 and 11:00 AM.

For Western blot analysis, rats (N = 8 for each experimental group) were quickly decapitated with small animal guillotine (Harvard Apparatus, Holliston, MA) and cortical brain areas were taken out for immediate synaptosomal isolation as previously described (Horvat et al., 2010). In short, cortices were homogenized with 20 strokes in Potter-Elvehjem glass Teflon homogenizer (Sigma-Aldrich Co., St Louis, MO) at 900 rpm in ice-cold isotonic medium (0.32 M sucrose, 5 mM Tris-HCl, pH 7.4) and centrifuged at 1000 *g* for 10 min at 4 °C. Supernatants were decanted and stored on ice, while pellets were additionally resuspended in 10 ml of the same medium and centrifuged for a second time under similar conditions. Collected supernatants were furthermore centrifuged at 12,000 *g* for 25 min at 4 °C and pellets were resuspended in 5 mM Tris-HCl, pH 7.4. Protein concentration of samples was measured by the modified method of Lowry (Markwell et al., 1978) using bovine serum albumin (BSA) as a standard.

To prepare for Fluoro-Jade B staining that labels degenerating neuronal somata and their processes, rats (N = 2–3 animals for each experimental group) were deeply anesthetized with an overdose i.p. injection of 5% chloral hydrate, perfused transcardially with saline (4 ºC, 200 ml) and then with 4% paraformaldehyde (PFA). Brains were rapidly removed, placed on ice, fixed in 4% PFA for 24 h at 4 °C, cryoprotected in a sucrose gradient in 0.2 M phosphate-buffered saline (in 10 %, 20 % and 30 % sucrose for 24 h at 4 °C), frozen in isopentane, cooled on dry ice and stored at -70 °C.

Additional groups of sham and 2VO animals (N = 8 animals for each experimental group) were deeply anesthetized with an overdose i.p. injection of 5% chloral hydrate and perfused transcardially with saline (4 °C, 200 ml) for DNA fragmentation assay. Cerebral cortices from the individual animals were rapidly removed and placed on ice. All samples were immediately frozen in liquid nitrogen and stored at -70 °C.

Western blotting

Equal amounts (20 μg) of total protein from each sample were separated in 10 % or 12 % SDS-PAGE gel and transferred onto PVDF membranes (Imobilion-P membrane, Millipore, Billerica, MA). Membranes were blocked in TBS containing 5% non-fat milk (NFM) 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., Dallas, TX, 1 : 1000), anti-Bcl-2 (sc-492, Santa Cruz Biotechnology Inc., 1 : 1000), anti-procaspase 3 (sc-7148, Santa Cruz Biotechnology Inc., 1 : 1000), anti-PARP (#9542, Cell Signaling Technology Inc., Danvers, MA, 1 : 1000). Goat polyclonal anti-β-actin antibody (sc-1615, Santa Cruz Biotechnology Inc., 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., 1 : 5000) for Bcl-2, procaspase 3 and PARP; horseradish-peroxidase-conjugated donkey anti-mouse antibody (sc-2318, Santa Cruz Biotechnology Inc., 1 : 5000) for Bax and with horseradish-peroxidase conjugated donkey anti-goat antibody (sc-2033, Santa Cruz Biotechnology Inc., 1 : 5000) for β-actin. The antigen-antibody complex was detected using the enhanced chemiluminescence (ECL) system (Amersham Bioscience, Piscataway, NJ). The control male group was taken as 100 % and changes were calculated with respect to this value.

Fluoro-Jade staining and image analysis

Brains were cut and every 3rd coronal section (16 or 25 µm thick) was mounted on a slide, allowed to dry overnight and stored at -20 °C. The slides containing cortical areas were first immersed in a basic alcohol solution consisting of 5% NaOH in 70% ethanol, dH_2O and incubated in 0.06% $KMnO_4$ solution for 10 min; then transferred for 20 min into a 0.0001% solution of Fluoro-Jade B (Chemicon International, Millipore, Billerica, CA) dissolved in 0.1% acetic acid and rinsed by three changes of dH_2O for 1 min per change. Cells stained with Fluoro-Jade B were detected as individual shiny green spots clearly distinct from the background. The number of degenerating neurons labelled by Fluoro-Jade B was counted in three fields under the area of the screen (0.38 mm^2) in five sections of the regions of interest per animal (Drakulić et al., 2013) by a researcher who was unaware of the surgical procedure. Cortical sections from all groups were run in the same assay, using sham-operated rats of each investigated time point as a control for neurodegeneration.

Several slides were additionally immersed in 0.01% Hoechst 33258 (Acros Organics, Fair Lawn, NJ) staining solution for 10 min and coverslipped with glycerol. The sections were examined with an Axio Observer Microscope Z1 (Zeiss, Jena, Germany) using a filter system suitable for visualizing fluorescein isothiocyanate.

DNA fragmentation assay

DNA fragmentation assay was performed as previously described by Drakulic et al. (2013). Briefly, frozen cortical brain areas were homogenized in lysis buffer containing 5 mM Tris-HCl (pH 8.0), 20 mM ethylenediamine tetraacetic acid (EDTA) and 0.5% Triton X-100. Homogenates were then centrifuged at 27,000 *g* for 20 min at 4 °C to separate intact chromatin in the pellets from fragmented DNA in the supernatant fractions. Pellets were resuspended in 0.5 N perchloric acid (PCA), whereas 5.5 N PCA was added to the supernatant fractions to a final concentration of 0.5 N. Samples were heated at 90 °C for 15 min and centrifuged at 1500 *g* for 10 min at 4 °C to remove proteins. The supernatant fractions were reacted with diphenylamine for 16–20 h at room temperature and absorbance was measured at 600 nm. The level of DNA fragmentation in control samples is expressed as percentage of total DNA appearing in the supernatant fraction whereas the effects of cerebral hypoperfusion are reported as quantity of control fragmentation. A suitable control male group was taken as 100 % and changes were calculated with respect to this value.

