**Biological Markers and Guided Therapy, Vol. 5, 2018, no. 1, 75 - 84**
**HIKARI Ltd, www.m-hikari.com**
**https://doi.org/10.12988/bmgt.2018.81010**

# Study of the Expression Pattern of mRNA Hsp70 and the Level of $HSP_{70}$ Protein in Experimental Subtotal Ischemia and in the Contrast of Pharmacological Correction of $HSP_{70}$ Modulators

**I. F. Belenichev*, Yu. V. Bila and A. M. Kamyshniy**

Department of Pharmacology and Medical Formulation State Medical University, Zaporozhye, Ukraine
&
Mayakovsky av. 26, Zaporozhye, State Medical University
Post code: 69035, Ukraine
*Corresponding author



**Abstract**

The research is devoted to the current issue of medicine - optimization of the treatment of cerebral strokes. It was studied the neuroprotective effect of $HSP_{70}$ modulators- tamoxifen, melatonin, HSF-1 and glutamine. The research study was performed on 148 Wistar rats. An acute cerebrovascular disturbance was modeled by bilateral ligation of the common carotid arteries under thiopental anesthesia (40 mg / kg). Within 4 days, each group received treatment with one of the selected drugs (tamoxifen 1 mg / kg, melatonin 5 mg / kg, HSF-1 200 μl / kg, glutamine 25 mg / kg, piracetam 500 mg / kg). It was determined that on the $4^{th}$ day of the acute cerebrovascular disturbance a significant activation of oxidative stress occurs in contrast to an extreme decrease in the level of the $HSP_{70}$ protein and suppression of the expression of mRNKHsp70. The studied drugs reduced the distinct features of oxidative stress (according to the nitrotyrosine marker) and increased the level of HSP70 in contrast to increase in the expression of mRNKHsp70. The results identify the ability of tamoxifen, melatonin, HSF-1 and glutamine to modulate the activity of the HSPs system, which confirms that they

have $HSP_{70}$-mediated neuroprotection mechanisms and justifies the future prospects of $HSP_{70}$ regulation as an important target for neuroprotection.

**Keywords**: endogenous neuroprotection, mRNA Hsp70, tamoxifen, melatonin, glutamine, HSF-1, PCR-RT, nitrotyrosine

## Introduction

The prevalence of cerebrovascular diseases, including ischemic stroke, is at high level throughout the world. [1, 2, 3]. The search of new links of targets for pharmacological correction of the main stages of the cascade mechanisms of neurodestruction is constantly taking place.

A long-term study of the molecular and biochemical mechanisms of ischemic neurodestruction allowed us to identify the mechanisms of endogenous neuroprotection, aimed at increasing to neuron resistance to ischemia. [3,4]. HSP70 heat shock proteins play a special role in implementing these mechanisms. These proteins have antiapoptotic and cytoprotective effects. [3,5, 6]. At the Department of Pharmacology and Medical Formulation of ZSMU Research is being conducted to study the neuroprotective properties of HSP70 and the search for ways of pharmacological modulation of their activity. We obtained the data concerning GSH-dependent regulation of endogenous HSP70. [7,8]. In addition, recent research demonstrated the strong correlation of protein level of heat shock 70 with factor activity initiated with hypoxia HIF-1, which provides cell adaptation under hypoxic damage. [6, 9]
Of particular interest are drugs with a supposed ability to modulate the expression of HSP70. Our preliminary studies have determined the neuroprotective efficacy of modulators of the activity of HSP70: tamoxifen, melatonin, heat shock factor HSF-1 and glutamine. [3, 9].
Thus, the purpose of our research is to study the expression pattern of mRNAHsp70 and the protein level of HSP70 in experimental global ischemia and in contrast of applying HSP70 modulators: tamoxifen, melatonin, HSF-1 and glutamine.

The object and methods of research. The experimental part was performed on 148 Wistar rats that were kept in vivarium with natural light and had a standard nutrition. Experimental studies were conducted in accordance with the main provisions of the Council of Europe Convention on the Protection of Vertebrate Animals, which are used in experiments and other scientific purposes (Strasbourg, 1986), and others. [10,11]
An acute cerebrovascular accident (ACVA) by type of ischemic stroke was modeled by bilateral irreversible occlusion of the common carotid arteries under thiopental with sodium anesthesia (40 mg / kg). [12] Animals were divided into VII groups by randomization method: I - pseudo-operated rats (PO, n = 10); II - animals with ACVA (n = 10); III - ACVA + tamoxifen (Pharmaceutical Company

"Zdorovie ", Ukraine) (1 mg / kg) [13], (n = 10); IV - ACVA + melatonin (JSC "Kiev Vitamin Plant", Ukraine) (5 mg / kg) [14], (n = 10); V - ACVA + heat shock factor (HSF-1) (Sigma, USA) (200 μl / kg), (n = 10); VI - ACVA + glutamine (Sigma, USA) (25 mg / kg) [15], (n = 10); VII - ACVA + piracetam (PC "Borschahovsky Chemical and Pharmaceutical Plant", Ukraine) (500 mg / kg) [16], (n=10).

