Int J Clin Exp Med 2010;3(2):144-151
www.ijcem.com /IJCEM1002006

Original Article

Oxidative stress induced in rat brain by a combination of 3-nitropropionic acid and global ischemia

Dusan Dobrota[1], Tatiana N. Fedorova[2], Maria S. Stepanova[2], Eva Babusikova[1], Dagmar Statelova[4], Zuzana Tatarkova[1], Sergey S. Stvolinsky[2], Alexander A. Boldyrev[2,3]

[1]Department of Medical Biochemistry, Jessenius Faculty of Medicine, Comenius University, Martin, Slovak Republic; [2]Laboratory of Clinical Neurochemistry, Research Center of Neurology RAMS, Moscow, Russia; [3]International Center for Biotechnology, Lomonosov Moscow State University, Moscow, Russia; [4]Clinic of Stomatology and Maxillofacial Surgery, Jessenius Faculty of Medicine, Comenius University, Martin, Slovak Republic.

Received February 25, 2010, accepted May 11, 2010, available online May 15, 2010

Abstract: In our investigation, we describe the complex model of brain oxidative stress consisted of combination of experimental brain ischemia and energy metabolism violation induced by irreversible inhibitor of mitochondrial succinate dehydrogenase, 3-nitropropionate (3-NPA). 3-NPA causes selective degeneration of striatum neurons, which is extremely sensitive to energy deficit. This complex model allows revealing not only biochemical but also neurological symptoms in experimental animals that permits proper estimation of protective effect of different drugs on animal status. Combination of global ischemia induced by 3-vessel occlusion of major arteries supplys rat brain and subsequent 5-day reperfusion with intraperitoneal injection of 3-nitropropionic acid induces vigorous oxidative stress in brain tissues accompanied by evident neurological symptoms in Wistar rats. Such a combination of damaging factors may be considered as a new complex experimental model of brain oxidative stress permitting the evaluation of neuroprotective effect of potential therapeutic agents. Using this model, protective effect of neuropeptide carnosine was demonstrated which is in agreement with previous data.

Keywords: Brain global ischemia, 3-nitropropionic acid, oxidative stress, carnosine

Introduction

It has long been recognized that 3-NPA causes selective degeneration of striatum neurons, which is extremely sensitive to energy deficit [1]. The protective effect of neuropeptide carnosine has also been demonstrated in previous studies [2, 3]. For the development the new therapeutic strategies for treatment of the cerebrovascular deseases more than 100 different experimental models of brain ischemia on animals are used [4]. However these models are not completely match to clinical situation, because in human cerebrovascular diseases many pathological factors (like atherosclerosis, hypertension, etc.) are involved in brain damage development. For example, in spite of considerable biochemical disorders and high mortality of animals subjected to global ischemia, only poor neurological symptoms were revealed [5, 6]. In such cases, the neurochemical and morphological defects displayed in their brain cannot be verified by behavioral features. This circumstance embarrasses the evaluation of novel therapeutic drugs being potentially able to normalize brain metabolism after ischemic episode using both neurochemical and neurological parameters [6, 7]. Probably, it is one of the reasons why drugs that were effective on experimental models didn't show protective effect on clinical trials [8, 9]. Oxidative stress makes an important contribution in ischemic injury. Our goal was to make a model of brain oxidative stress, that allows revealing neurological symptoms and estimating protective action of new pharmacological drugs.

Recently we have noted that experimental brain ischemia aggravated by energy deficit induced by simultaneous administration of 3-nitropropionic acid, 3-NPA, reveals both neuro-

chemical deviations in the brain and a neurological deficit [10]. 3-NPA as an uncompetitive inhibitor of mitochondrial succinate dehydrogenase [11, 12] causes rapid suppression of energy metabolism in the brain, thus resulting in the release of excitotoxic neuromediators, development of oxidative stress, and death of the brain neurons [13]. During systematic administration of the neurotoxin, degeneration of neurons of the striatal area was noted being accompanied with specific locomotor and cognitive disorders close to those described in the Huntington disease [14, 15].