Statistical analysis

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

Results

Permanent common artery occlusion and apoptosis-related protein expression in the synaptic fraction

In the first set of experiments, the effects of cerebral hypoperfusion on protein activation and expression of pro- and anti-apoptotic molecules at different time points were examined in synaptosomal fractions obtained from the cerebral cortex of control and 2VO male and female rats.

Expression of Bcl-2 and Bax proteins

The results revealed a considerably reduced level of Bcl-2 protein in 2VO rats on postoperative days 3 (62.96 ± 3.53, $P < 0.001$ in males and 76.65 ± 2.78, $P < 0.01$ in females) and 7 (55.19 ± 4.79, $P < 0.001$ in males and 71.45 ± 4.04, $P < 0.01$ in females) (Fig. 1). The observed down-regulation of Bcl-2 was more prominent in male rats compared to females on postoperative days 3 ($P < 0.05$) and 7 ($P < 0.05$).

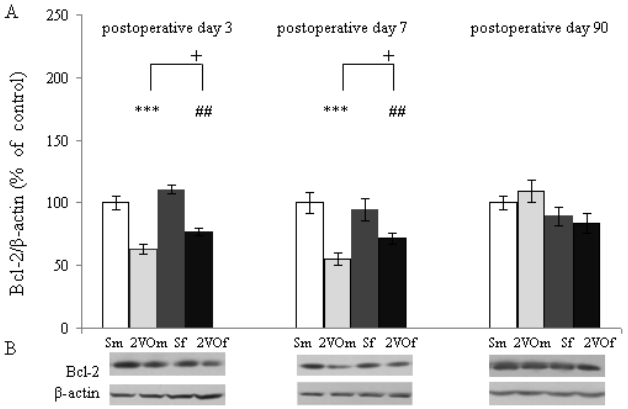

Fig. 1. The effect of cerebral hypoperfusion on Bcl-2 protein abundance in male and female rat cortical synaptosomal fraction on postoperative days 3, 7 and 90

Panel **A**: The relative abundances of Bcl-2 in cortical synaptosomal fraction of sham-operated (S) and bilaterally occluded (2VO) male (m) and female (f) rats were expressed as percent of appropriate male control (mean ± SEM). Intergroup statistical analysis on postoperative day 3 ***$P < 0.001$, Sm vs. 2VOm; ##$P < 0.01$, Sf vs. 2VOf; postoperative day 7 ***$P < 0.001$, Sm vs. 2VOm; ##$P < 0.01$, Sf vs. 2VOf. Intragroup statistical analysis on postoperative day 3 +$P < 0.05$, 2VOm vs. 2VOf; and postoperative day 7 +$P < 0.05$, 2VOm vs. 2VOf.

Panel **B**: Representative Western blots of Bcl-2 protein in cortical synaptosomal fraction of sham-operated (S) and bilaterally occluded (2VO) male (m) and female (f) animals at three investigated time points.

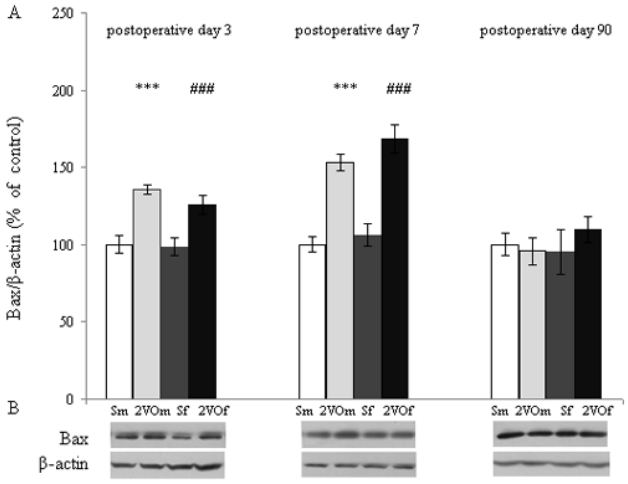

Fig. 2. The effect of cerebral hypoperfusion on Bax protein abundance in male and female rat cortical synaptosomal fraction on postoperative days 3, 7 and 90
Panel **A**: The relative abundances of Bax protein in cortical synaptosomal fraction of sham-operated (S) and bilaterally occluded (2VO) male (m) and female (f) rats were expressed as percent of appropriate male control (mean ± SEM). Intergroup statistical analysis on postoperative day 3 ***P< 0.001, Sm vs. 2VOm; ###P < 0.01 Sf vs. 2VOf; and postoperative day 7 ***P < 0.001, Sm vs. 2VOm; ###P < 0.01 Sf vs. 2VOf. Intragroup statistical analysis showed no considerable differences between male and female rats.
Panel **B**: Representative Western blots of Bax protein in cortical synaptosomal fraction of sham-operated (S) and bilaterally occluded (2VO) male (m) and female (f) animals at all three investigated time points.

