Original Article

www.anatomy.org.tr
Recieved: April 14, 2008; Accepted: June 4, 2008; Published online: October 31, 2008
doi:10.2399/ana.08.039

anatomy
International Journal of Experimental and Clinical Anatomy

Ipsilateral and contralateral cortical apoptosis in rats after unilateral middle cerebral artery occlusion

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Abstract

Cerebral ischemia and subsequent reperfusion is known to induce irreversible tissue damage with the consequence of more or less pronounced impairments. The underlying cellular and molecular mechanisms however are far from being understood today. In the present study, we employed the well established rat model MCAO (middle cerebral artery occlusion), which uses a unilateral ligature of the middle cerebral artery for a defined period of time, prior to reperfusion. Animals develop symptoms, comparable to stroke in humans. Brains of such animals were used for histochemical and biochemical evaluation of markers, indicating programmed cell death (apoptosis) as evidenced by presence of tissue transglutaminase stained cells, DNA fragmentation and presence of cytochrome c in both cerebral hemispheres ipsilateral and contralateral to occlusion.

Key words: cerebral ischaemia; reperfusion; apoptosis; cytochrome c; DNA fragmentation; tissue transglutaminase

Anatomy 2008; 2: 39-48, © 2008 TSACA

Introduction

Stroke is the third leading cause of death in the developed countries and the leading cause of major adult disability. It was projected that, if the age-specific rates of stroke remain unchanged, the overall number of strokes in the United States will increase from approximately 700,000 in 2002 to 1136,000 in 2025, owing to the rising percentage of the population in older groups. Among the stroke patients, 85-90% of the cases are ischemic stroke with a predominant cause of cerebral arterial thrombosis.[1,2]

Brain injury following transient or permanent focal ischaemia (stroke) develops from a complex series of pathophysiological events that evolve over time and space.[3] These pathophysiological processes that contribute to neuronal death are discussed independently, but they are inter-related and dependent on each other to some extent. For example, most of the effects of increased intracellular calcium (Ca^{2+}) are secondary to energy failure and (Ca^{2+}) in turn triggers many reactions leading to generation of free radicals. Free radicals can aggravate processes leading to increased intracellular (Ca^{2+}).[4]

The mechanisms underlying the ischaemic damage include; energy failure and loss of ion homeostasis, excitotoxicity, loss of calcium homeostasis, acidosis, cytokines and inflammation, alteration in systems regulating protein synthesis, local microvascular response to ischaemia-reperfusion (I/R), generation of free radicals, changes in genes expression and cell death either apoptotic or necrotic cell death.[5,6]

Reperfusion injury was considered by many authors as an essential cause of damage following transient cerebral ischaemia. Reperfusion is the restoration of blood flow after a period of ischaemia.[7] Reperfusion is essential for saving the ischaemic brain tissue from irreversible infarction. However, it can place the ischaemic tissue at risk of further cellular necrosis.[8] It can save the ischaemic tissues if occlusion of the artery is corrected within 3-4 hours, therefore, the neurons in the penumbra can recover completely. However, if reperfusion is performed very late after the insult, it can produce deleterious effects due to several factors as; aggravation of the vasogenic oedema, disruption of blood brain barrier (BBB), free radical and toxic oxygen production, release of inflammatory mediators and adhesion of leucocytes to the endothelial cells causing microcirculatory occlusions which contributes to the occurrence of no-reflow phenomenon, that defined as the inability to reperfuse the infarcted areas or delayed decrease in CBF that occur after reperfusion.[5]

Brain cells that are compromised by excessive glutamate-receptor activation, Ca^{2+} overload, and oxygen radicals or by mitochondrial and DNA damage can die by necrosis or apoptosis. The decision in part, depends on the nature and intensity of the stimulus, the type of cells, and the stage it has reached in its life cycle or development. Necrosis is the predominant mechanism that follows acute, permanent vascular occlusion, whereas in milder injury, cell suicide becomes unmasked and death resembles apoptosis, particularly within the ischaemic penumbra occurs.[9]

Several studies, however, reported the occurrence of various functional alterations within the structurally intact brain regions, ipsilateral and contralateral to the focal ischaemia. These changes were termed as remote effects. Remote effects in turn, were divided into ipsilateral or contralateral effects; these contralateral effects were attributed to the extensive interhemispheric connections which existed in the rat cortex. Additionally, other mechanisms such as cerebral oedema, free radical formation or general stress response may contribute to the remote effects.[10,11]

