Available online at www.elixirpublishers.com (Elixir International Journal)

Pharmacy

Elixir Pharmacy 76 (2014) 28286-28292

ISSN: 2229-712X

New xanthine derivative B-YR-2 as antioxidant modulator of post-stroke damage of sensorimotor cortex neurons in rats

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ARTICLE INFO

Article history:
Received: 2 September 2014;
Received in revised form:
25 October 2014;
Accepted: 4 November 2014;

Keywords
Ischemic stroke, Antioxidants,
Xanthine derivatives, Neuroprotection,
Cerebroprotective action,
Frontal cortex.

ABSTRACT

Acute ischemic stroke is a major cause of long-term disability and the second leading cause of mortality worldwide. One of main parts of it pathogenesis is production of reactive oxygen species. The goal of this study was to evaluate the neuroprotective effect of the new xanthine derivative B-YR-2 (hydrazide of 1,3-dimethyl-8-N-benzylaminoxanthinyl-7-acetic acid), which demonstrated high antioxidant qualities in vitro, in comparison with thiotriazoline, mexidol and citicoline. Injection of B-YR-2 compound to the rats with experimental stroke had the most pronounced neuroprotective effect. B-YR-2 increased number of glial cells and neurons in the cerebral cortex and stimulated their morphofunctional activity.

Introduction

Stroke is a major cause of long-term disability and the second leading cause of mortality worldwide resulting in approximately 6 million deaths every year [1,2]. The main pathophysiological processes following ishemic stroke are bioenergetic failure, loss of cell ion homeostasis, acidosis, increasing of intracellular calcium levels, excitotoxicity, reactive oxygen species-mediated toxicity, cytokine-mediated cytotoxicity and activation of neuronal and glial cells [1,3].

Neurons have high content of oxidizable substrates and an elevated metabolic rate, that's why neural tissues are highly sensitive to reactive oxygen species (ROS) attack. An additional release of ROS that takes place during reperfusion and restoration of the blood flow, could cause a delayed neuronal death [4-6]. Accumulation of ROS or oxidative stress plays a pivotal role in neurodegeneration associated with ischemia and other neurodegenerative diseases [7-11].

Among the activators of increased ROS-formation (from the electron transport chain, NADPH oxidases etc) during cerebral ischemia are Ca^{2+} ions. The increased concentration of intracellular Ca^{2+} ions can activate nuclear and cytosolic proteases such as endonuclease and calpains [1,12,13].

Abnormal accumulation of Ca^{2+} ions perturbs the electron transport chain of mitochondria, and causes electron leakage. In turn, the electrons can react with oxygen to produce superoxide anion (O^{2-}) [14,15]. The increase of cytosolic Ca^{2+} can also activate protein kinase C, which activates NADPH oxidase and stimulates production of O^{2-} anion [1,16-18]. Increased production of O^{2-} can lead to the formation of additional free radicals: hydrogen peroxide, hydroxyl radical and peroxynitrite (by reacting with nitric oxide produced by neuronal and endothelial NO synthase) [19,20]. In addition, ROS can stimulate transcription factors to cause neuronal and glial damage by modulating caspase-mediated apoptosis [21,22]. Thus, antioxidants (compounds able to decrease the level of ROS) can be used as neuroprotectors during ischemic stroke.

Substituted xanthine derivatives represent an important class of pharmacologically active compounds that are known to exhibit various pharmacological activities, including antioxidant properties [23,24]. The goal of this study was to evaluate the neuroprotective effect of the new xanthine derivative B-YR-2 (hydrazide of 1,3-dimethyl-8-N-benzylaminoxanthinyl-7-acetic acid, Fig.1), which demonstrated high antioxidant qualities in vitro [25], in comparison with other neuroprotectors, such as thiotriazoline [26], mexidol [27] and citicoline [28].