On the 4th day, the animals were taken out of the experiment under thiopental anesthesia (40 mg / kg). The brain areas located in the sensorimotor zone of the cortex were used, which were homogenized using a SilentCrusher S (Heidolph) homogenizer in sucrose buffer (250mM, EDTA 1mM, pH 7,4) t = + 40 C. The next differential centrifugation in a refrigerator centrifuge "Sigma 3-30k" (Germany) at 14000 g, t = + 40C, cytosolic and mitochondrial fractions were isolated.[12]

The feature of oxidative stress was determined in the cytosolic fraction of the brain by the nitrotyrosine marker using the enzyme immunoenzymometric kit from Hycultbiotech (Netherlands).

The level of heat shock protein HSP70 was determined in the cytosolic and mitochondrial fractions of the brain by immunoenzymometric analysis using an Enzo kit (Sweden).

For the analysis of gene expression, the polymerase chain reaction method with reversible transcription in real time (PCR - RT) was used.

Isolation of total RNA from rat tissue was performed using the Trizol RNA Prep100 kit (IZOGEN, Russia), which contains the following reagents: Trizolreagent and ExtraGene E.

The CFX96 ™ Real-Time PCR Detection Systems amplifier (Bio-RadLaboratories, Inc., USA) and the reagent kit for PCR-RV in the presence of SYBR Green R-402 (Sintol, Russia) were used to determine the expression level of the studied genes. The final reaction mixture for amplification contained dye SYBR Green , SynTaq DNA polymerase with inhibiting the activity of the enzyme by antibodies, 0,2 μl of direct and reversible specific primers, dNTP-deoxynucleoside triphosphates, 1 μl of matrix (cDNA).

The actin gene, beta (Actb) was used as a reference gene to determine the relative value of the change in the expression level of the studied genes.

We used the comparative Ct method (ΔΔCt method) to express the relative level of gene expression. Statistical analysis of PCR - RT data were performed using CFX Manager ™ software (Bio-Rad, USA). All amplification reactions were carried out on individual samples in triplicate. The results of real-time PCR analysis of Hsp70 were expressed as relative normalized expression of the indicated mRNA.

The results were processed using the statistical package of the licensed program STATISTICA® for Windows 6.0 (StatSoftInc.), Microsoft Excel 2010. Data are presented as arithmetic mean and standard error (M ± SEM). The significance of the differences (p) in the experimental data was calculated using Student's criterion, with parametric distribution, or by the Mann-Whitney U-criterion, with non-parametric values. Analysis of (ANOVA) was used to compare the independent

variables in more than two samples with a normal distribution or the Kruskal-Wallis criterion for a distribution other than normal. Differences were considered statistically significant at $p \leq 0,05$.

## Results and Discussion

Because of ligation of the common carotid arteries for 4 days in experimental animals, the expression of HSP70 was inhibited, which was reflected in a decrease in the level of HSP70 protein and expression of m RNA of Hsp70 against the background of activation of nitrosative stress. Thus, the level of nitrotyrosine in the control group increased by 553.5% relative to the PO group. (table1). At the same time, the heat shock protein concentration in the control group sharply decreased in the cytosol by 9.7 times and in the mitochondrial fraction by 3.2 times relative to the PO group (Table 2). Inhibition of expression of m RNA of Hsp70 was also observed by 3.2 times relative to PO.

Our data do not contradict other studies that show that cerebral ischemia leads to the formation of glutamate excitotoxicity, activation of iNOS and the development of nitrosative stress. An increase in NO and its intermediates leads to a disturbance of adaptation processes in the brain tissue [3], the production of c-fos, HSP70, HIF-1 is also reduced. [3, 6]

The studied drugs reduced the level of nitrotyrosine: tamoxifen by 69.4%, melatonin by 66.4%, heat shock factor HSF-1 by 69.9%, glutamine by 60.1%, piracetam by 63.3% relative to the control group (table 1). The obtained data confirm the ability of the studied drugs to prevent the development of hypoxic damage and to provide neuroprotective effects.