Animal stroke models have been used extensively in studying the pathophysiology of cerebral ischemia and in screening novel neuroprotective agents that may be applied to treat patients with strokes.[12] Desirable animal models are those that replicate features of human cerebrovascular syndromes. Rodent stroke models are divided into two types: focal and global cerebral ischemic models. The focal cerebral ischemic models are divided into permanent and transient models according to the stroke researcher's design. Of these methods for replicating ischemia, the use of MCAO is the most prevalent. Permanent MCAO can be produced using one of the following three techniques: direct ligation of the MCA by craniotomy, and intraluminal suturing without craniotomy, either via the ECA or via the CCA.[13,14]

Materials and Methods

This study was performed on 40 male adult albino rats. Animals were housed individually at room temperature and standard condition with free access to standard laboratory diet, tap water ad libitum. All experimental procedures were conducted according to a protocol approved by the Governmental Animal Care Committee, Germany.

The animals were assigned to four experimental groups:

Group 1: Ten sham-operated rats were kept on standard diet serve as control.

Group 2: 10 rats were subjected to cerebral ischaemia for 30 minutes.

Group 3: 10 rats were subjected to cerebral ischaemia for 60 minutes.

Group 4: 10 rats were subjected to cerebral ischaemia for 90 minutes.

The animals in the experimental groups were subjected to transient middle cerebral artery occlusion. The animals were fasted overnight prior to surgery with free access to tap water. After complete anaesthesia, rats were placed on their back and a ventral midline cervical incision was made to isolate both sides of CCA. Clamping the left CCA by microvascular clips produced transient focal cerebral ischaemia. A 4-0 silicone- coated filament was introduced into the internal carotid artery between

16 and 17.5 mm (as measured from carotid bifurcation) until a faint resistance was felt. The filament was secured in this position to occlude the left middle cerebral artery. After occlusion times of 30, 60 and 90 minutes, the vascular clips and the intraluminal thread were removed. Both neck and cranial skin incision were closed. After surgery, animals were allowed to awake, and then they were transferred to their cages and reperfused for 7 days.[10,11]

In animals receiving sham operation, all surgical procedures were the same as above, but no arterial occlusion was performed. The rats were killed at the 7th day of reperfusion by prolonged ether anaesthesia. Rats were perfused through the left ventricle with 200 ml of 4% paraformaldehyde diluted in isotonic phosphate buffer (PBS) (0.1 mol/l, pH 7.2). Brains were then removed, kept for 2 days in the same fixative solution. Brains were cut on a vibratome (Cambridge instruments) into frontal sections of 100 µm thickness. Sections were stored in PBS for further use at 4 C°.

The sections were then used for estimation of the following parameters: (All chemicals were obtained from Sigma Chemical Co., Germany unless otherwise stated).

1. Estimation of DNA fragmentation by terminal deoxynucleotidyl transferase mediated dUTP-biotin nick end labeling (TUNEL method)

Vibratome sections were processed and immunocytochemistry was performed with a mouse anti- BrdU monoclonal antibody (Progen- Heidelberg) and goat anti-mouse secondary antibody as described by Aschoff et al.[15]

2. Anti- tissue- transglutaminase staining

Because sole confirmation of DNA fragmentation is not a sufficient marker for apoptosis, immunohistochemistry for type II tissue- transglutaminase was performed in addition to detection of DNA fragmentation. Vibratome sections were incubated with anti-tissue-transglutaminase type II (anti type II transglutaminase, goat polyclonal antibody, at a dilution of 1:1000 with 1% rabbit serum in phosphate buffered saline) for 24 h at 4 C°. The sections were then incubated with secondary antibody, rabbit antigoat antibody (at a dilution of 1: 100 with PBS) for 1 h at room temperature. Third antibody was then used which is goat peroxidase-antiperoxidase (in a dilution of 1:100 with PBS) for 1 h at room temperatureas described by Aschoff et al.[16]

3. Estimation of cytochrome c by immunoassay

In ELISA plate 20 µl of the homogenate was applied to each well. Sheep anticytochrome c antibody was added, (diluted with PBS triton, i.e. 0.1 M phosphate buffer PH 7.2 containing 0.5% tritonX-100 PBST 1:1000) to each well, and was incubated over night at 4 C°. The second antibody biotin labeled anti-sheep immunoglobulin was applied (diluted 1:100 with PBS triton) for 30 minutes at room temperature. Then third antibody streptavidin horseradish- peroxidase was added, (diluted 1: 100 with PBS triton) for 30 minutes.