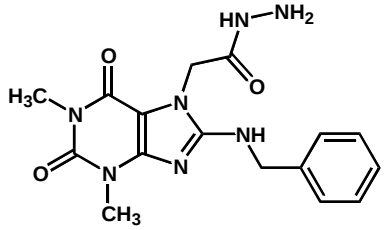

Figure 1. Hydrazide of 1,3-dimethyl-8-N-benzylamino-xanthinyl-7-acetic acid – B-YR-2

Materials and methods

Animals

The experiments were performed on white Wistar rats of both sexes with the body weight of 150-200 g. Animals were obtained from the nursery of the Institute of Pharmacology and Toxicology of Ukraine. The duration of an acclimatization period (quarantine) was 14 days for all objects. The behavior and general condition of each animal was assessed every day. Twice a day the animals were monitored for morbidity and mortality. Animals not complying with the experimental criteria were excluded from the study during the quarantine. Cages with animals (6 rats per cage) were placed in separate rooms with the natural day/night light cycle.

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Air temperature and relative humidity were monitored every day and maintained between 19-25 °C and 50-70%, respectively. The diet consisted of the cornmeal, bread, and root vegetables (beets, carrots).

All experimental procedures and operative interventions were done in accordance with WMA Statement on Animal Use in Biomedical Research.

Acute toxicity

For definition of the median lethal dose of B-YR-2 compound we used Kerber method in Loit modification and K. K. Sidorov classification [29,30]. Several doses of xanthine derivative were injected intragastrically as a water suspension through the metal catheter into 5 groups of laboratory animals (6 rats per group). Animals that survived the surgery were observed during two weeks after injection.

Stroke model

In order to model the acute stroke we selected the global incomplete cerebral ischemia model, which was shown to be the most adequate in terms of clinical implications [31]. The acute stroke condition was induced by bilateral ligation of common carotid arteries performed under the ethaminal-sodium anesthesia (40 mg/kg), with implication of surgical approach by means of separation of carotid arteries and single-step silk deligation [31].

Animals were divided on 6 experimental groups and 11 subgroups: 1) group I_A – animals with CVA without the treatment (control group), which were gathered from the experiment on the 4th day after the surgery (20 rats); 2) group I_B – animals with CVA without the treatment (control group), which were gathered from the experiment on the 18th day after the surgery (25 rats); 3) group II_A – animals with CVA treated with thiotriazoline during 4 days following the surgery (10 rats); 4) group II_B – animals with CVA treated with thiotriazoline for 18 days after the procedure (10 rats); 5) group III_A – animals with CVA treated with mexidol for 4 days after the surgery (10 rats); 6) group III_B – animals with CVA treated with mexidol for 18 days following the surgery (10 rats); 7) group IV_A – animals with CVA treated with B-YR-2 compound for 4 days following the procedure (10 rats); 8) group IV_B – animals with CVA treated with B-YR-2 compound for 18 days following the surgery (10 rats); 9) group V_A – animals with CVA treated with citicoline for 4 days following the surgery (10 rats); 10) group V_B – animals with CVA treated with citicoline for 18 days following the surgery (10 rats); 11) group IV was represented by the pseudo-operated animals (intact group, 10 rats).

The compound B-YR-2 was injected once a day as a water suspension intragastrically through the metal catheter. We used 100 mg/kg as the curative dose (1/10 LD_{50}) during the whole experiment. Thiotriazoline was injected according to the same schedule at a dose of 50 mg/kg intragastrically. Curative dose of mexidol and citicoline was 100 mg/kg. Animals of control and intact groups received physiological solution (saline) with the Tween-80 according to the same schedule.

Neurological impairment

The neurological impairment was assessed by the scale stroke-index by C.P. McGrow [32]. Severity of the condition was defined by the sum of the relevant points as follows: 3 points – a mild degree of impairment, 3 to 7 points – an average degree, and 7 points or more corresponded to a severe degree of impairment. During experimental observations of animals, we recorded the following parameters as manifestations of neurological deficit: paresis, paralysis of limbs, tremors, circling, ptosis, side position, mobility and the ability to retain on a rod of 15 cm in diameter rotating with a speed of 3 rev/min. The animals were tested daily and the number of points was recorded during each assessment.