In this article, we characterized rat brain global ischemia aggravated with systemic administration of 3-NPA using both behavioral and biochemical parameters. In order to evaluate the meaning of such a model of brain ischemia we have evaluated the therapeutic effect of neuropeptide carnosine known as the antioxidant brain protector [2, 16].

Materials and methods

Experiments were made using 68 male Wistar rats of 23-26 weeks old (315-350 g weight) maintained under standard conditions. Animals were treated and handled according to the guidelines of the European Union Council. The animals were housed in a well-ventilated animal room under a light/dark cycle of 12:12 h (light on from 06:00 to 18:00 h). The animals had free access to food and water.

Three-vessel global ischemia of rat brain [5, 7] was performed as a 2-step procedure: at the first day, the left *arteria vertebralis* was electrocauterized, and at the next day, 15 min occlusion of both carotid arteries was carried out with a subsequent 5 days reperfusion. On the last day of the experiment, the animals were decapitated under light anesthesia.

At the beginning of the experiment, the animals were randomly distributed into the following groups: 1 – intact animals (n=8); 2 – sham-operated animals (n=8); 3 – sham-operated animals treated with 3-NPA (30 mg/kg per day intraperitoneally starting from 3rd day of experiment) (n=10); 4 – the animals subjected to global brain ischemia (n=11); 5 – the animals subjected to global brain ischemia in combination with 3-NPA injection (30 mg/kg per day starting from 3rd day of experiment) (n=15); 6 – the animals subjected to global brain ischemia in combination with 3-NPA (30 mg/kg per day starting from 3rd day of experiment) and carnosine (100 mg/kg) (n=14).

The following protocol for carnosine treatment was used: the above mentioned dose was injected intraperitoneally in the very beginning of the re-perfusion, and after 4, 8, and 24 hrs after operation, then once a day until the end of experiment. All substances were dissolved in physiological solution, and the same amount (0.5 ml) of physiological solution was injected to the animals of the control groups.

Neurological symptoms were evaluated by using a scale of features [7] (**Table 1**) revealing in rats after 3-NPA injection [11, 12]. It was made immediately after the animals were woken up from anesthesia, then after 4, 8, and 24 hrs and every other day during the entire reperfusion period.

On the 7th day of the experiment, the animals were decapitated and the brain prepared using iced physiological solution. The homogenate for Fe^{2+}-induced chemiluminescence was obtained from 0.3 g of both hemispheres using phosphate buffer (60 mM KH_2PO_4 and 105 mM KCl, pH 7.45). The mitochondrial fraction was isolated using the differential centrifugation procedure [17] and the samples obtained were

Table 1. Scale of neurological symptoms

Huntington's disease symptoms	Points
Decrease of moving activity, pose defects	1
Weakness, languor	2
Rigidity of back extremities and the tail, rotation	3
Paresis of back and later extremities	4
Total suppression of locomotion	5