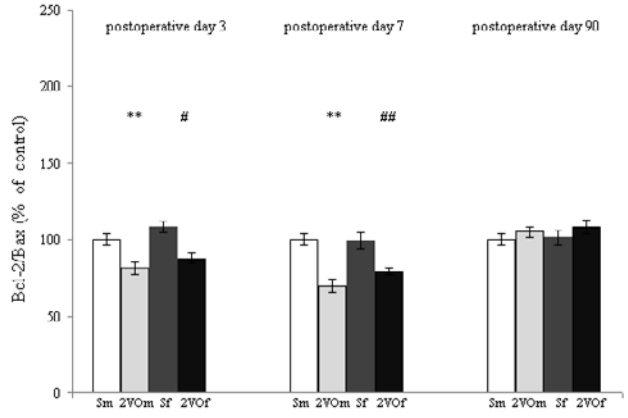

Fig. 3. The effect of cerebral hypoperfusion on the relative ratio of Bcl-2 to Bax in male and female rat cortical synaptosomal fraction on postoperative days 3, 7 and 90
The relative Bcl-2/Bax protein ratio in cortical synaptosomal fraction of sham-operated (S) and bilaterally occluded (2VO) male (m) and female (f) rats was expressed as percent of appropriate male control. The results are expressed as the mean ± SEM. Intergroup statistical analysis on postoperative day 3 **P < 0.01, Sm vs. 2VOm; ##P < 0.05, Sf vs. 2VOf and on postoperative day 7 **P < 0.01, Sm vs. 2VOm; ##P < 0.01, Sf vs. 2VOf.

As shown in Fig. 2, there was a significant increase of Bax expression in both genders on postoperative days 3 (135.69 ± 3.09, P < 0.001 in males and 125.73 ± 6.23, P < 0.001 in females) and 7 (153.09 ± 5.51, P < 0.001 in males and 168.46 ± 9.27, P < 0.001 in females) with no significant difference between male and female experimental groups at all investigated time points.

On postoperative day 90, the expression of both studied proteins was not significantly different between experimental groups in male and female rats (Figs. 1, 2).

Fig. 3 illustrates that Bcl-2/Bax ratio was decreased following the 2VO insult on postoperative days 3 (81.28 ± 4.23, P < 0.01 in males and 87.82 ± 3.51, P < 0.05 in females) and 7 (69.65 ± 4.32, P < 0.01 in males and 79.62 ± 2.34, P < 0.01 in females).

Cerebral hypoperfusion induced formation of active fragments of caspase 3

The state of cerebral hypoperfusion markedly resulted in an increase of procaspase 3 content in cortical synaptosomal fractions isolated on days 3 (125.39 ± 5.72, P < 0.01 in males and 159.56 ± 7.86, P < 0.01 in females) and 7 (132.15 ± 6.64, P < 0.001 in males and 164.78 ± 7.38, P < 0.001 in females) (Fig. 4). The observed up-regulation of procaspase 3 was more promi-

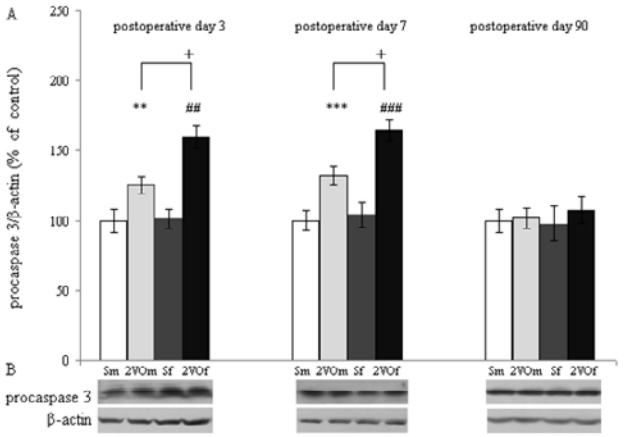

Fig. 4. The effect of cerebral hypoperfusion on procaspase 3 protein abundance in male and female rat cortical synaptosomal fraction on postoperative days 3, 7 and 90
Panel **A**: The relative abundances of procaspase 3 in cortical synaptosomal fraction of sham-operated (S) and bilaterally occluded (2VO) male (m) and female (f) rats were expressed as percent of appropriate male control (mean ± SEM.) Intergroup statistical analysis on postoperative days 3 **P < 0.01, Sm vs. 2VOm; ##P < 0.01 Sf vs. 2VOf; and on postoperative day 7 ***P < 0.001, Sm vs. 2VOm; ###P < 0.001 Sf vs. 2VOf. Intragroup statistical analysis on postoperative days 3 +P < 0.05, 2VOm vs. 2VOf; and on postoperative day 7 +P < 0.05, 2VOm vs. 2VOf.
Panel **B**: Representative Western blots of procaspase 3 in cortical synaptosomal fraction of sham-operated (S) and bilaterally occluded (2VO) male (m) and female (f) animals at three investigated time points.