The plate then was air-dried and evaluated using grey level reading software, flat Scan and a desktop scanner. Mean values of grey scale density were established from quadruplicate samples and standard curves were plotted (as previously described by Jirikowski, Groping et al., and Ekes et al.[17-19]).

Results

Operative results

Group 1 (Sham operated animals): Showed no infarction.

Group 2: After 30 minutes of MCAO occlusion, 3 animals showed no macroscopic evidence of infarction and 7 animals showed cortical infarcts; 2 of them had both cortical and subcortical infarcts. After 60 minutes of left CCA occlusion all animals developed cortical infarction and 4 of them had both cortical and subcortical infarcts. The same findings were observed after 90 minutes of ischaemia. The infarct, however, developed in both cerebral hemispheres in 2 of the studied animals.

Cytochrome c immunoassay (ng/µg protein) in the experimental groups in left and right cerebral hemisphere (Figures 1 and 2)

Cytochrome c was estimated in left cerebral hemisphere homogenate. Group I (sham operated group) had a mean value of 0.33±0.16 (ng/µg protein). In group II, no

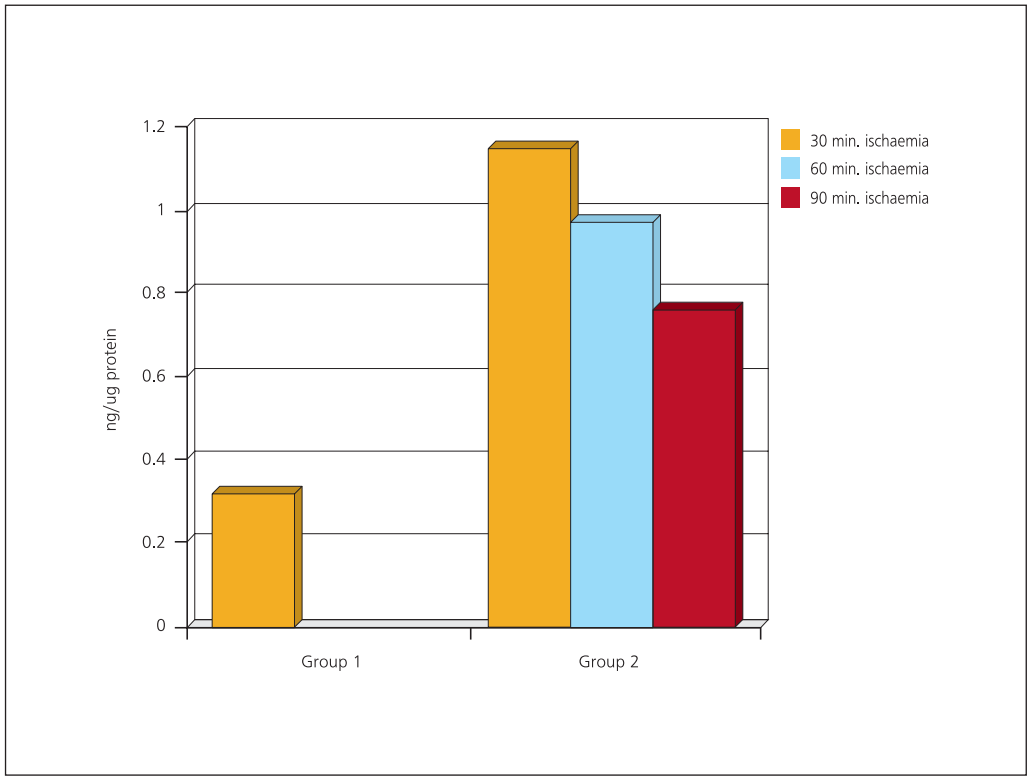

Figure 1. Cytochrome c assay in the experimental groups.