Morphometric analysis

For assessment of pharmacocorrection results we collected brain tissue from experimental animals on the fourth day (subgroups A) and on the eighteenth day (subgroups B) after the surgery. Brain tissue was fixed with 10% Bouin's fluid for 24 hours, and was embedded in paraffin blocks. These blocks were used for preparation of 5-micron frontal histological sections of the postcentral gyrus (somatosensory cortex). To study the morphological and functional state of neurons of IV-V cortical layers and for specific detection of RNA, histological sections were deparaffinized and then stained by gallocyanin - chrome alum (Einarson's Method) [33]. Images of the cerebral cortex were obtained by microscope Axioskop (Zeiss, Germany), using an 8-bit CCD-camera COHU-4922 (COHU Inc., USA) and then were processed by the computer image analysis system VIDAS-386 (Kontron Elektronik, Germany). Morphometric analysis of brain cells was performed in an automatic mode using the VIDAS-2.5 software (Kontron Elektronik, Germany) [34].

The following parameters were defined:

- density of neurons, glial cells, apoptotic and destructured neurons (the number of cells per 1 mm^2 of area of cerebral cortex section);
- surface area of cell bodies of normal, apoptotic and destructured neurons (μm^2);
- RNA concentration in normal, apoptotic and destructured neurons (absorbance units, E_{OD}), which was calculated as the logarithm of the ratio of the optical density of the cell body to the optical density of the intercellular substance.

Immunohistochemical determination of the quantity of c-Fos-positive neurons

The brain tissue of experimental animals was fixed in Carnoy's fluid for 24 hours and was embedded in paraffin blocks, which were used for preparation of 14-micron histological sections of IV-V layer of the sensorimotor cortex.

For assessment of expression of early response genes c-Fos, histological brain sections were deparaffinized, rehydrated, thrice washed with phosphate buffer for 5 minutes (pH = 7.4) and incubated with 2N hydrochloric acid solution for 30 minutes (T = 37 ^oC). Then sections were washed twice with phosphate buffer for 5 minutes (pH 7.4), twice for 5 minutes with a borate buffer by Holmes (pH = 8.4), four times for 5 minutes with the phosphate buffer (pH = 7.4) and incubated with 0.1% trypsin solution in the phosphate buffer for 30 minutes (T = 37 ^oC). After incubation, sections were washed four times for 5 minutes with phosphate buffer (pH = 7.4) and then incubated for 24 hours in a humid chamber (T = 4-6 °C) with the primary rabbit polyclonal antibodies IgC (1:500) to the c-Fos protein (sc-253-G) manufactured by Santa Cruz Biotechnology, Inc. (USA). After incubation, sections were washed four times for 5 minutes with the phosphate buffer (pH = 7.4) and incubated for 1 hour (t = 37 ° C) with the goat secondary antibody to a fragment of mouse IgG, conjugated to a fluorescent dye (FITC, Sigma-Aldrich company, cat. № F 2266). After the final wash with the phosphate buffer (pH 7.4), sections were embedded in a glycerol-phosphate buffer (9:1). Intensity of c-Fos expression was determined by the density of c-Fos-positive cells in sections with the camcorder COHU-4922 (USA) of the epifluorescence microscope Axioskop (Ziess, Germany). Obtained material was processed by the computer image analysis system VIDAS-386 (Kontron Elektronik, Germany).

Statistical analysis

The statistical data analysis was carried out with the help of the software STATISTICA® for Windows 6.0 (StatSoft Inc. AXXR712D833214FAN5) [35]. The data is presented as the sample mean ± the standard error of the mean. The fidelity of differences between experimental groups was estimated with the help of Student's t-test and Fisher's exact test.

Results

The acute toxicity

Intragastric injection of B-YR-2 in a dose of 900 *mg/kg* resulted in a 100% mortality of the animals within a day following the treatment, whereas a dose of 100 *mg/kg* did not cause death. Injections of intermediate doses (300, 500 and 700 *mg/kg)* caused death of 33.3%, 50% or 83.3% of animals respectively (Table 1). The obtained experimental data showed that the test compound did not show significant toxicity, and its LD_{50} was 500 ± 10 mg/kg.