**Table 1. Level of nitrotyrosine in the brain tissue of animals with stroke**

| Group of animals (n=10) | Nitrotyrosine, nmol/g in protein |
|---|---|
| Pseudo-operated animals (PO) | 10,19±0,80 |
| Animals with ACVA | 66,59±13,1* |
| Animals with ACVA + tamoxifen | 20,39±0,96** |
| Animals with ACVA + melatonin | 22,4±1,91** |

**Table 1. (Continued): Level of nitrotyrosine in the brain tissue of animals with stroke**

| | |
|---|---|
| Animals with ACVA + HSF-1 | 20,04±1,77** |
| Animals with ACVA + glutamine | 26,63±2,47** |
| Animals with ACVA + piracetam | 24,46±2,13** |

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

The most pronounced increase in the level of heat shock protein 70 in the conditions of the ACVA caused the course assignment of the heat shock factor HSF-1. Thus, the level of HSP70 in the cytosolic fraction increased 11.3 times, in mitochondria 2.6 times relative to the control. (table 2), while the expression of m RNA of Hsp70 was 4.1 times higher than in the control. (Table 3) These changes can be explained by the fact that HSF-1 is a natural stress-inducible transcription factor of the HSPs family. Under conditions of accumulation of products of oxidative and nitrosative stress, trimerization of HSF-1 occurs with detachment of inactivated HSP70 molecules from the latter. The activated HSF-1 complex is sent to the nucleus and, combining with the elements of heat shock (HSE), starts the process of transcription of the Hsp70 genes.

With the introduction of tamoxifen, there was an increase in the level of HSP70 in cytosolene by 5.1 times, in the mitochondrial fraction by 1.5 times relative to the control group. (Table 2) We assume that the interaction of tamoxifen with estrogen receptors (ER) leads to the detachment of heat shock protein 70 from the latter molecules, which subsequently has a cytoprotective effect. [17]. The level of Hsp70 mRNA expression increased 2.5 times relative to the control (Table 3), which indicates the direct participation of tamoxifen in the transcription signaling of Hsp70 mRNA according to the feedback principle. Thus, as HSP70 is used in antiapoptotic processes, tamoxifen triggers the transcription of Hsp70 mRNA.

The introduction of melatonin led to an increase in the concentration of HSP70 relative to the control in the cytosol by 2.6 times, and in the mitochondria 1.2 times. (Table 2), and the expression of Hsp70 mRNA was increased by 2.6 times relative to the control. (tab.3) This effect of melatonin is explained by its antioxidant properties. [14] Melatonin reduces the number of free radicals and, as a result, prevents total DNA damage. [14,18] In addition, it was found that the interaction of melatonin with the MT1 and MT2 receptors prevents the translocation of NF-κB into the nucleus, inhibits the expression of iNOS and reduces the level of pro-inflammatory cytokines TNF-α, IL-1β, IL-6, and others. [18, 19] The increase in HSP70 under the action of melatonin is explained by its effect on nuclear transcription processes.

On the background of glutamine injection, the chaperone protein level increased 1.5 times in the cytosol, and 1.1 times in the mitochondrial fraction relative to the control (Table 2), and Hsp70 mRNA expression increased 1.7 times (Table 3). It is known that glutamine is a precursor for the synthesis of glutathione SH. [20] The latter is a component of the nonenzymatic thiol-disulfide antioxidant system, which regulates apoptosis, proliferation, redox homeostasis, cellular signaling, etc. [7, 8, 18] Introduction of glutamine normalizes the redox ratio GSH/GSSG forms of glutathione, which triggers the expression of adaptation genes and prevents protein damage, including HSP70. [14, 18] It is also known that glutathione regulates HSP70 [7, 8]

**Table 2. The concentration of HSP70 protein in the cytosolic and mitochondrial fractions of the brain with stroke.**

| The group of animals (n = 10) | $HSP_{70}$, cytosolic fraction, ng/ml | $HSP_{70}$, mitochondrial fraction, ng/ml |
|---|---|---|
| Pseudo-operated animals (PO) | 16,83±0,64 | 8,60±0,58 |
| Animals with ACVA | 1,73±0,11* | 2,72±0,19* |
| Animals with ACVA + tamoxifen | 8,81±0,51** | 4,15±0,20** |
| Animals with ACVA + melatonin | 4,55±0,27** | 3,3±0,19** |
| Animals with ACVA + HSF-1 | 19,50±1,05** | 7,08±0,23** |
| Animals with ACVA + glutamine | 2,61±0,16** | 3,01±0,17** |
| Animals with ACVA + piracetam | 2,24±0,17** | 2,80±0,19 |