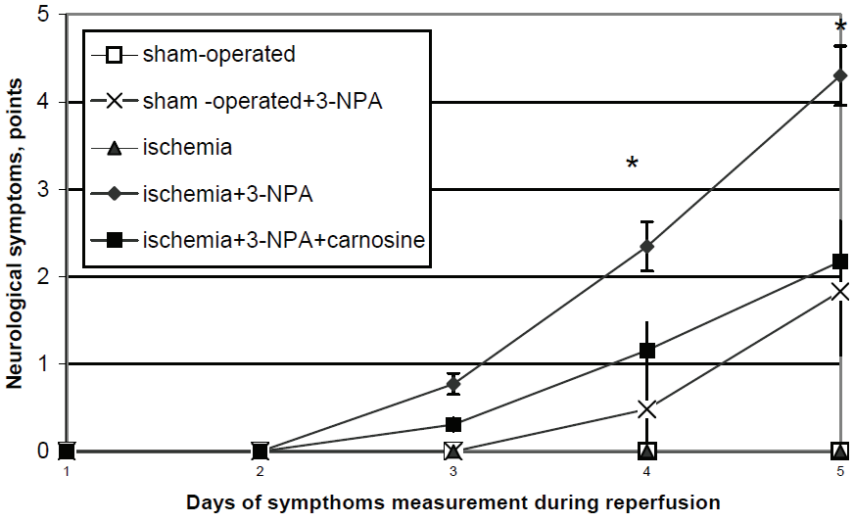

Figure 1. Neurological symptoms in the experimental animals studied (ordinate – neurological points, abscissa – days after ischemic episode); "*" corresponds to statistically significant difference ($p<0.05$) from groups 3 and 6.

stored (no more than 2 weeks before analyses) at -80°C. Grey matter of both cerebral hemispheres (about 0.8 g) was homogenized in 0.32 M sucrose containing 5 mM HEPES pH 7.4 at a ratio of 9 ml of homogenization medium/1 g of tissue in a Potter homogenizer with a Teflon pestle. The homogenate was centrifuged at 3600 rpm for 10 min to obtain the supernatant. 3 ml of supernatant was pipetted over 4 ml 1.2 M sucrose (each sample per 2 tubes) and centrifuged at 25000 rpm for 20 min in Beckman SW41T centrifuge. The two residues were suspended in 0.5 ml of 0.32 M sucrose and frozen at -80 °C.

In the mitochondrial fraction superoxide dismutase, SOD, monoamine oxidase B, MAO B, and succinate dehydrogenase, SDG were measured. SOD was determined by the suppression of nitro blue tetrazolium reduction in the presence of superoxide anion generated by xanthine oxidase; enzyme units corresponded to the amount of the enzyme providing 50% inhibition of nitro blue tetrazolium reduction [18]. MAO B was monitored using benzyl amine as a substrate [6]. SDG was determined as a rate of reduction of 2,6-dichlorophenolindophenol in the presence of phenazine methosulfate during enzymatic oxidation of sodium succinate [19].

In the brain homogenate, the Fe^{2+}-induced chemiluminescence was monitored giving the ability to determine the level of lipoperoxides preformed, the rate of Fe^{2+}-induced oxidation and the oxidative resistance of the samples (by measuring the lag-period between the addition of Fe^{2+}-ions and the beginning of the chemiluminescence reaction) [20].

Protein was measured by Lowry [21]. Statistic analysis of the data was made using one-way analysis of variance (ANOVA) followed by the Tukey-Kramer and Duncan test. Results were expressed as a mean ± S.D.

Results

In the experiments presented, control (sham-operated) animals (Group 2) as well as the animals subjected to global brain ischemia (Group 4) possessed no neurological symptoms. Both appearance and behavior of these animals were not distinct from those of intact rats (**Figure 1**). Mortality was noted only in Groups 3, 5, and 6 (**Table 2**). In the Group 3 rats, mortality appeared on the 4th day after the 3-NPA injection. In the Group 5 rats (combination of global ischemia with 3-NPA injection), the mortality rate was only a little bit higher (33% compared with 30%, statistically insignificant) than in Group 3, suggesting that the main reason for the mortality was the 3-NPA injection. Animal mortality was observed at the 4th-5th days of 3-NPA injection. However, the Group 5 animals were characterized as having the highest level of neurological symptoms (**Figure 1**). Typical neurological symptoms (pose defects, rigidity of back extremities and the tail, paresis of back and later on upper extremities, rotation, total suppression of locomotion and loss of body weight) appeared on