Table 1. Results of acute toxicity test in rats following intragastric injection of compound B-YR-2 after 2 weeks of observation

Dose, mg/kg	100	300	500	700	900
Proportion of survived rats on the 14th day, %	100	66.7	50	16.6	0

Influence on survival and progression of neurological deficit

Double-sided common carotid arteries ligation caused serious neurological changes in animals organisms, e.g. paralysis, paresis, ptosis, with maximum manifestation on the fourth day post-surgery. So, the average score of uncured group of animals for this period corresponded to a serious degree of neurological symptoms according to the C.P. McGraw scale (Table 2).

Table 2. Influence of B-YR-2, thiotriazoline, mexidol and citicoline on survival and progression of neuralgic deficit of animal in different periods on time after CVA

Group	**Average score according to C.P. McGrow scale**		**Proportion of survived /operated rats on the 4th day, %**
	Subgroup A (4th day)	**Subgroup B (18th day)**	
I	17,66 ± 2,02	4,3 ± 0,33	(14/45) 31
II	7,16 ± 0,59*	2,2 ± 0,47*	(14/20) 70*
III	6,21 ± 0,47*	2,7 ± 0,11*	(14/20) 70*
IV	6,6 ± 0,6*	1,9 ± 0,28*	(16/20) 80*
V	8,8 ± 0,8*	3,6 ± 0,42	(10/20) 50
VI	0,4 ± 0,11		(10/10) 100

Remark: * - $p \leq 0,05$ in relation to control

On the forth day of observation 31% of animals of the control group survived and their average score by the McGrow scale was 17.66, which corresponds to severe neurological symptoms (Table 2). Treatments with B-YR-2 compound, thiotriazoline, mexidol or citicoline had different neuroprotective effects, with B-YR-2 compound demonstrating the most significant effect. This xanthine derivative reduced the occurrence of neurological symptoms and mortality by approximately 2.6 fold in B-YR-2-treated animals compared to the untreated group.

Morphometric analysis

Ischemia resulted in a significant decrease of the neuron density in the cortex compared to intact animals (Table 3, Figs 2,3). At the same time, there was a significant decrease in the surface area of neuron cell bodies with an increase in their RNA content compared to intact animals. Ischemia did not significantly affect the density of glial cells in the cerebral cortex, but caused a significant increase in the area of glial cells with high RNA content (Table 4).

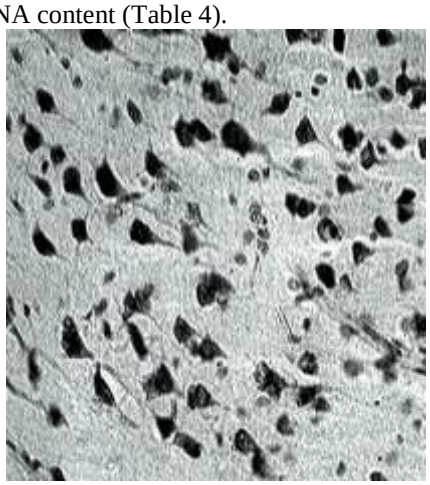


Figure 2. Neurons of the sensorimotor area of the frontal cortex of rats of intact group (group VI)

This was indicative of a compensatory increase in the functional state of glial cells in response to ischemic brain damage. In the cortex of animals in the control group the number of apoptotic and destructed neurons was 3.8 fold higher than in the cortex of intact group. It reached 17,45 ± 0,8% of the total number of cells on the 4th day (neurons and glial cells) and 14,36 ± 0,74% on the 18th day post-sugrery (Table 5).

Quantity of c-Fos-positive neurons

Acute cerebral ischemia resulted in a decrease of the c-Fos protein on the 4th day following the surgery (the number of the c-Fos-positive cells was decreased by 3.6 fold) (Table 6, Figs. 4,5). On the 18th day of experiment we observed some recovery processes. The number of c-Fos-positive neurons increased in the IV-V layers of the sensorimotor cortex, but the c-Fos content remained on the low level.