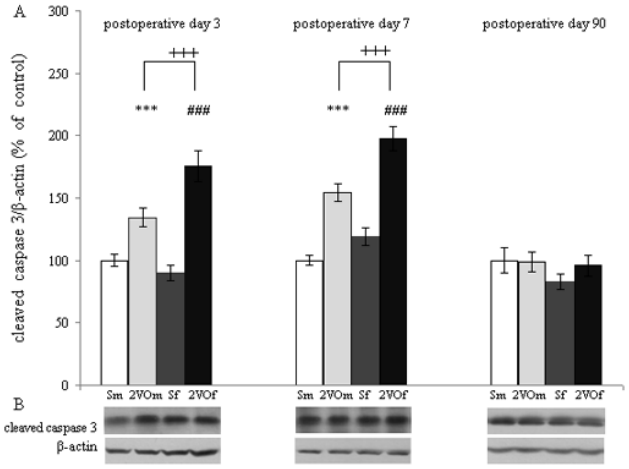

Fig. 5. The effect of cerebral hypoperfusion on cleaved caspase 3 protein abundance in rat cortical synaptosomal fraction on postoperative days 3, 7 and 90
Panel **A**: The relative abundances of cleaved caspase 3 in cortical synaptosomal fraction of sham-operated (S) and bilaterally occluded (2VO) male (m) and female (f) rats were expressed as percent of appropriate male control (mean ± SEM). Intergroup statistical analysis on postoperative day 3 ***$P < 0.001$, Sm vs. 2VOm; ###$P < 0.001$ Sf vs. 2VOf; and on postoperative day 7 ***$P < 0.001$, Sm vs. 2VOm; ###$P < 0.001$ Sf vs. 2VOf. Intragroup statistical analysis on postoperative day 3 +++$P < 0.001$, 2VOm vs. 2VOf; and on postoperative day 7 +++$P < 0.001$, 2VOm vs. 2VOf.
Panel **B**: Representative Western blots of cleaved caspase 3 in cortical synaptic fractions of sham operated (S) and bilaterally occluded (2VO) male (m) and female (f) animals at three investigated time points.

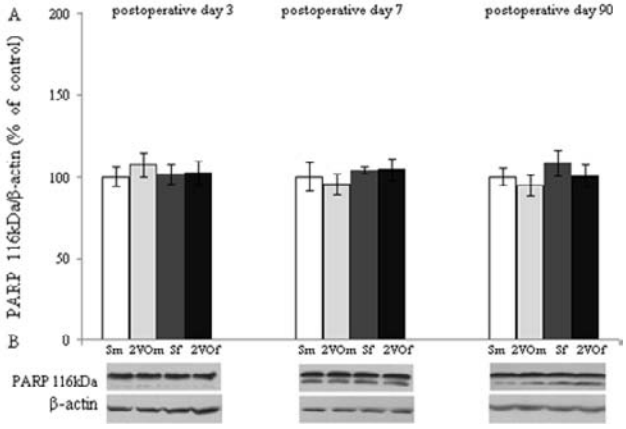

Fig. 6. The effect of cerebral hypoperfusion on PARP full-length protein (116 kDa) abundance in rat cortical synaptosomal fraction on postoperative days 3, 7 and 90
Panel **A**: The relative abundances of PARP full-length protein in cortical synaptosomal fraction of sham-operated (S) and bilaterally occluded (2VO) male (m) and female (f) rats were expressed as percent of appropriate male control (mean ± SEM).
Panel **B**: Representative Western blots of PARP full-length protein in cortical synaptosomal fractions of sham-operated (S) and bilaterally occluded (2VO) male (m) and female (f) animals at three investigated time points.

nent in female rats when compared to males on postoperative days 3 ($P < 0.05$) and 7 ($P < 0.05$). On the other hand, the expression of this protein on post-lesion day 90 was similar between control and 2VO groups in both genders.

On postoperative days 3 and 7, a significant increase of caspase 3 active fragment of 17 kDa was detected in 2VO samples of both genders (on postoperative day 3 134.46 ± 7.43, $P < 0.001$ in males and 175.46 ± 12.49, $P < 0.001$ in females; while on postoperative 7 it was 154.46 ± 6.64, $P < 0.001$ in males and 197.47 ± 9.59, $P < 0.001$ in females) (Fig. 5). The observed generation of protein fragments resulting from caspase 3 activity was more pronounced in females on postoperative days 3 ($P < 0.001$) and 7 ($P < 0.001$). There was no significant difference in the expression of 17 kDa subunits in the cerebral cortex of both female and male rats 90 days following the 2VO insult (Fig. 5).

Changes in PARP proteolysis induced by cerebral hypoperfusion

No significant difference in the expression of 116 kDa full-length PARP was detected at all investigated time points following cerebral hypoperfusion (Fig. 6). However, quantification of the 89 kDa cleavage product showed considerable augmentation on postoperative days 3 (143.15 ± 6.38, $P < 0.001$ in males and 195.34 ± 6.07, $P < 0.001$ in females) and 7 (149.27 ± 4.45, $P < 0.001$ in males and 202.06 ± 12.82, $P < 0.001$ in females), which was more evident in females on postoperative days 3 ($P < 0.001$) and 7 ($P < 0.001$). At the latest investigated time point, its expression was near control values in all 2VO groups (Fig. 7), which is consistent with our findings for caspase 3 protein expression.

Time course of cerebral hypoperfusion-induced neurodegeneration

Figs. 8, 9, 10 reveal an increase in the number of Fluoro-Jade B-labelled cells in the cerebral cortex, particularly in prefrontal cortex of control and 2VO male and female rats on postoperative days 3 and 7, while on post-lesion day 90 it was similar between groups in both genders.