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

**Table 3. The expression level of Hsp70 mRNA in the brain tissue of animals with stroke**

| The group of animals (n = 10) | Expression of Hsp70 mRNA |
|---|---|
| Pseudo-operated animals (PO) | 3,15±0,21 |
| Animals with ACVA | 1,00±0,11* |
| Animals with ACVA + tamoxifen | 2,49±0,36** |
| Animals with ACVA + melatonin | 2,08±0,14** |
| Animals with ACVA + HSF-1 | 4,07±0,41** |
| Animals with ACVA + glutamine | 1,73±0,17** |
| Animals with ACVA + piracetam | 1,10±0,06 |

* - $p \leq 0,05$ in relation to PO

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

## Conclusions

1. On the 4th day of the ACVA simulation in experimental animals, a decrease in Hsp70 mRNA expression by a factor of 3.2 and a concentration of HSP70 protein in the cytosolic by 9.7 times and 3.2 times in the mitochondrial brain fraction is observed, which indicates a breakdown of the mechanisms of endogenous neuroprotection and leads to oxidative brain damage, as evidenced by an increase in nitrotyrosine by 553.5% relative to pseudo-operated animals.
2. Treatment of HSF-1 (200 μl/kg), tamoxifen (1 mg/kg), melatonin (5 mg/kg) and glutamine (25 mg/kg) reduces the severity of oxidative stress, as evidenced by a decrease in nitrotyrosine level by 69.9 %, 69.4%, 66.4% and 60.1%, respectively, relative to the control.
3. Introduction of HSF-1 (200 μl/kg), tamoxifen (1 mg/kg), melatonin (5 mg/kg) and glutamine (25 mg/kg) increased the level of heat shock protein HSP70 by 11.3 times, 5.1 time, 2.6 times and 1.5 times in the cytosolic fraction, respectively, relative to the control group, which indicates the ability of the studied drugs to modulate HSP70-mediated mechanisms of endogenous neuroprotection.
4. Introduction of HSF-1 (200 μl/kg), tamoxifen (1 mg/kg), melatonin (5 mg/kg) and glutamine (25 mg/kg) increased the level of heat shock protein HSP70 2.6 times, 1.5 times, 1.2 times and 1.1 times in the mitochondrial fraction, respectively, relative to the control group, which indicates the mitoprotective properties of the studied drugs, which are mediated by the modulation of HSP70-mediated mechanisms of endogenous neuroprotection.
5. For the first time, the ability of tamoxifen, melatonin, HSF-1 and glutamine to increase the expression of Hsp70 mRNA under conditions of modeling of an acute violation of cerebral circulation has been established.

6. The most pronounced increase in the expression of Hsp70 messenger RNA was caused by the heat shock factor HSF-1 (4.1 times relative to the control group), which is explained by its function of the transcription factor.
7. Experimental data confirm the presence of tamoxifen, melatonin, HSF-1 and glutamine HSP70-dependent mechanisms of regulation of endogenous neuroprotection and justifies their use as neuroprotective agents in the treatment of stroke.

## References

[1] T.S. Mishchenko, Epidemiology of Cerebrovascular Diseases and Organization of Care for Patients with a Brain Stroke in Ukraine, *Ukrainian Newsletter of the Psychoneurological*, **25** (2017), no. 1, 22-24.

[2] D. Mozaffarian, E.J. Benjamin, A.S. Go et al, Executive summary: Heart Disease and Stroke Statistics-2016 update: A report from the American Heart Association, *Circulation*, **133** (2016), 447.

[3] I.F. Belenichev, V.I. Cherny, S.V. Pavlov, *Neuroprotection and Neuroplasticity*, The Publishing House Zaslavsky, Kyiv, 2016.

[4] I.S. Chekman, I.F. Belenichev, N.O. Gorchakova et al, Antioxidants: Clinical and Pharmacological Aspect, *Ukrainian Medical Journal*, **1** (2014), no. 1, 22-28.

[5] F.R. Sharp, X. Zhan, D. Liu, Heat Shock Proteins in Brain: Role of Hsp70, Hsp 27 and HO-1 (Hsp32) and Their Therapeutic Potential, *Translational Stroke Research*, **4** (2013), no. 6, 685–692. https://doi.org/10.1007/s12975-013-0271-4

[6] I.F. Belenichev, The role of heat shock proteins in the implementation of molecular biochemical mechanisms of neuroprotection, *Pharmacology and Medicine Toxicology*, **6** (2013), no. 36, 72-80.