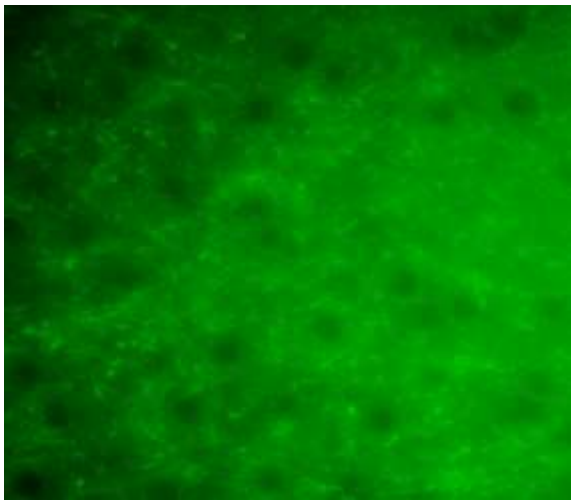
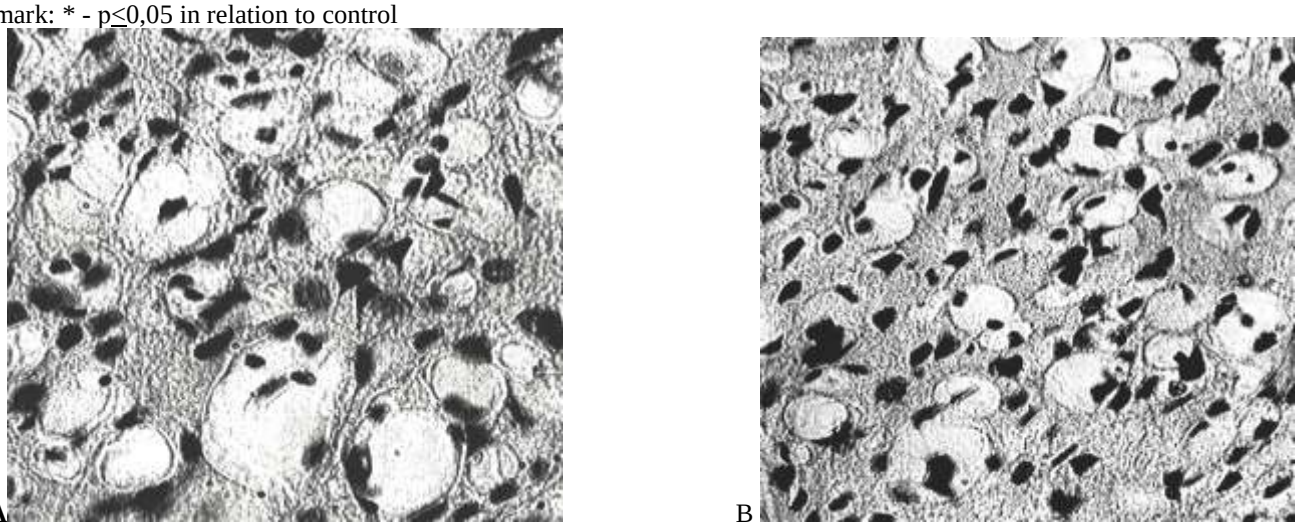


Figure 4. Content of c-Fos-positive cells in brain of rats of intact group (group VI)

Table 3. Characteristic of neurons of IV-V layers of cortex of brain of rats with ishemic stroke

