Introduction

Brain ischemia, as a local interruption of blood flow in the brain, is becoming the most common death cause in developed countries; while the transient global ischemia, caused usually by cardiopulmonary arrest, is a more significant cause of cerebral stroke today. Various external and internal factors can cause ischemia, and despite advances in the research field of brain ischemia in experimental rat models, no effective therapy has been established [1]. In order to find an appropriate neuroprotective treatment, various experimental studies have been conducted in the rat brain by causing ischemic changes induced by occlusion of blood vessels in the brain, hypotension and hypovolemia [2]. The most common way was the occlusion of four blood vessels (four-vessel occlusion) [3]. Occlusion of the four major blood vessels of the brain (two carotid and two vertebral arteries) creates the conditions for the occurrence of ischemic changes. So far, various studies have been conducted in an attempt to reduce the consequences of ischemic attacks on the functioning of neural pathways. Ferreira et al. pointed out the significant potential of fish oil in reducing amnesia after a transient ischemic attack, but not in chronic cerebral hypoperfusion [4]. On the other hand, in their research, the River-Auty et al. failed to confirm the hypothesis according to which the cannabinoid CB2 receptor agonists should improve the condition of neurons in rats after brain ischemia [5]. In this area, experimental studies are still performed in order to improve the condition and function of neurons after ischemic attack.
At the onset of ischemia, there is an expression of the so-called group of early genes or proto-oncogenes (immediate early gene expression) [6].

**The role of Proto-Oncogenes and their Importance**

Proto-oncogenes are genes which are rapidly and temporarily expressed as an early cellular response to various stimuli, and they are also called a “gate of genomic response”. Ischemia as a stimulus leads to a post-ischemic expression regulated by transcription factors, the important mediators between the extracellular and cellular response signal in the form of long-term changes in the cell phenotype.

So far, about forty proto-oncogenes have been found, most notably the human homologs of retroviral oncogenes – the Fos family (c-fos, fos-B, fra-1, fra-2) and the Jun family (c-jun, jun-B, jun-D). The regional and occasional models of proto-oncogene induction are related to gene expression regulated by neurotransmitter, which implies the transcription in physiological and pathological conditions. The physiological stimuli lead to a discrete production of expression of these genes. However, pathological causes (tumor proliferation, cardiac disorder, occlusion of blood vessels of the brain, etc.) lead to the induction of expression of these genes and their proteins in the wide regions of the brain [7], even beyond the initial damaged areas, which serves as a marker of early neuronal activity [8].

Different models of global ischemia are used in experiments, such as Pulsinellini’s method [9], which we also applied, but it is very difficult to set clear barrier between a lethal and reversible injury caused by various models of global cerebral ischemia. In animal models, we can perceive an ischemic tolerance, transient cerebral ischemia, which protects the brain from subsequent ischemic attacks. It functions by inducing endogenous neuroprotectants, based on the program of post ischemic genetic expression, which is regulated by the transcription factors of early genes [10]. This functional-protectoral role of the proto-oncogenes depends on the degree of created ischemic tolerance. A prolonged activation of c-fos gene leads to the programmed cell death [11]. In their research, Jee et al. have shown that the antidepressant fluoxetine can inhibit apoptosis of hippocampus neurons caused by transient global ischemia, protecting the integrity of the blood-brain barrier [12]. Hua-Juan et al. suggest the need to examine the therapeutic potential of chlorpromazine as a neuroprotective agent in the treatment of ischemic stroke [13].

The increase in stress factors, neurotrophic and early genes in response to stress as a condition of mild stroke may indirectly prevent lethal ischemic insult by opening the adenosine triphosphate (ATP)-sensitive potassium channels [14]. The level of ATP, as the most important parameter for cell survival, directly affects the intensity of the stroke, and therefore the type of cell death caused by experimentally induced ischemia. This biochemical parameter cannot be below 20% [15].

Global ischemia activates an early gene as a trigger of transcription factor, which plays the role in signal transduction from extracellular signal to long-term changes in cellular phenotype. All early genes, including c-fos gene, can be activated by different harmful stimuli, such as cocaine, heroin, and other psychoactive drugs [16], “heat stress”, adrenalectomy, intracortical injection of nerve growth factor (NGF), some medicines, and reduced diet. Jungenitz et al. showed that the induction of c-Fos gene can be encouraged by high frequency stimulation in their research on hippocampal granule cells [17]. Expression of c-Fos proteins can be inhibited by conditioned fear [18] and noise [19].

Since the role of c-Fos protein in neurodegenerative and neuroprotection is controversial, it is the subject of many studies. There are different in vivo and in vitro studies showing that prolonged induction of c-fos proto-oncogene leads to neuronal death after ischemia, and that this proto-oncogene and its products cause diverse neuronal damages in addition to the neurodegenerative nature of c-Fos induction [20]. On the other hand, the expression of c-Fos protein is considered essential to the recovery of the neurons after ischemia in certain cases [21].