[7] S. V. Gorbacheva, I. F. Belenichev, L. I. Kucherenko, Mechanisms of endogenous neuroprotection using modulators of the thiol-disulfide system under conditions of experimental cerebral circulation disorders, *Pharmacology and Medicine Toxicology*, **1** ( 2016), 24-30.

[8] I. F. Belenichev, Yu. V. Bila, The relationship between the concentration of HSP70, the activity of the thiol-disulfide system and the degree of neurological disorders in the simulation of acute cerebral ischemia, *Bulletin of Biology and Medicine*, **1** (2017), no. 135, 86-91.

[9] Yu.V. Bila, I.F. Belenichev, A.M. Kamyshniy, Peculiarities of impaired expression of Hif-1α and Hsf-3α matrix RNA under conditions of acute cerebrovascular accident and during the use of HSP70 modulators, *Pharmacology and Medicine Toxicology*, **3** (2018), no. 59, 3-9.

[10] Ethics of the doctor and human rights: the provisions on the use of animals in biomedical cases, *Experimental and Clinical Physiology and Biochemistry*, **2** (2003), no. 22,108–109.

[11] Law of Ukraine. On the Protection of Animals from Cruel Treatment [Electronic Resource] // Bulletin of the Verkhovna Rada of Ukraine (BIS). - 2006. - Access mode: http://zakon1.rada.gov.ua/laws/show/3447-15

[12] I. S. Chekman, Yu.I. Gubsky, I.F. Belenichev, *Preclinical Studies of Specific Activity of Potential Neuroprotective Drugs*, Kyiv, SPhC MoH of Uktaine, 2016.

[13] S. V. Pavlov, I. F. Belenichev, Molecular-biochemical aspects of the neuroprotective effect of a selective estrogen receptor modulator - tamoxifen under conditions of modeling of acute cerebral ischemia, *Neurochemistry*, **31** (2014), no. 1, 36-41. https://doi.org/10.7868/s1027813313040079

[14] S. M. Beni, R. Kohen, R. J. Reiter, Dun-Xian Tan, Esther Shohami, Melatonin-induced neuroprotection after close head injury is associated with increased brain antioxidants and attenuated late-phase activation of NF-kappaB and AP-1, *FASEB J.*, **18** (2004), 144 –151. https://doi.org/10.1096/fj.03-0323fje

[15] G. P. Zabot, G. F. Carvalhal, N. P. Marroni, R. Hartmann, V. Duval da Silva, H. Fillmann, Glutamine prevents oxidative stress in a model of mesenteric ischemia and reperfusion, *World Journal of Gastroenterology*, **20** (2014), no. 32, 11406-11414. https://doi.org/10.3748/wjg.v20.i32.11406

[16] U. Keil, I. Scherping, S. Hauptmann, K. Schuessel, A. Eckert, W. Muller, Piracetam improves mitochondrial dysfunction following oxidative stress, *British Journal of Pharmacology*, **147** (2006), 199–208. https://doi.org/10.1038/sj.bjp.0706459

[17] S. Pavlov, I. Belenichev, Molecular and Biochemical Aspects of the Neuroprotective Effect of the Selective Estrogen Receptor Modulator Tamoxifen in a Model of Acute Cerebral Ischemia, *Neurochemical Journal*, **8** (2014), no. 1, 28-32. https://doi.org/10.1134/s1819712413040077

[18] D. Alonso-Alconada, A. Álvarez, O. Arteaga, A. Martínez-Ibargüen, E. Hilario, Neuroprotective Effect of Melatonin: A Novel Therapy against Perinatal Hypoxia-Ischemia, *International Journal of Molecular Sciences*, **14** (2013), no. 5, 9379-9395. https://doi.org/10.3390/ijms14059379

[19] E. Esposito, S. Cuzzocrea, Antiinflammatory Activity of Melatonin in Central Nervous System, *Current Neuropharmacology*, **8** (2010), no. 3, 228-242. https://doi.org/10.2174/157015910792246155

[20] M.I. Amores-Sánchez, M. A. Medina, Glutamine, as a Precursor of Glutathione, and Oxidative Stress, *Molecular Genetics and Metabolism*, **67** (1999), no 2, 100 – 105. https://doi.org/10.1006/mgme.1999.2857

**Received: October 15, 2018; Published: November 7, 2018**