J. Fac. Pharm. Ankara / Ankara Ecz. Fak. Derg., 42(1): 14-21, 2018
Doi: 10.1501/Eczfak_0000000598

ORIGINAL ARTICLE / ÖZGÜN MAKALE

INFLUENCE OF THE FIXED COMBINATION OF GLYCINE WITH THIOTRIAZOLINE ON ENERGY METABOLISM PARAMETERS IN BRAIN IN CONDITIONS OF EXPERIMENTAL CEREBRAL ISCHEMIA

GLİSİN İLE TİYOTRİAZOLİN SABİT KOMBİNASYONUNUN DENEYSEL SEREBRAL İSKEMİ ŞARTLARINDA BEYİN ENERJİ METABOLİZMASI GÖSTERGELERİNE ETKİSİ

Lyudmila KUCHERENKO[1,2], Igor BELENICHEV[1], Ivan MAZUR[1,2], Olga KHROMYLOVA[1]*, Natalia PARNIUK[1]

[1]Zaporozhye State Medical University, Head of the Department of Pharmaceutical Chemistry, 69035, Zaporozhye, Ukraine
[2]SPA Farmatron

ABSTRACT

Objective: *The perspective direction of primary neuroprotection in cerebral ischemia is the correction of the imbalance of excitatory and inhibitory neurotransmitter systems by activating natural inhibitory processes. There is evidence of the ability of anti-oxidant thiotriazoline to potentiate the therapeutic effect of neuro-metabolic cerebroprotectors. Therefore, it is interesting to create a new combined drug based on glycine and thiotriazoline. The purpose of this study is to investigate the effect of glycine, as well as its combination with thiotriazoline, on the parameters of hydrocarbon-energy processes and oxidative metabolism under the conditions of simulation of acute cerebrovascular disorder (ACVD).*

Material and Method: *To create ACVD, a classic model consisting of simultaneous ligation of common carotid arteries was used in 50 Wistar male rats. All drugs were administered intraperitonally for four days starting with anesthesia recovery of rat groups. The content of adenyl nucleotides, pyruvate, lactate, malate, isocitrate and activities of succinate dehydrogenase, cytochrome C-oxidase, glutamate decarboxylase, GABA-transferase were determined in the homogenates of brain cortex by biochemical methods.*

Result and Discussion: *Our results showed that the combination of glycine with thiotriazoline is better than such reference drugs like pyracetam and glycine, according to degree of effect on the indicators of energy metabolism of the brain, indicating the relevance of further study of the proposed combination.*

* **Corresponding Author / Sorumlu Yazar:** Olga KHROMYLOVA
e-mail: hromyleva.olga@gmail.com

Submitted/Gönderilme: 20.12.2017 **Accepted/Kabul:** 08.02.2018

Keywords: *glycine; neuroprotective action; stroke; thiotriazoline; transmitter amino acids*

ÖZ

Amaç: *Serebral iskemide primer nörokoruma uyarıcı ve inhibitor nörotransmiter sistemleri arasındaki bozulan dengenin doğal inhibitor yolakların aktivasyonu ile düzeltilmesi yaklaşımına dayanır. Antioksidan bir madde olan tiyotriazolinin nöro-metabolik serebral koruyucuların terapötik etkilerini arttırdığına ilişkin kanıtlar bulunmaktadır. Bu nedenle glisin ve tiyotriazolini birlikte içeren yeni bir ilaç kombinasyonu oluşturmak ilginç olabilir. Bu çalışmada glisinin, ve tiyotriazolin ile kombine kullanımının akut serebrovasküler bozukluk (ASVB) oluşturulmuş rat modelinde hidrokarbon-enerji prosesleri ve oksidatif metabolizma parametreleri üzerindeki etkilerinin araştırılması amaçlanmıştır.*

Gereç ve Yöntem: *ASVB, klasik yöntem olan ana karotis arterlerin simültane ligasyonu ile gerçekleştirilmiş ve deneylerde 50 erkek Wistar rat kullanılmıştır. Uygulamalara gruplardaki ratlar anesteziden çıktıktan hemen sonra intraperitonal olarak başlanmış ve 4 gün boyunca devam edilmiştir. Beyin korteklerinde adenil nükleotidler, piruvat, laktat, malat ve isositrat düzeyleri, suksinat dehidrogenaz, sitokrom C-oksidaz, glutamat dekarboksilaz ve GABA-transferaz aktiviteleri biyokimyasal yöntemlerle saptanmıştır.*

Sonuç ve Tartışma: *Glisin ve tiyotriazolinin birlikte kullanımının, beyin enerji metabolizması göstergeleri üzerine referans ilaçlar olan pirasetam ve glisinden daha etkili olması, bu kombinasyon üzerinde araştırmaların devam etmesi gerekliliğini göstermektedir.*

Anahtar kelimeler*: amino asit transmitterler; glisin; inme; nörokoruyucu etkinlik; tiyotriazolin*

INTRODUCTION

Cerebrovascular diseases are widespread throughout the world and are among the most dangerous for the population. High indicators of mortality and disability of patients have caused great interest in this pathology over the past decades. Brain strokes often cause death, complete or partial disability, and significant decrease in the quality of life of patients [1]. From this perspective, it is extremely important to prevent death of nerve cells, protect them from damage in ischemia, restoration of impaired blood flow with pathological changes in blood circulation [2, 3].