Table 2. Mortality in the animal groups studied

Groups of animals	Mortality	
	amount	%
1. Intact (n=8)	0	0
2. Sham-operated (n=8)	0	0
3. Sham-operated+3-NPA (n=10)	3	30
4. Ischemia (n=11)	0	0
5. Ischemia+3-NPA (n=15)	5	33
6. Ischemia+3-NPA+carnosine (n=14)	1	7

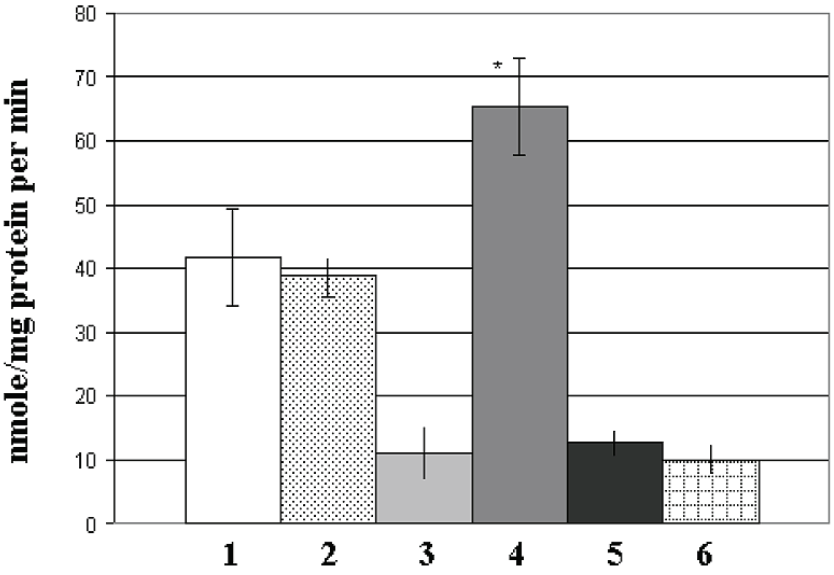

Figure 2. SDG activity (nmole succinate/mg protein per min) in brain mitochondria of the animals studied. 1 – intact animals, 2 – sham-operated animals, 3 – sham-operated+3-NPA, 4 – ischemia, 5 – ischemia+3-NPA, 6 – ischemia+3-NPA+carnosine; "*" corresponds to statistically significant difference ($p<0.05$) from group 1.

the 3rd day after the 3-NPA injection and became stronger with time (**Figure 1**).

Carnosine treatment was very efficient – it protected the ischemic animals from both mortality (see **Table 2**) and marked neurological symptoms (**Figure 1**).

The analysis of enzyme activity showed the following changes. Succinate dehydrogenase in brain mitochondria was unchanged in Group 2 animals (compared to intact control) but sufficiently increased under experimental ischemia (Group 4, **Figure 2**). When 3-NPA was injected, significant 4 fold suppression of mitochondrial succinate dehydrogenase was found confirming that an energy deficit was an evident cause of the neurological deficit which appeared. Such an effect of 3-NPA does not depend on experimental conditions (experimental ischemia or carnosine treatment).

In **Figure 3** the values of mitochondrial SOD in several groups of animals studied are presented. It is seen that in the sham operation (Group 2), 3-NPA treatment (Group 3) or the experimental ischemia (Group 4) revealed little effect on SOD, whereas a combination of experimental ischemia with 3-NPA (Group 5) sufficiently increased SOD activity. Carnosine normalized the SOD level (Group 6).