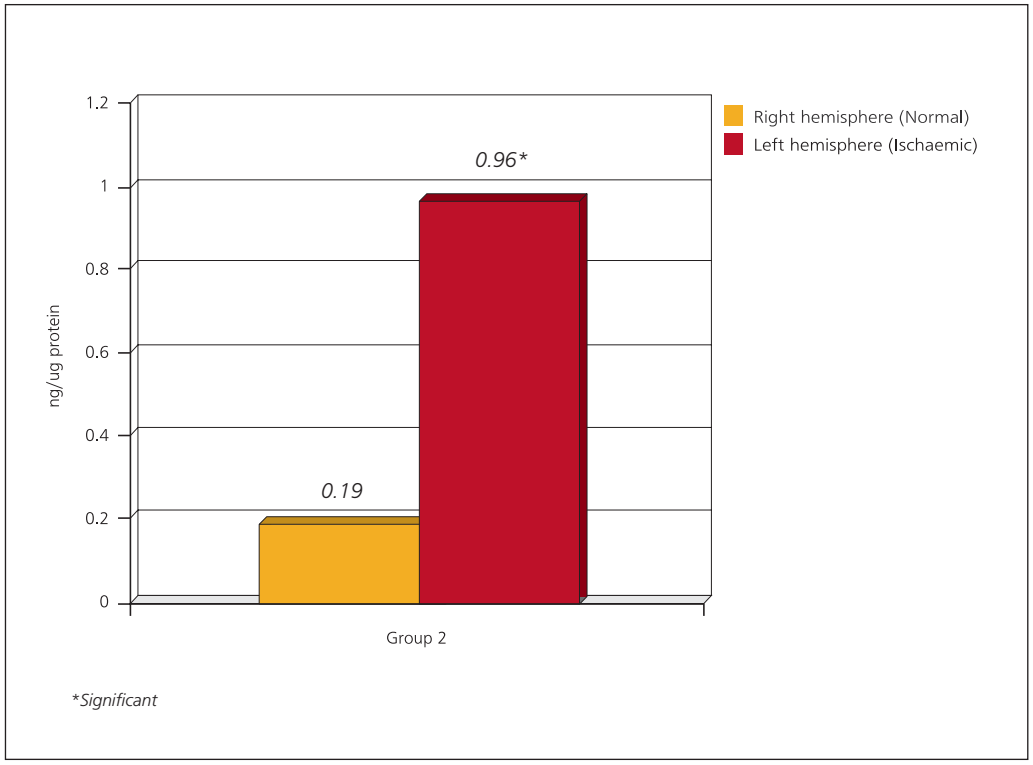

Figure 2. Cytochrome c (ng/µg tissue protein) in the right and left cerebral hemispheres in group II.

significant difference was detected in the mean values of cytochrome c after 30, 60 and 90 minutes of ischaemia. The mean values were 1.15±0.58 ng/µg protein, 0.99±0.96 ng/µg protein and 0.74±0.32 ng/µg protein respectively (F= 0.92, P= 0.41). The mean value of cytochrome c in the right cerebral hemisphere (normal hemisphere) in groups II was 0.19±0.13 ng/µg protein. t-test was done to compare the mean value of cytochrome c content in both sides the brain. Cytochrome c content was significantly higher in the left hemisphere in group II, t= 5.93.

Tissue transglutaminase positive cells in the studied animals (Figures 3-5)

In group I, single immune-stained transglutaminase (tTG) positive cell was seen in the supra-optic nucleus of the hypothalamus (SON). Most probably it was a microglia with a mean diameter of 15-20 µm (the microglia normally showed the process of turnover). Moreover, these animals also showed tTG positive cells in the periventricular nucleus of the hypothalamus (PVN), these cells were in close proximity to blood vessels and they appeared to be perivascular microglia.

In group II, animals subjected to 30 minutes ischaemia; tTG positive cells were intense in the cortical areas (especially peri-infarct zone), piriform cortex and the motor cortex. Moreover, few scattered cells were observed in the amygdala and SON. After 60 minutes of ischaemia, there was a reduction of tTG staining in peri-infarct zone. However, very pronounced labeling was seen in the hypothalamus, thalamus specially the periventricular nucleus, paraventricular nucleus and the SON. The labeling was positive on the contralateral side as well. With prolonged duration of ischaemia to 90 minutes in the same group, the tTG positive cells were diminished in the striatum and peri-infarct zone. Similarly, the cortex showed no stained cells. However, SON contained intensely labelled cells, and the paraventricular nucleus showed few positive cells bilaterally.