A very low degree of neuroapoptosis was observed in all control samples from both genders, as revealed by a minimal number of stained cells (Figs. 8, 9, 10). The most pronounced increase in the number of Fluoro-Jade B-positive cells was apparent in the prefrontal cortex three days following 2VO insult in both male (5.12 ± 0.66, $P < 0.05$) and female (4.73 ± 0.54, $P < 0.05$) rats; their numbers peaked on day 7 (in males 13.87 ± 0.76, $P < 0.05$; and in females 12.51 ± 0.66, $P < 0.05$), while no evident change in their number was detected on the day 90 (Figs. 8, 9, 10). As our results demonstrate, rats

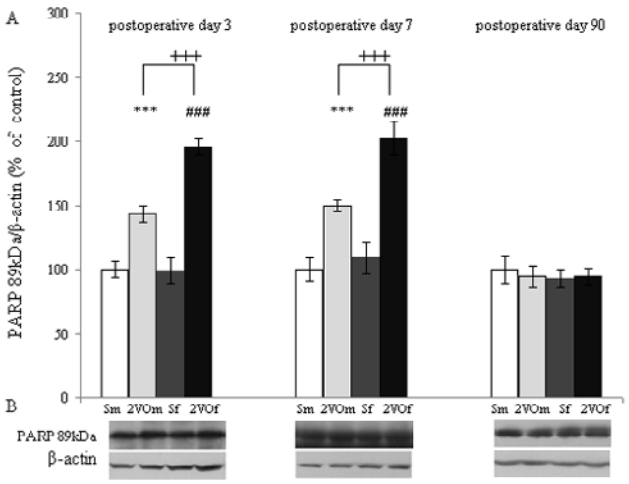

Fig. 7. The effect of cerebral hypoperfusion on cleaved PARP protein (89 kDa) abundance in rat cortical synaptosomal fraction on postoperative days 3, 7 and 90

Panel **A**: The relative abundances of cleaved PARP protein in cortical synaptosomal fraction of sham-operated (S) and bilaterally occluded (2VO) male (m) and female (f) rats were expressed as percent of appropriate male control (mean ± SEM). Intergroup statistical analysis on postoperative day 3 ***$P < 0.001$, Sm vs. 2VOm; ###$P < 0.001$ Sf vs. 2VOf; and on postoperative day 7 ***$P < 0.001$, Sm vs. 2VOm; ###$P < 0.001$, Sf vs. 2VOf. Intragroup statistical analysis on postoperative day 3 +++$P < 0.001$, 2VOm vs. 2VOf; and on postoperative day 7 +++$P < 0.001$, 2VOm vs. 2VOf.

Panel **B**: Representative Western blots of cleaved PARP protein in cortical synaptosomal fractions of sham-operated (S) and bilaterally occluded (2VO) male (m) and female (f) animals at three investigated time points.

from both gender groups were uniformly susceptible to 2VO insult at all investigated time points.

Furthermore, to confirm the presence of cell death in cortical neurons, following Fluoro-Jade B staining, the sections were double-stained with a blue fluorescent DNA stain, Hoechst 33258. In control groups a few cells containing compact foci were detected, while in 2VO animals the number of these cells was significantly increased at both early time points, which indicated that cells were undergoing apoptosis (Figs. 8, 9).

Time course of cerebral hypoperfusion-induced apoptotic DNA fragmentation

As shown in Fig. 11, in response to cerebral hypoperfusion, there was a considerable increase of the content of apoptosis-specific DNA fragments in both male and female animals on postoperative days 3 (136.35 ± 4.50, $P < 0.01$ in males and 134.20 ± 4.39, $P < 0.01$ in females) and 7 (175.30 ± 5.81, $P < 0.001$ in males and 172.55 ± 5.35, $P < 0.001$ in females), while on day 90 it was unchanged. Moreover, there was no significant difference between gender groups at all investigated time points (Fig. 11).

Discussion

Although previous studies have shown that neurons undergo apoptosis after cerebral ischaemia (Li et al., 1995; Ni et al., 1998), to our knowledge there are no available data concerning the time- and gender-specific effects of cerebral hypoperfusion on the occurrence of apoptotic changes and finally apoptosis in the cerebral cortex. The current study has started to address this matter by examining the gender response and time window of the implication of Bcl-2 family members along with caspase 3 and PARP in this process in synaptosomal fraction. Our results revealed several interesting findings. First, the time-specific up-regulation of Bax as well as time- and gender-associated changes in the protein levels of procaspase 3, accompanied with increased cleavage of procaspase 3 and PARP as well as reduced levels of Bcl-2, emerged in the synaptosomal fraction as a consequence of persistent reduction of cerebral blood flow. Second, time- but not gender tissue-specific damage consistent with appearance of neurodegeneration was observed in this particular brain area.

The activation of caspase cascade, especially initiation of caspase 3, represents a crucial step in intrinsic mitochondrial-mediated apoptotic cell death in different models of ischaemia (Gill et al., 2002). The augmentation in the procaspase 3 mRNA level was previously demonstrated after focal or global ischaemia in rats (Ni et al., 1998) and in the 2VO model on postoperative days 1–3 (Tomimoto et al., 2003), while up-regulation of the caspase 3 protein level was observed in the period from day 1 to 30 post surgery (Tomimoto et al., 2003). In cortical synaptosomes, caspase activation arises following different apoptotic insults within 1–2 h and peaks later (Mattson and Duan, 1999), which is consistent with our findings.

The literature highlights a large number of caspase substrates such as procaspases (Nunez et al., 1998), Bcl-2 family members (Cheng et al., 1997), PARP (Yang et al., 2004), cytoskeletal proteins such as actin and spectrin (Mashima et al., 1997), protein kinases (Datta et al., 1997), amyloid precursor protein (Barnes et al., 1998), presenilins (Kim et al., 1997), etc. The cleavage of some caspase 3 substrates contributes to biochemical and morphological changes associated with apoptosis (e.g., nuclear disintegration and membrane blebbing), whereas degradation of others modifies function of different cellular systems that further modulate the apoptotic process (e.g., calcium influx and mitochondrial function) (Mattson and Duan, 1999). Hence, the observed time-dependent and gender-specific elevated levels of cleaved caspase 3 and PARP, along with the alterations in the expression of Bcl-2 family members, illustrate the involvement of caspase 3 in the specific changes that occur during cerebral hypoperfusion.