The role of c-Fos proto-oncogene was examined by several types of experimental models in the rat brain, each securing global transient ischemia, followed by unilateral or bilateral cerebral ischemia. Initial studies of behavior of hippocampus cells [22] and the vulnerable neurons of Cornu Ammonis (CA1) as well as neurons slightly more resistant to hypoxia-dentate gyrus and CA2-4 have shown that the level of c-Fos protein drops all parts of the hippocampus up to 6 hours after the reperfusion. Its level increases in the presence of N-acetyl-O-methylidopamine (NAMD), which can be used for pharmacological purposes [9]. Sanz et al. published studies with similar findings [23].

After the blood pressure had decreased by 30% during the period of 60 minutes, immunohistochemical reaction was recorded in the following structures: nc. tractus solitarii, area postrema, nc. parabrachialis lateralis, nc. paraventricularis et supraopticus, nc. amygdalae centralis [24].

The striatal neurons were studied in immature rat brains, and it was found that the induction of this protein started 0 to 12 hours after ischemia. Caudoputamen of adult brain was examined on the type of unilateral, focal ischemia and the c-Fos protein level was found to decrease 4 hours after reperfusion [25].
On the hypoxia model of immature rat brain during unilateral ligature it has been proved that the fastest messenger ribonucleic acid (mRNA) expression was in the ipsilateral cortex, and then in all other vulnerable regions, i.e. hippocampus and striatum [26]. The increase in the level of c-Fos protein appeared in the contralateral hemisphere in hypoxia, and it was shown that different parts of the brain c-Fos are immunopositively activated [8].

The most comprehensive study of transient brain ischemia was done by Wassel and Volpe [27]. The increase in c-Fos expression in global ischemia can be preventive, causing the so-called preconditioning. Tolerance to ischemia achieved in this way protects the vulnerable regions of the brain [28].

See et al. obtained similar results in their study of using proton magnetic resonance spectroscopy, with which they confirmed the reduction of neuronal damage by the method of ischemic and hypoxic preconditioning [29].

Ischemia and Anterior Amygdaloid Area and Nc. Accumbens

Anterior amygdaloid area and nc. accumbens were the two brain structures of the examined brain regions which were among the most prominent ones in the expression of c-Fos protein according to semiquantitative method. These two regions belong to the limbic part of the brain, which is the backbone and morphological basis of higher nervous activity, responsible for behavior, affects, and emotional behavior. In the limbic system, the impulses, which are sent from the neocortex, have been modified and thus processed are sent to the hypothalamus and the hypothalamus affects the function of vegetative functions and character of behaving. The results of our study should show whether these brain structures are somehow protected from the consequences of ischemic attack by specific molecular and biochemical way.

The aim of the study was to determine the expression of c-Fos protein in the anterior amygdaloid area and nc. accumbens in various models of global ischemia.

Material and Methods

Distribution of c-Fos protein was examined in 10 adult male Wistar rats, weighing 200 to 635 g. Ischemia was created by the method of Pulsinelli:

- total ischemia ligation of four blood vessels (electrocauterization of the vertebral artery, with bilateral ligation of carotid artery by paraffined thread for 10 min, followed by perfusion lasting 60 min), marked as R-group, 4 animals
- tolerant ischemic attack (electrocauterization of vertebral artery with bilateral ligation of carotid arteries by paraffined thread for 4 minutes, and then repeated after 72 hours for 10 min), marked as T-group, 4 animals,
- the first control group in which there was no intervention, one animal;
- the second control group for tolerant ischemia (vertebral electrocauterization with ligation of both carotid arteries for 4 minutes without 10-minute religature, one animal.

The experimental R-group included a subgroup of R1 and R2, in which we observed anterior amygdaloid area, and nc. accumbens, respectively. In the T1 and T2 subgroup, from the experimental T-group, we observed anterior amygdaloid area, and nc. accumbens, respectively.

The rats were anesthetized with ketamine on the first day (100 mg/kg), when electrocauterization of the vertebral artery was done. The rat was anesthetized with halothane (1.5–2.0%) the following day, and both of its common carotid arteries were ligated with paraffined thread to avoid the damage to the vessel wall when tightening the thread.

In the total ischemic group, the occlusion of the carotid arteries lasted 10 minutes, after which the blood recirculation was achieved by cutting the thread and after 60 min the animals were sacrificed. In the tolerant group, 72 hours after the first occlusion, the common carotid artery was occluded for 10 min and 60 min after the occlusion, the animals were executed in under anesthesia with ketamine (100 mg/kg), and intracranial brain became reperfused with 4% paraformaldehyde.

After the overnight fixation, the tissue was in cryoprotective, 20% sucrose, and then cut by free-floating technique, the incision being 50 µm thick. In this study, we used the immunohistochemical technique of avidin-biotin-peroxidase, as well as semiquantitative technique as a method of detection of the examined protein. The sections were washed with 0.1 M phosphate buffer, and incubated in Triton X-100 and 10% normal goat solution. This was followed by 48 h incubation with the specific c-Fos antibody (Santa Cruz, 1:100 000). The next step was the incubation in the rabbit secondary antibody for 60 min, and then avidin-biotin-peroxidase was added for the same time. Elution was carried out with 0.1 M phosphate buffer (pH 7.26). Antigen-antibody complex was visualized by 3,3'-diamino benzidine and 0.03% of H₂O₂. The preparations were observed and analyzed by the Leica microscope. Photos were taken by Analisis program and analyzed by Scion J program, converting images into binary representation.