Group	Neuronal density, cell quantity /mm^2		Neuronal bodies area, μm^2		RNA content in neurons, E_{OD}	
	Subgroup A (4th day)	Subgroup B (18th day)	Subgroup A (4th day)	Subgroup B (18th day)	Subgroup A (4th day)	Subgroup B (18th day)
I	1065 ± 27	1082 ± 19	64,19 ± 0,9	62,12 ± 1,08	12,4 ± 0,2	11,5 ± 0,6
II	1081 ± 29	1117 ± 32*	62,97 ± 1,14	65,12 ± 0,94*	11,26 ± 0,16*	11,19 ± 0,28
III	1182 ± 24*	1235 ± 18*	67,78 ± 1,13*	68,67 ± 1,08*	11,76 ± 0,11*	11,23 ± 0,15
IV	1278 ± 17*	1307 ± 22*	69,52 ± 0,65*	70,82 ± 0,72*	11,17 ± 0,12*	10,67 ± 0,17*
V	1069 ± 38	1163 ± 26*	63,46 ± 0,8	68,71 ± 0,93*	11,37 ± 0,18*	11,03 ± 0,23
VI	1281 ± 34	1292 ± 31	75,21 ± 1,12	74,87 ± 1,32	9,69 ± 0,15	9,72 ± 0,14

Remark: * - p≤0,05 in relation to control



A B

Figure 3. Neurons of the sensorimotor area of the frontal cortex of rats of group I (A – subgroup A, B – subgroup B)

Table 4. Characteristic of glial cells of IV-V layers of cortex of brain of rats with ishemic stroke

Group	Density of glial cells, cell quantity/mm^2		Area of glial cell bodies, μm^2		RNA content in glial cells, E_{OD}	
	Subgroup A (4th day)	Subgroup B (18th day)	Subgroup A (4th day)	Subgroup B (18th day)	Subgroup A (4th day)	Subgroup B (18th day)
I	396 ± 11	410 ± 11	21,2 ± 0,11	21,8 ± 0,17	4,05 ± 0,02	4,03 ± 0,02
II	476 ± 14*	493 ± 12*	22,5 ±0,21*	23,9 ± 0,21*	4,13 ± 0,02*	4,16 ± 0,02*
III	469 ± 17*	485 ± 15*	22,8 ± 0,14*	23,7 ± 0,17*	4,11 ± 0,03*	4,14 ± 0,02*
IV	465 ± 12*	478 ± 11*	23,4 ± 0,15*	25,0 ± 0,14*	4,17 ± 0,03*	4,21 ± 0,01*
V	497 ± 19*	507 ± 14*	21,7 ± 0,28	24,9 ± 0,31*	4,25 ± 0,02*	4,29 ± 0,03*
VI	418 ± 21	421 ± 14	20,5 ± 0,19	20,7 ± 0,24	3,34 ± 0,07	3,31 ± 0,05

Remark: * - p≤0,05 in relation to control

Table 5. Density of apoptotic and destrucrted neurons of IV-V layers of cortex of brain of rats with ishemic stroke

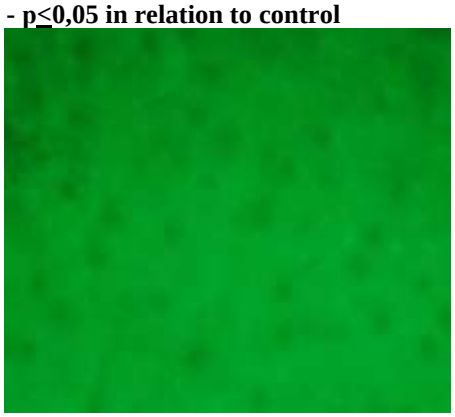
Group	Density of cells on 1 mm^2		Percentage of apoptotic cells, %	
	Subgroup A (4^{th} day)	Subgroup B (18^{th} day)	Subgroup A (4^{th} day)	Subgroup B (18^{th} day)
I	294 ± 18	287 ± 18	17,45 ± 0,8	14,36 ± 0,74
II	162 ± 16*	112 ± 10*	9,7 ± 0,41*	7,5 ± 0,49*
III	174 ± 11*	128 ± 13*	7,8 ± 0,51*	6,7 ± 0,35*
IV	149 ± 5*	107 ± 9*	6,8 ± 0,6*	5,7 ± 0,65*
V	438 ± 29*	153 ± 18*	16,3 ± 1,7	6,4 ± 0,52*
VI	107 ± 9	110 ± 7	4,5 ± 0,53	4,3 ± 0,61