Global ischemia increased the mitochondrial MAO B (by 15% compared with sham-operated rats) and 3-NPA did not affect this enzyme. In

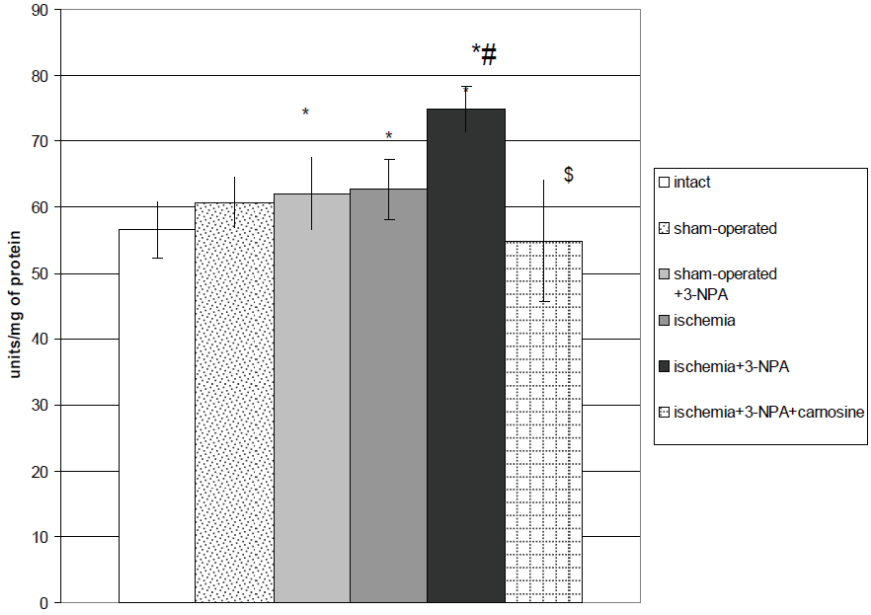

Figure 3. SOD (units/mg of protein) in brain mitochondria of the animals studied. "*" corresponds to statistically significant difference ($p<0.05$) from group 1; "#" corresponds to statistically significant difference ($p < 0.05$) from group 2, 3, 4 and group 6, "$" corresponds to statistically significant difference ($p<0.05$) from group 4, 5, other explanations as in Figure 2.

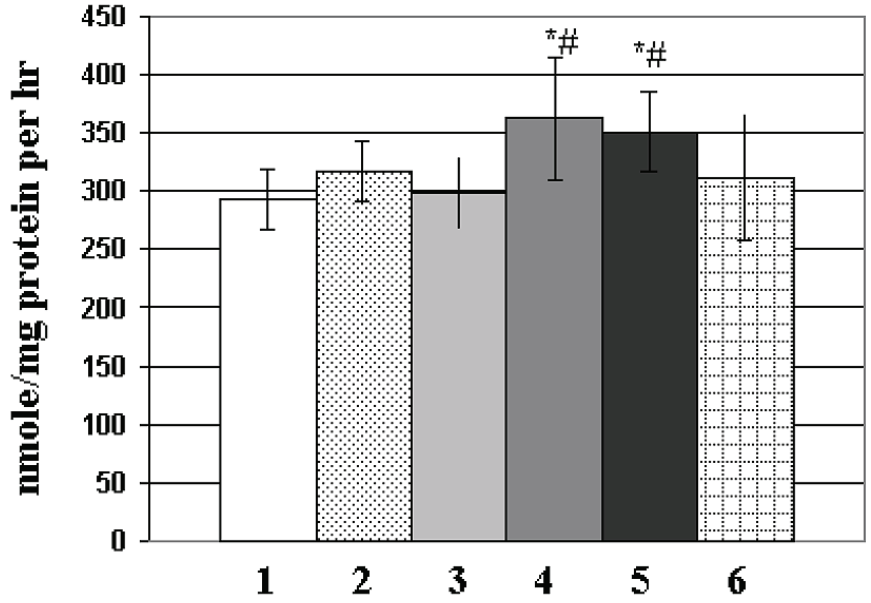

Figure 4. MAO B activity (nmole benzaldehyde/mg protein per hr) in brain mitochondria of the animals studied. "*" corresponds to statistically significant difference ($p<0.05$) from group 1; "#" corresponds to statistically significant difference ($p<0.05$) from group 2, other explanations as in Figure 2.

Group 6 animals, MAO B did not differ from that in Groups 1 and 2 (**Figure 4**).