Results

The observed c-Fos protein was found in both experimental groups, with no difference in coloration. The photos of stained preparations of examined structures were converted into binary representations in order to obtain numerical values of some
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Minimum and maximum values were obtained by semiquantitative method of determining the degree of staining neurons in the examined areas of the brain (Figure 1).

The R and T values of samples were grouped according to the analyzed parameters based on the measured values and shown in graphs. The order of column values is presented in such a way that every two columns (one pair of columns) are the left and the right side of the same location. We noticed an uneven expression of observed protein on the left and right in the same rats (graphs 1 and 2).

A clear dominance of T numeric values was noticed by grouping the values of coloration degree of c-Fos immunoreactive neurons at the location R1/T1 (anterior amygdaloid area). Grouping the values of staining degree of c-Fos immunoreactive neurons at the location R2/T2 (nc.) shows that the numerical values of T brains are grouped around the middle and the end points of a series take R values.

Discussion

All the medical conditions leading to a reduced blood flow not only through the brain but also through the heart, kidneys and other organs are common epidemiological problems that should be researched in this way.

Recent studies in this area deal with the expression of c-Fos protein after ischemic attack mainly in the hippocampus of rats, without taking some other parts of the limbic system as subject of their interest, such as the area amygdaloidea anterior and nc. accumbens. Our experiment was a part of a larger study of all structures of the adult brain in hypoxia, which relied on previous research conducted by scientists such as Nytrai et al. [6], Cho et al. [10] Soriano et al. [25], Xie et al. [30] and Johansen et al. [14], whose studies marginally presented results on cells of other (non-hippocampal) regions.

Semiquantitative method showed a high degree of neuron discoloration in the anterior amygdaloid area, which was determined by measuring the degree of discoloration and shown in the photos. The similar results were obtained examining the parietal cortex and the olfactive tubercle by Puškaš et al. [31].

Nc. accumbens showed a distinct expression of c-Fos protein in the T-group of rats, which is clearly seen as a graphic shift of T-values to the right side, to a higher value. However, the absolute values indicative of greater expression of the R-group of rats were

Table 1. The level of staining of neurons in the examined subgroups according to the semiquantitative method

<table>
<thead>
<tr>
<th>Subgroup</th>
<th>Minimal values</th>
<th>Maximal values</th>
</tr>
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<tbody>
<tr>
<td>R1</td>
<td>20.00</td>
<td>46.00</td>
</tr>
<tr>
<td>T1</td>
<td>23.00</td>
<td>127.00</td>
</tr>
<tr>
<td>R2</td>
<td>10.00</td>
<td>413.00</td>
</tr>
<tr>
<td>T2</td>
<td>21.00</td>
<td>73.00</td>
</tr>
</tbody>
</table>

Figure 1. Binary representation of the coloring of preparation of anterior amygdaloid area by c-Fos protein in rat brain ischemia. 20x magnification.

Slika 1. Binarni prikaz prebojenosti preparata area amygdaloidea anterior c-Fos proteinom pri ishemiji mozga pacova. Uvećanje 20 x.

Figure 2. Binary representation of the coloring of preparation of nc. accumbens by c-Fos protein in rat brain ischemia. 20x magnification.

Slika 2. Binarni prikaz prebojenosti preparata nc. accumbens c-Fos proteinom pri ishemiji mozga pacova. Uvećanje 20 x.
obtained by semiquantitative method for determining the degree of staining of neurons. This contradiction of results can be explained by technically poor adhesion of antibodies or by other technical artifacts.

The graphs clearly show the grouping of R and T treated brains against the intensity of the reaction, where the T group showed a more prominent reaction and more apparent grouping towards higher values.

The results of our study are correlated with the research of Seo et al. who, using proton magnetic resonance spectroscopy, came to the conclusion that ischemic and hypoxic preconditioning could reduce the extent of neuronal damage in ischemia of the affected part of the brain [29].

Our research should continue into several directions - to observe the brain in different stages of gestational development and adult age, to determine the degree of reactivity of c-Fos protein in the unilateral ligation and in the direction of pharmacological quantification of c-Fos protein in relation to the NAMD, as a potential therapeutic agent for brain ischemia in humans.

**Conclusion**

Based on our research, we concluded that there is a statistically significant difference of the observed c-Fos protein in the group of rats belonging to the group with total ischemia of compared to the group with tolerant ischemic attack, and in both examined structures of the brain. In this way, the expression of the investigated protein where the organ is “accustomed” to the reduced flow of oxygen and nutrients is prominent, which confirms its importance for the survival of neurons.

**References**