In response to cerebral hypoperfusion, we found more pronounced protein expression of procaspase 3, increase of active cleaved caspase 3 and augmentation of caspase-generated 89 kDa PARP fragment in synaptic

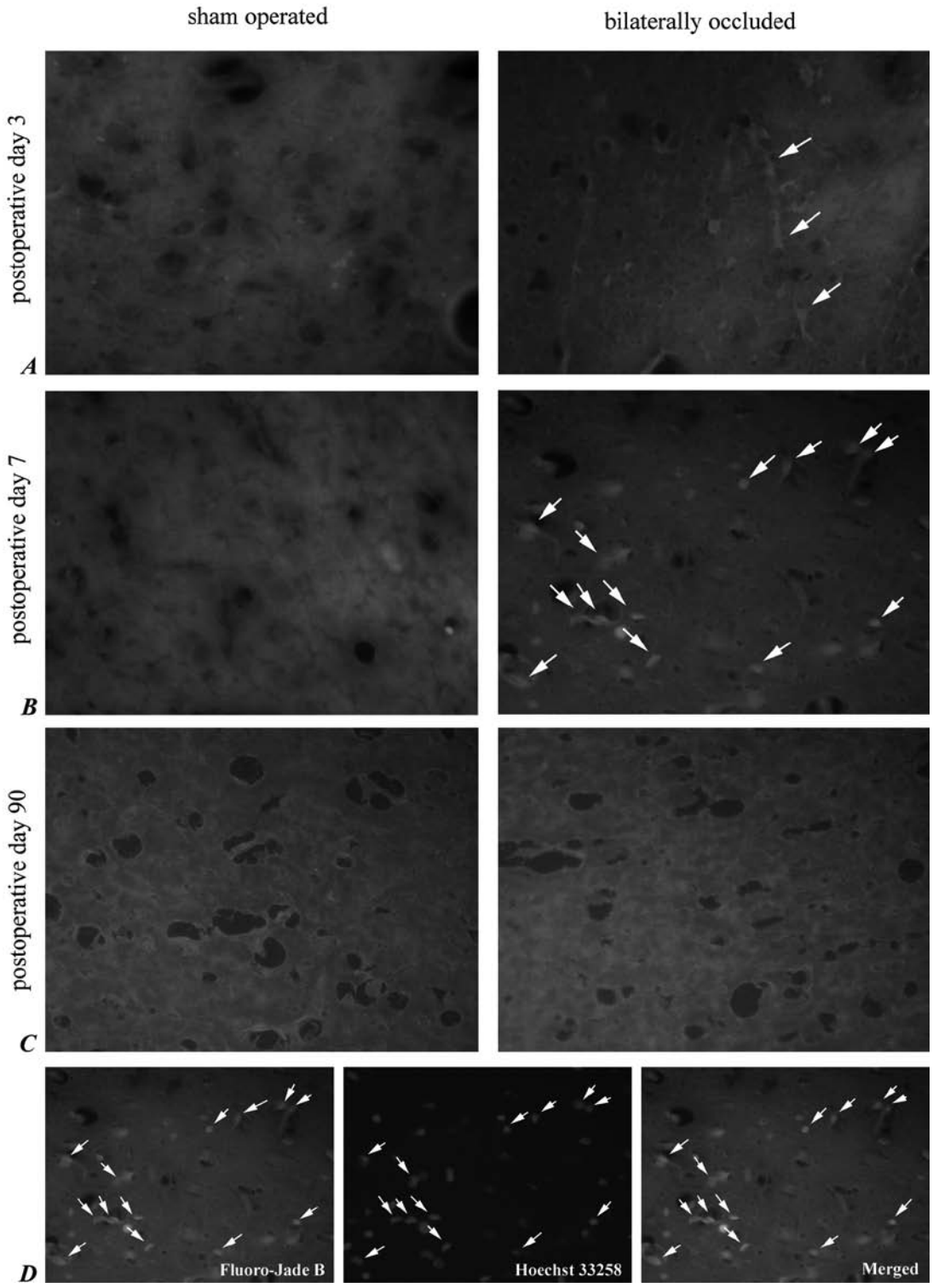

Fig. 8. Fluoro-Jade B staining of adult male Wistar rat brains at three different time points following permanent common carotid occlusion (2VO)
Fluoro-Jade B staining of adult male rat brains, section prefrontal cortex, 3 (**A**), 7 (**B**) and 90 (**C**) days following sham operation and bilateral occlusion. Male prefrontal cortical sections were additionally stained with Hoechst 33258, a blue fluorescent DNA stain, alongside Fluoro-Jade B (**D**). Some neurons revealed bright clumps of fragmented and condensed chromatin, typical of apoptotic nuclei, as well as positive green staining for Fluoro-Jade B.

terminals of females compared to males. These results strongly support gender-specific cellular-related response and more powerful activation of caspase-dependent pathway in females versus males, which is in agreement with findings of other studies that investigated different models of ischaemia (McCullough et al., 2005; Zhu et al., 2006; Liu et al., 2009). Therefore, our data represent a novel and valuable observation for the