DNA fragmentation in the studied groups (Figures 6 and 7)

DNA fragmentation was detected by the presence of TUNEL positive nuclei. None of the sham operated

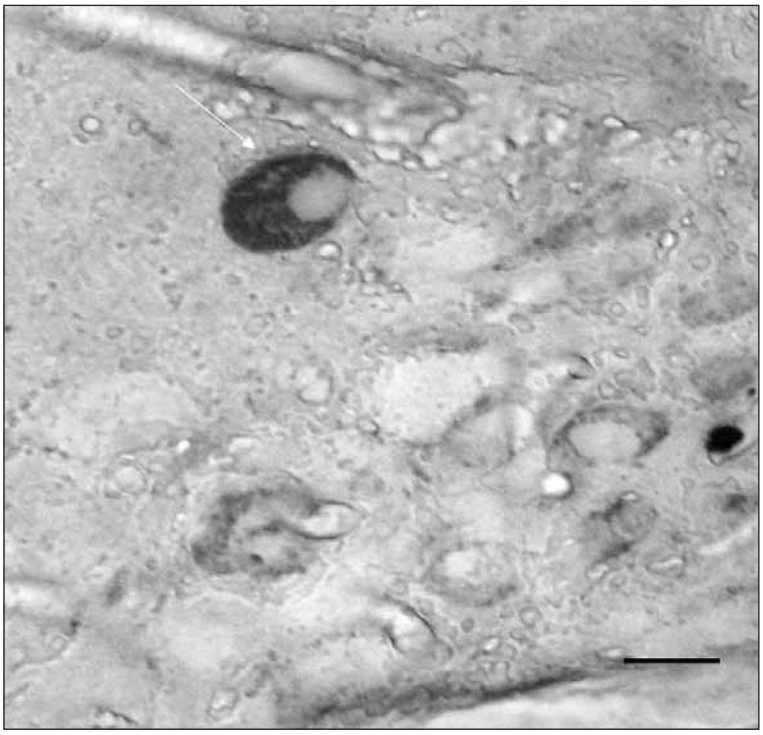

Figure 3. tTG positive cells in supraoptic nucleus of a sham operated animal (group I). Scalebar= 10 µm.

animals had showed any labeled cells. However, in group II, rats subjected to 30 minutes ischaemia, exhibited TUNEL positive nuclei (nuclei showed DNA fragmentation) in the peri-infarct zone, while the rest of the brain was devoid of staining. Increased duration of ischaemia to 60 and 90 minutes reduced the TUNEL positive nuclei in the peri-infarct zone. However, TUNEL positive cells were observed in the ipsilateral and contralateral sides of the motor cortex.

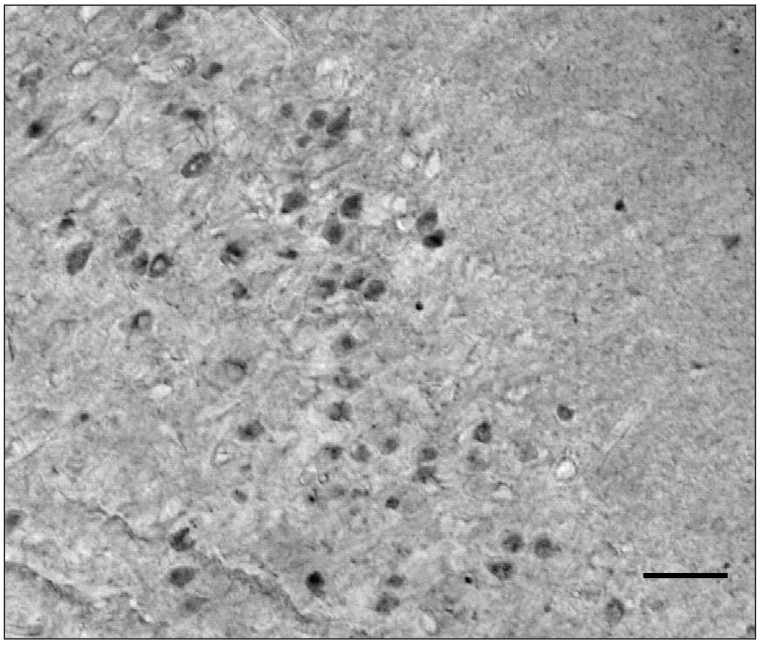

Figure 4. tTG positive cells after 60 minutes ischaemia in the contralateral cortex in group II. Scalebar= 50 µm.