Table 3 contains the results of chemiluminescence analysis of Fe^{2+}-induced oxidation of lipids in brain samples from several experimental groups. It is seen that the Group 3 animals (sham-operated and 3-NPA treated) did not differ from Groups 1 and 2. At the same time, Groups 4 and 5 rats were characterized by an elevated level of hydroperoxides, a shortened lag-period of oxidation and a dramatic increase in the rate of oxidation compared to the animals in Groups 1 and 2. For Group 5 rats, these parameters were much more pronounced which was consistent with the stronger suffering of these animals under the combined damage induced by both ischemia and 3-NPA. This data indicates more pronounced symptoms in these rats (see **Figure 1**).

Group 6 animals being treated with both 3-NPA and carnosine did not significantly differ statistically from the Groups 1 and 2 animals (intact

Table 3. Parameters of Fe^{2+}-induced oxidation of brain homogenate of the animals studied

№	Groups of animals	Parameters of Fe^{2+}-induced lipid peroxidation in brain homogenates		
		Hydroperoxides pre-formed, arbitary units	Lag period, sec	Rate of oxidation, units per min
1	Intact (n=8)	161±28	122±16	103±4
2	Sham-operated (n=8)	167±30	116±10	94±3
3	Sham-operated + 3-NPA (n=7)	201±32	115±7	98±2
4	Ischemia (n=8)	248±12(*)	83±10 (*)	152±7 (*)
5	Ischemia+3-NPA (n=7)	272±29(*)	86±9 (*)	175±2 (*)
6	Ischemia + 3-NPA +Carnosine (n=10)	200±20	120±7 (**)	91±4 (**)

and sham-operated) while for these animals the slightly increased level of lipid hydroperoxides was found. This data proves that carnosine compensates to the antioxidant stability of the brain exhausted by the combined action of experimental ischemia and 3-NPA. This conclusion is in total agreement with the positive effect of carnosine on neurological symptoms (**Figure 1**) and the viability (**Table 2**) of the animals.

Discussion

Development of free radical damage of brain tissues during ischemia is accompanied by neuronal degeneration [5, 22, 23]. Rats usually demonstrate definite resistance to ischemic damage. Thus, although in most cases of experimental brain ischemia there are indications of sufficient metabolic changes, they are not accompanied by neurological symptoms or mortality [7].

The combination of 3-NPA and the experimental brain ischemia resulted in stronger neurological symptoms (**Figure 1**), which correlated with metabolic disorders in the brain. Under these conditions, the level of lipid hydroperoxides was increased in the brain (by 63% compared to Group 2), the lag-period of Fe^{2+} -induced lipid peroxidation was shortened (by 26% compared to Group 2) and the rate of oxidation was correspondently increased (see **Table 3**).

All these observations pointed to the decrease in the efficiency of the antioxidant defense in the brain under the combined attack of ischemic processes and 3-NPA. The effect on the brain of experimental ischemia itself developed in a similar direction but was much less pronounced.

The neurological symptoms, which appeared under the combined action of the experimental brain ischemia and the 3-NPA are indicative of the degeneration of the dopaminergic neurons in the striatum, along with an energy deficit which is characteristic of 3-NPA action. This model, therefore, may be considered as a useful tool in estimating the efficiency of drugs potentially suitable for treating the Huntington disease and relative pathologies.

It is important to note that experimental ischemia (Group 4) is accompanied by the increase in the level of SDG and MAO B in the brain mitochondria (**Figure 2**) while the combination of ischemia and 3-NPA treatment in addition increases SOD. These modulations of metabolism however do not prevent the accumulation of lipid peroxides (**Table 3**). Elevated levels of oxidized lipid products in the brain are also in correlation with the high MAO B activity in Group 4 compared to Group 3. It is known that MAO B activation occurs during the reperfusion period [24], which can accelerate lipid peroxidation by the products of MAO B reaction – semialdehyde and superoxide anion (or hydrogen peroxide) [13]. The increased expression of MAO B is induced by the accumulation of extracellular dopamine during the disorder of its re-uptake and the death of dopaminergic neurons [24]. These facts may be interpreted to be an insufficient

adaptive response of the ischemic brain to attempt to neutralize the oxidative stress induced by the experimental ischemia.