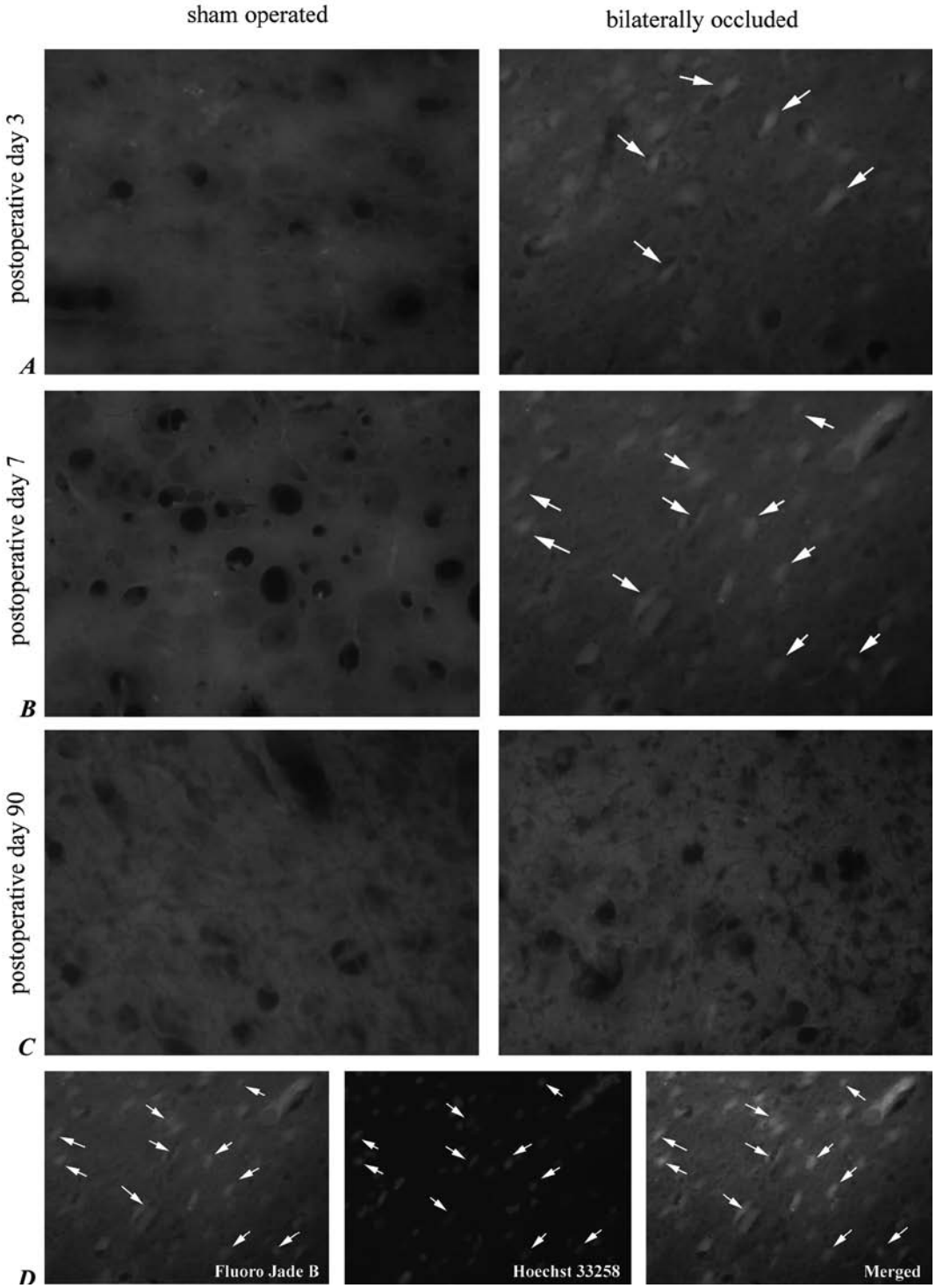

Fig. 9. Fluoro-Jade B staining of adult female Wistar rat brains at three different time points following permanent common carotid occlusion (2VO)
Fluoro-Jade B staining of adult female rat brains, section prefrontal cortex, 3 (**A**), 7 (**B**) and 90 (**C**) days following sham operation and bilateral occlusion. Female prefrontal cortical sections were additionally stained with Hoechst 33258, a blue fluorescent DNA stain, alongside Fluoro-Jade B (**D**). Some neurons revealed bright clumps of fragmented and condensed chromatin, typical of apoptotic nuclei, as well as positive green staining for Fluoro-Jade B.

2VO model that requires further investigation and might contribute to better understanding of ischemic mechanisms in both genders.

Several studies demonstrated the significance of Bcl-2 family members and their relative ratio in the progression of various diseases associated with impairment of cerebral blood flow. For instance, the increase of Bax and decrease of Bcl-2 was detected after focal and global ischaemia (Krajewski et al., 1995). After forebrain ischaemia, the expression of Bax precedes DNA frag-

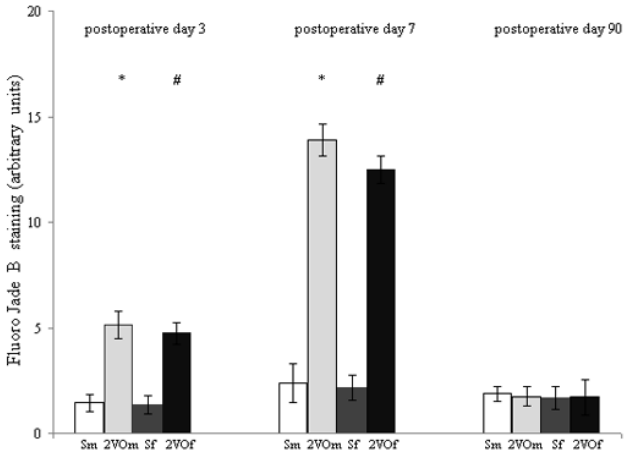

Fig. 10. Fluoro-Jade B staining of adult male and female Wistar rat brains at three different time points following permanent common carotid occlusion (2VO)
Quantitative analysis of Fluoro-Jade B staining of adult male and female rat prefrontal cortical sections. Data are presented as means ± SEM. A probability value of *P < 0.05 was considered significant.