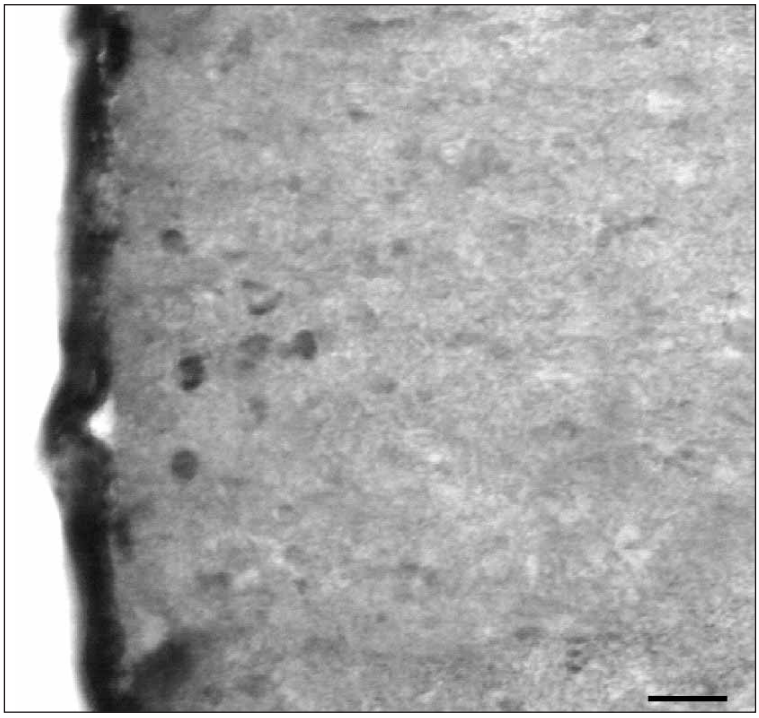

Figure 5. tTG positive cells in the ipsilateral cortex in group II. Scalebar= 25 µm.

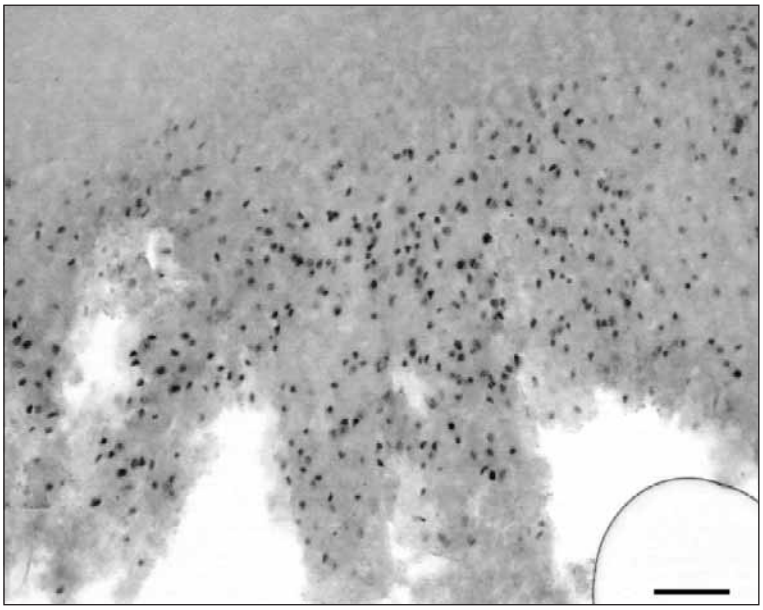

Figure 6. TUNEL-stained nuclei in the peri-infarct zone after 30 minutes ischaemia. Scalebar= 150 μm.