In the literature there were several articles published demonstrating an increase in activity of both cytosolic and mitochondrial SOD in response to 3-NPA injection [25-27]. However, in terms of high mortality of the animals treated with 3-NPA, such adaptive reaction proves to be unsuccessful. At the same time, support of the antioxidant system with the injection of natural neuropeptide carnosine seems to be very effective. Carnosine significantly decreases the mortality of the rats (**Table 2**), decreases the tissue level of lipid peroxides and restores resistance of the brain towards Fe^{2+}-induced lipid peroxidation (**Table 3**), although it is not able to prevent SDG inhibition by 3-NPA (**Figure 2**). Only carnosine may restore the antioxidant state of the brain very efficiently so that SOD does not receive the signal to be stimulated so that it really does not take place (**Figure 3**).

Carnosine was found to possess a number of useful properties suggesting that it is the natural brain protector against oxidative stress [3]. In our experiments, carnosine also demonstrated high protection efficiency, which permits the recommendation of this compound as an effective neuroprotector. On the whole, the complex research of oxidative stress on the brain using the model described gives an opportunity to estimate possible therapeutic effects on the brain using a number of novel perspective antioxidants.

Acknowledgements

The work is supported by RFBR (#06-04-49675, Russia) and the International Vysegrad Foundation, Slovak Republic (S-051-2005).

Please address correspondence to: Stepanova Maria, PhD, Research Center of Neurology RAMS, Moscow, Russia. Tel: +7(495)/4902409, Fax: +7 (495)/4902409, E-mail: stepanova.mar@gmail.com

References

[1] Akopian G, Crawford C, Beal MF, Cappelletti M, Jakowec WM, Petzinger GM, Zheng L, Gheorghe SL, Reichel CM, Chow R, and Walsh JP. Decreased striatal dopamine release underlies increased expression of long-term synaptic potentiation at corticostriatal synapses 24 h after 3 -nitropropionic-acid-induced chemical hypoxia. J. Neurosci 2008; 28: 9585 - 9597.

[2] Boldyrev AA. Carnosine is a natural antioxidant and neuroprotector: biological functions and possible clinical use. Free radicals, NO, and inflammation: Molecular, Biochemical and Clinical Aspects Edited by Tomasi A, Ozbren T. and Skulachev VP. IOS Press, 2003, pp. 203-217.

[3] Boldyrev AA. Protection of proteins from oxidative stress: a new illusion or a novel strategy? Ann NY Acad Sci 2005 1057: 193-205.

[4] Hossmann K-A. Experimental models for the investigation of brain ischemia. Cardiovasc Res 1998; 39: 106-120.

[5] Pulsinelli WA, Brierley JV. A new model of bilateral hemispheric ischemia in the unanaesthetized rat. Stroke 1979; 10: 267-272.

[6] Gallant S, Kukley M, Stvolinsky S, Bulygina E, Boldyrev A. Effect of carnosine on rats under experimental brain ischemia. Tohoku J Exp Med 2000; 191: 85-99.

[7] Fedorova TN, Stvolinsky SL, Dobrota D, Boldyrev AA. Therapeutical effects of carnosine in experimental brain ischemia. Problems Biol Med Pharm Chem (in Russian) 2002; 1: 41-44.

[8] del Zoppo GJ. Why do all drugs work in animals but none in stroke patients? 1. Drugs promoting cerebral blood flow. J Int Med 1995; 237: 79–88.

[9] Grotta J. Why do all drugs work in animals but none in stroke patients? 2. Neuroprotective therapy. J Int Med 1995; 237: 89–94.