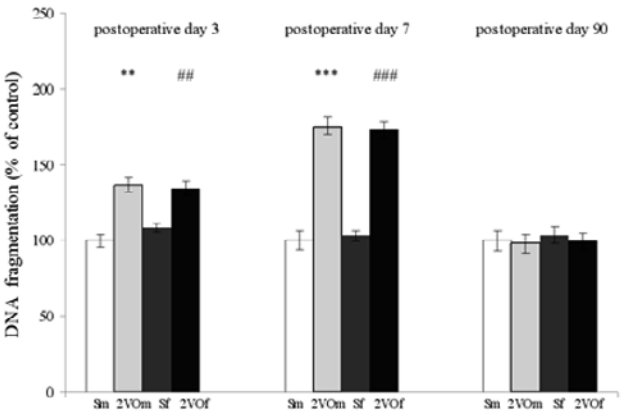

Fig. 11. Measurement of DNA fragments with the diphenylamine (DPA) colorimetric assay
Results are presented as a percent of control fragmentation. T = fragmented DNA, B = intact DNA obtained from male (m) and female (f) rats subjected to sham operation (S) and bilateral occlusion (2VO) on postoperative days 3, 7 and 90 days. Intergroup statistical analysis on postoperative day 3 **P < 0.01, Sm vs. 2VOm; ##P < 0.01 Sf vs. 2VOf; postoperative day 7 ***P < 0.01, Sm vs. 2VOm; ###P < 0.01 Sf vs. 2VOf.

mentation in the hippocampus of gerbils (Hara et al., 1996), while in rat a simultaneous increase of Bax expression accompanied with a decrease of Bcl-2 protein in the same neuronal population is associated with cell death following the ischemic insult (Isenmann et al., 1998). Our results suggest that the observed modified levels of Bcl-2 and Bax proteins as well as decrease of Bcl-2/Bax protein ratio, associated with up-regulation of caspase 3 and cleavage of PARP in synaptic terminals, are crucial events in apoptotic occurrence on postoperative days 3 and 7 in both genders.

Given that Bcl-2 is an oestrogen-responsive gene in the brain tissue, its expression is elevated in oestrous and oestradiol-treated rats (Garcia-Segura et al., 1998), and injury-induced changes of these proteins are prevented by oestradiol (Won et al., 2005), we are tempted to speculate that female gonadal hormones (oestradiol and progesterone) might vary the levels of this molecule in ischemic conditions and also alleviate the injury-induced down-regulation of Bcl-2 observed in females when compared to males.

Various physiological and pathological settings consider signalling events in synaptic terminals as decisive in either promoting (e.g., activation of glutamate receptors in postsynaptic spines) or preventing (e.g., activation of neurotrophic factors in presynaptic terminals) neuronal cell death (Mattson and Duan, 1999). According to the presented findings, the investigated proteins that cause local functional and morphological alterations could activate the apoptotic biochemical cascade in a time- and gender-specific manner regionally in synaptic compartments. To intensify our observations, a few additional experiments were conducted. They point to time-specific appearance of apoptotic cells in cerebral cortex following cerebral hypoperfusion. On postoperative days 3 and 7, elevated numbers of Fluoro-Jade B positive cells followed by double staining with Hoechst 33258 and increased DNA fragmentation were proportional to the duration of cerebral hypoperfusion in both male and female rats. Since the presence of apoptotic cells is eliminated within a few hours and days (Li et al., 1995), the obtained data demonstrate that the initiation of apoptosis is ongoing increasingly over several days following the actual insult, while these sinister stimuli appear to exert detrimental effects over a prolonged but definite period of time.

Although our findings provided evidence of a time-specific extent of ischemic damage, they did not provide evidence of gender-associated response. We assume that several other pathways on postoperative days 3 and 7 in synaptic terminals, besides the activation of caspase 3, are involved in biochemical and morphological changes that contribute to the apoptosis occurrence in males.

As expected, compared to control rats, male and female animals with cerebral hypoperfusion on post-lesion day 90 demonstrated no significant alterations in the expression of investigated apoptotic molecules as well as the lack of apoptosis. Our findings have confirmed that the cell damage could be decreased as time elapses following the injury, most likely by the activation of adaptive and compensatory mechanisms, normalization of oxygen and glucose levels through cerebral blood flow restoration (Farkas et al., 2007).

In conclusion, the current study directly indicates that cerebral cortex exerts a time-specific tissue response to persistent reduction of cerebral blood flow. Our findings imply that the time-dependent increase of caspase 3, along with cleavage of PARP and apoptosis, are inversely related to the levels of Bcl-2 and equally related to the levels of Bax protein. Furthermore, there were no gen-

der differences in the extent of apoptosis; however, the mechanisms of neuronal cell death occurrence were gender-dependent. The activation of the caspase-dependent pathway was more prominent in females, whereas additional apoptotic pathways are most likely involved in males. The activation of these signalling pathways should be further and better investigated.

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