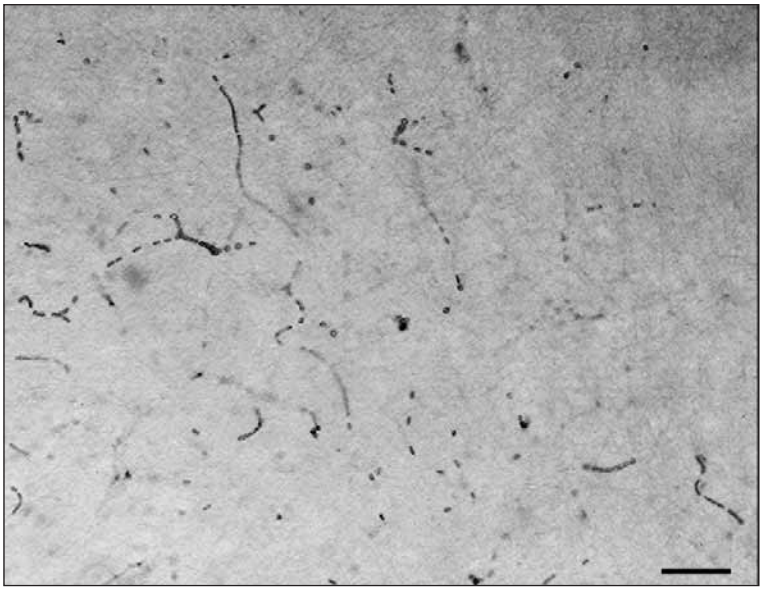

Figure 7. Positive stained nuclei with DNA fragments in the ipsilateral cortex. Scalebar= 150 μm.

Discussion

Regarding the operative findings

In the present study, ipsilateral infarcts were reported in 28 of animals subjected to common carotid artery (CCA) and middle cerebral artery (MCA) occlusion for 30, 60 and 90 minutes and reperfused for 7 days. The most striking finding was the development of infarcts in the contralateral hemisphere in 2 of the studied animals. Redecker et al.[11] reported the occurrence of various functional alterations within the structurally intact brain regions, ipsilateral and contralateral to the focal ischemia. These changes were termed as remote effects. Remote effects in turn, were divided into ipsilateral or contralateral effects; these contralateral effects were attributed to the extensive interhemispheric connections which existed in the rat cortex. Additionally, other mechanisms such as cerebral oedema, free radical formation or general stress response may contribute to the remote

effects.[10] On the other hand, these effects may be viewed as a form of (diaschisis), the term diaschisis was initially coined by Von Monakow[20] to describe transient remote effects after central nervous system injury attributable to the deafferentation of regions connected to the damaged area.

Other contributing factor to the development of infarct and apoptosis in the contralateral hemisphere is the role played by the neurotrophic factors (growth factors) in maintaining the vitality of the neurons. These neurotrophic factors are permanently produced in the nerve cells; they are essential for survival of the target cell and maintain the synaptic contact between neurons. Death of neurons by ischaemia leads to lack in neurotrophic factors production and therefore, death of the neighboring cells (anterograde degeneration).[21]

The effect of ischaemia on cytochrome c content of the brain

The mitochondria are the key intracellular site for sensing various death-inducing stimuli and transmitting them to the executional process of apoptosis. Release of stable intermembrane protein such as cytochrome c and apoptosis inducing factor from the mitochondria into the cytosol is a crucial step in the mitochondrial apoptotic signaling pathway.[22]

The current study revealed the presence of cytochrome c in the sham operated animals (group 1), this could be due to that cytochrome c is naturally present in the space between the outer and inner membrane of mitochondria, where it functions in the respiratory chain.[23] In the present study, cytochrome c mean value was significantly higher in animals subjected to I/R (group 2) compared to sham operated group which prove its role in the apoptotic pathway. Cytochrome c in our study showed an early phase of increased production after 30 minutes ischaemia then gradually decreased with 60-90 minutes of ischaemia in animals subjected to I/R.

Several studies were in accordance with our finding and approved the role of cytochrome c as an early marker of apoptosis being detectable in the cytosol as early as 2 hours after reperfusion[24] or at 6-24 hours after ischaemia and was found in the region corresponding to the infarct zone.[25] Whereas, Ouyang et al.[26] demonstrated that cytochrome c release occurred as late as 36 hours after ischemia. Other investigators approved the presence of cytochrome c immunoreactivity in the ischaemic core which was prominent at 24 hour of reperfusion.[27]