Remark: * - p≤0,05 in relation to control

Table 6. Content of c-Fos-positive cells in brain of rats with ishemic stroke

Group	Subgroup	Content of c-Fos-positive cells
I	A (4^{th} day)	4,0 ± 0,7
	B (18^{th} day)	19,0 ± 0,54
II	A (4^{th} day)	7,6 ± 0,5*
	B (18^{th} day)	25,8 ± 0,86*
III	A (4^{th} day)	8,2 ± 0,66*
	B (18^{th} day)	26,0 ± 0,7*
IV	A (4^{th} day)	9,4 ± 0,66*
	B (18^{th} day)	27,3 ± 0,7*
V	A (4^{th} day)	5,2 ± 0,58
	B (18^{th} day)	19,2 ± 0,66
VI	–	15,4 ± 1,07

Remark: * - p≤0,05 in relation to control



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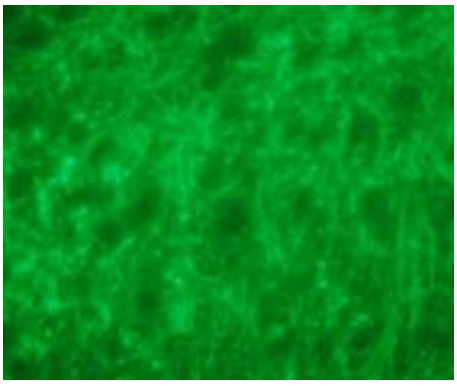


B

Figure 5. Content of c-Fos-positive cells in brain of rats of group I (A – subgroup A, B – subgroup B)

Discussion

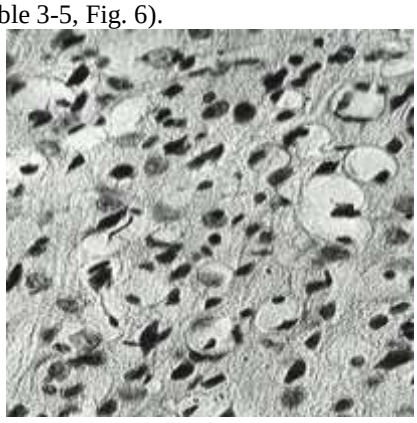
The treatment of animals that underwent bilateral ligation of common carotid arteries with B-YR-2, thiotriazoline, mexidol, and citicoline resulted in a reduction of development of neurological deficit. The treatments accelerated the restoration of the neurological status, improved learning processes and memory, and reduced mortality.

Experimental therapy of animals with stroke with citicoline led to an increase of the neuronal density in the cortex by 1.5 fold on the 4th day of ischemia. In the prolonged periods of ischemia, citicoline injection reduced the density of neurons and their RNA content compared with the control group of animals. However, the number of apoptotic and destructed neurons of animals of V_A subgroup did not differ from the control group, but in the long-term ischemia conditions citicoline treatment significantly reduced the percentage of apoptotic neurons and contributed to maintaining a high functional activity of glia. Thus, citicoline has no neuroprotective effect in the acute period of stroke. But in the recovery period, this drug significantly increased the density of the neurons and glial cells, that led to an increase in the total cell density by 1.5 fold relative to control values, and reduced the percentage of apoptotic neurons by 2.2 fold.

Thiotriazoline caused an increase in the density and total area of glial cells both in the acute and recovery phases. Also, after thiotriazoline injection we observed an increase in the RNA content of glial cells, which indicates an increase in gene activation, protein synthesis, and functional activity of these cells. Anti-ischemic action of thiotriazoline is based on its ability to enhance the compensatory activation of anaerobic glycolysis, to reduce the degree of inhibition of oxidative processes in the Krebs cycle, and to maintain an intracellular ATP depot that stabilizes the metabolism of neurons [26]. Thiotriazoline reduced the production of reactive oxigen species in mitochondria by utilizing the reduced forms of pyridine nucleotides and presercing the oxidative energy production. Thiotriazoline prevented oxidative modification of proteins, receptors, ion channels, enzymes, and transcription factors by reducing overproduction of peroxynitrite and superoxide radical.