[10] Dobrota D, Fedorova T, Stvolinsky S, Babusikova E, Likavcanova K, Drgova A, Strapkova A, Boldyrev A. Carnosine protects the brain of rats and mongolian gerbils against ischemic Injury: After-Stroke-Effect. Neurochem Res 2005; 30 (10): 1283-1288.

[11] Beal MF. Neurochemistry and toxin models in Huntington's disease. Curr Opin Neurol 1994; 7: 542-547.

[12] Guyot MC, Hantaraye P, Dolan R, Palfi S, Maziere M, Brouillet E. Quantifiable bradykinesia, gait abnormalities and Huntington's disease-like striatum lesions in rats chronically treated with 3 -nitropropionic acid. Neuroscience 1997; 79: 45 -56.

[13] Maragos WF, Tillman PA, Jakel RJ. Chlorgyline and deprenyl attenuate striatal malonate and 3-nitropropionic acid. Brain Research 1999; 834: 168-172.

[14] Borlongan CV, Koutouzis TK, Randall TS, Freeman TB, Cahill DW, and Sanberg PR. Systemic 3-NPA: Behavioral deficits and striatal damage in adult rats. Brain Res Bull 1995; 36(6): 549-556.

[15] Andreassen OA, Ferrante RJ, Hughes DB, Klivenyi P, Dedeoglu A, Ona VO, Frielander RM, Beal MF. Malonate and 3-nitropropionic acid neurotoxicity are reduced in transgenic mice expressing a caspase-1 dominant-negative mutant. J Neurochem 2000; 75: 847-852.

[16] Boldyrev AA, Song R, Lawrence D, Carpenter DO. Carnosine protects against excitotoxic cell death independently of effects on reactive oxygen spe-

cies. Neuroscience 1999; 94: 571-577.
[17] Dodd PR, Hardy JA, Oakley AE, Edwardson JA, Perry EK, and Delaunoy JP. A rapid method for preparing synaptosomes: comparison, with alternative procedures. Brain Res 1981; 226 (1-2): 107-118.
[18] Misra HP, Fridovich J. The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. Biochemistry 1972; 247: 3170-3175.
[19] Powell CS, Jackson RM. Mitochondrial complex I, aconitase, and succinate dehydrogenase during hypoxia-reoxygenation: modulation of enzyme activities by Mn-SOD. Am J Physiol Lung Cell Mol Physiol 2003; 285: L189-L198.
[20] Fedorova TN, Boldyrev AA, Gannushkina IV. Lipid peroxidation during experimental brain ischemia. Biochemistry (Moscow) 1999; 64: 94-98.
[21] Lowry OH, Rosebrough NJ, Farr AL, and Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951; 193(1): 265-275.
[22] Nakano S, Kogure K, Fujikura H. Ischemia-induced slowly progressive neuronal damage in the rat brain. Neuroscience 1990; 38(1): 115-124.
[23] Lipton P. Ischemic Cell Death in Brain Neurons. Physiol Rev 1999; 79(4): 1431-1568.
[24] Ghukasyan TG, Petrosyan AA, Shirinyan ME, Shirinyan EA. Catecholaminergic system of the brain during ischemia. Neurochemistry (Moscow) 2000; 17(1): 13-22.
[25] Fu YT, He FS, Zhang SL, Zhang JS. Lipid peroxidation in rats intoxicated with 3-nitropropionic acid. Toxicon 1995; 33(3): 327-331.
[26] Binienda ZK, Ali SF. Neuroprotective role of L-carnitine in the 3-nitropropionic acid induced neurotoxicity. Toxicol Lett 2001; 125(1-3): 67-73.
[27] McCracken E, Dewar D, and Hunter AJ. White matter damage following systemic injection of the mitochondrial inhibitor 3-nitropropionic acid in rat. Brain Res 2001; 892(2): 329-335.