Moreover, our results had demonstrated the presence of cytochrome c in the normal hemisphere of all the studied groups, which was significantly lower than the left (ischaemic) side. This finding could be attributed to two factors; first, total cytochrome c was estimated in our study in the mitochondria and cytosol; therefore, it must be existed in the normal hemisphere as a normally occurring protein. The second possibility is the occurrence of apoptotic lesion in the hemisphere contralateral to the lesion as evidenced by the presence of collateral damage with the occurrence of retro-grade and antero-grade degeneration. Cao et al.[25] had contradicted our observation by suggesting the absence of cytochrome c immuno-reactivity in the contralateral (normal) hemisphere at any time point, the same finding was reported by Prakasa et al.[27]

Effect of I/R on tissue transglutaminase content of the brain

Tissue transglutaminase is a protein cross liking enzyme that has been reported to play a role in physiological and pathological conditions, as during apoptosis, in cell differentiation, in mediating axonal growth and regeneration and in cell adhesion. Recent finding have provided evidence that tTG level increased significantly during apoptosis.[28] tTG, therefore, can be considered as a sure apoptotic marker. Transglutaminase positive cells as indicated by stained cytoplasm were found in the sham operated animals in the present study, most probably these cells were peri-vascular microglia which are supposed to exhibit turn over in their normal physiological conditions.[29] However, this is not the case with the neurons, i.e. the presence of tissue-transglutaminase positive neurons must be considered pathological. Moreover, the current study, revealed the presence of neurons with stained cytoplasm (positive for tissue-transglutaminase) in the peri-infarct zone in animals

subjected to I/R with 30 minutes ischaemia. Prolongation of ischaemia to 60 minutes was associated with increased intensity of labeling specially around the third ventricle (thalamus, hypothalamus) which is likely to be induced by substances that are present in the CSF after the ischaemic cortical lesion. This could be cytokines like IL-2 or IL-6, which are known to induce apoptosis.[3,30] However, exposure of our studied animals to 90 minutes occlusion of the left CCA and left MCA reduced the stained cells in the peri-infarct zone probably due to increased necrosis, where the necrotic cells can not exhibit any activity. At the same time, tTG positive cells were observed in the supraoptic nucleus and the contralateral hemisphere.

Effect of cerebral ischaemia on DNA fragmentation

DNA fragmentation is considered to be the final step of apoptosis; therefore, nuclei with fragmented DNA are apoptotic nuclei.[29] Although, demonstration of DNA fragmentation alone is not sufficient proof of end stage cell damage or apoptosis, because in some cases TUNEL positive nuclei can occur in absence of apoptosis, if there is extensive DNA damage during necrosis, and in proliferating cells. Therefore, DNA fragmentation could be considered as a proof of apoptosis in the brain since there is no proliferating neuron any more.[31,32]

None of the sham operated animals in our study showed TUNEL positive cells. However, Renolleau et al.[29] reported the occurrence of TUNEL in control rats during the neonatal age, which corresponds to programmed cell death that occur during development. In the second group of animals in the present study (which was subjected to different durations of ischaemia), there were intense TUNEL labelled cells after 30 min. of ischaemia especially in the peri-infarct zone and around the third ventricle. Prolongation of the ischaemic period (60 and 90 min.) was associated with reduction in the number of stained cells in the peri-infarct zone. They observed that, the appearance of TUNEL positive nuclei after reperfusion can start as early as 4 hours of reperfusion in the fronto-parietal cortex, increased up to 24 hours, and remained stable until 96 hours.[29] A progressive decrease in the of apoptotic cells was observed from 7-30 days. Furthermore, a prolonged presence of TUNEL positive nuclei from 6-30 days after reperfusion suggests that cell damage in these neurons is a persisted and ongoing process. These findings were contradicted by Zeng et al.[33] who reported that, the morphological appearance of apoptosis is likely to be missing if the histochemical study is conducted 3-4 days after ischemia.

Therefore, the assumption that apoptosis occurs after an ischaemic insult raises the possibility that interventions directed against apoptosis would constitute a therapeutic strategy that may have attractive features;

1. Synergy with anti-excitotoxic intervention and

2. A prolonged therapeutic window. The ischaemic apoptosis occurs in delayed fashion after an ischaemic challenge, it is therefore possible that blockade of apoptosis may have a longer window of opportunity 1-2 h typically observed with calcium or glutamate antagonist drugs. As a matter of fact the protease inhibition offers a 6-h window of opportunity in focal cerebral ischaemia.[34,35]

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Conflict of interest statement: No conflicts declared.