An injection of mexidol had more pronounced neuroprotective effect. The analysis of morphological parameters of the experimental group that received mexidol revealed an increase in density and surface area of neuronal cell bodies. An increase in the RNA content of neurons after mexidol treatment was indicative of the stimulation of gene activity and of the activation of the translation process in neurons. Such effect of mexidol was related to his activating influence on the antioxidant enzyme system. Mexidol inhibited processes of lipid, protein and nucleic acid oxidation, activated antioxidant enzymes, promoted sparing consumption of an endogenous antioxidant α-tocopherol, and contributed to maintaining the structural and functional integrity of neuronal membranes.

In our study, B-YR-2 compound had the most pronounced neuroprotective effect among the antioxidant compounds analysed (Table 3-5, Fig. 6).



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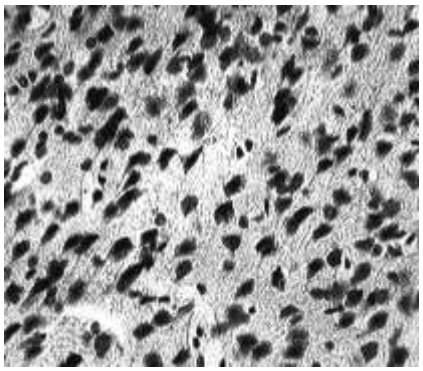
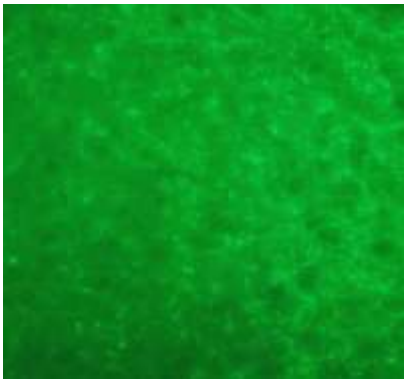


B

Figure 6. Neurons of the sensorimotor area of the frontal cortex of rats of group IV (A – subgroup A, B – subgroup B)

Its action on glia during stroke manifested in an increase in a number of glial cells and neurons in the cerebral cortex and stimulation of their morphofunctional activity (an increase in the RNA content). Such effect of this xanthine derivative is in accordance with its antioxidant properties that were demonstrated by the in vitro methods and described in our previous work [25,36].

The change in the c-Fos content in neurons in different periods of ischemia was associated with the prevalence of the type of cell death. Apoptosis led to an increase of the c-Fos content, and necrosis – to its decrease. Recovery period was characterized by the adaptation of the neurons and higher occurrence of an apoptotic type of cell death. An antioxidant treatment during stroke (with B-YR-2, mexidol, thiotriazoline) increased the c-Fos protein content (Table 6, Fig. 7), reduced the intensity of necrosis of neurons with simultaneous enhancement of apoptosis.



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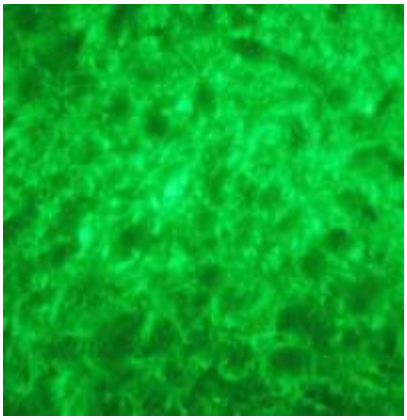

B

Figure 7. Content of c-Fos-positive cells in brain of rats of group IV (A – subgroup A, B – subgroup B)

Treatment with antioxidants not only reduced the percentage of cell death, but also changed the morphological type of neuronal death via an increase in apoptosis. Apoptotic cell death is an optimal process of cell life termination. It's characterized by stabilization of the cell membrane and utilization of the cell contents by the formation of apoptotic bodies and phagocytosis, without the development of the inflammatory response.

In conclusion, the neuroprotective effect of antioxidants during stroke provides solid grounds for further research. Xanthine derivative B-YR-2, a molecule with the most pronounced neuroprotective effect among studied compounds, is a particularly promising avenue for further in-depth investigation.

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