The promising direction of primary neuroprotection in cerebral ischemia is the correction of imbalance of excitatory and inhibitory neurotransmitter systems by activating natural inhibitory processes [4]. In this regard, the natural inhibitory neurotransmitter glycine and its role in the mechanisms of acute cerebral ischemia [5] are attracting attention. Traditionally, glycine was thought to exhibit neurotransmitter properties at the spinal cord. GABA and glycine are equivalent neurotransmitters that provide protective inhibition of the central nervous system. Glycine is also a coagonist of glutamate NMDA receptors and is required for their normal functioning in submicromolecular concentrations. There is evidence of the ability of antioxidant thiotriazoline to potentiate the therapeutic effect of neurometabolic cerebroprotectors [6]. Therefore, it is interesting to create a new combined drug based on glycine and thiotriazoline. This work is an integral part of the joint integrated work of the Department of Pharmaceutical Chemistry of the Zaporizhzhya State Medical University and TOV Scientific-Production Association "Farmatron" regarding the creation of new drugs based on combinations of derivatives of 1,2,4-triazoles, which lasts more than 20 years [7, 8]. The

purpose of this study is to investigate the effect of glycine, as well as its combination with thiotriazoline, on the parameters of hydrocarbon-energy processes and oxidative metabolism under the conditions of simulation of acute cerebrovascular disorder (ACVD).

MATERIAL AND METHOD

50 "Wistar" male rats weighing 180-200 g were used in experiments from the kennel of the Institute of Pharmacology and Toxicology of the Academy of Medical Sciences of Ukraine. All manipulations were carried out in accordance with the "European Convention for the Protection of Vertebrate Animals Used for Experiments or for Other Scientific Purposes". The protocols of experimental studies and their results are approved by decision of the Commission on Bioethics of Zaporozhye State Medical University (Record No. 33 as of October 26, 2016).

To create an ACVD, a classic model consisting of simultaneous ligation of common carotid arteries was used. The operation was performed with ethaminal-sodium anesthesia (40 mg/kg). Through the incision on neck, the right and left carotid arteries were found and segregated, placed ligatures under them and ligated [9].

All animals were divided into 5 experimental groups (10 animals in each group): the first - intact (falsely operated rats, which, after anesthesia, which common carotid arteries were segregated without carrying out their ligation); the second one - rats with ACVD (control); third - rats with ACVD, which were received glycine every day for 4 days at a dose of 200 mg/kg; the fourth - rats with ACVD which were received glycine every day for 4 days in combination with thiotriazoline (4:1) at a dose of 200 mg/kg (in terms of glycine), the fifth - rats with ACVD, which were received pyracetam in dose of 500 mg/kg. All drugs were administered intraperitoneally every day, starting with anesthesia recovery of rats.

On the fourth day of the experiment, the animals were withdrawn from the experiment under the ethaminal-sodium anesthesia (40 mg/kg). Blood was quickly removed from brain, separated from the meninges and the studied pieces were placed in liquid nitrogen. It was then ground in liquid nitrogen to a powdered state and homogenized in a 10-fold volume of medium at (2°C) containing (in mmol): sucrose-250, tris-HCl-buffer-20, EDTA-1 (pH 7,4) [10]. At a temperature (+4°C), a mitochondrial fraction was isolated by differential centrifugation at a Sigma 3-30k (Germany) reefer centrifuge. To purify the mitochondrial fraction from large cell fragments, centrifugation was carried out within 7 minutes at 1000g, and then the supernatant was re-centrifuged within 20 minutes at 17000g. The supernatant was drained and stored at -80°C.

The content of pyruvate, lactate, malate, isocitrate, activities of succinate dehydrogenase, cytochrome C-oxidase, glutamate decarboxylase, GABA-transferase were determined in the

homogenates of cortex by biochemical methods. The brain quickly removed, cerebral cortex was isolated, which homogenized in liquid nitrogen. Protein-free extract was obtained by adding an accurate weigth of brain tissue morselized in liquid nitrogen to chloric acid (0.6M) followed by 5.0M potassium neutralization by carbonate [10]. Determination of the content of adenyl nucleotides, glycine, glutamate and γ-aminobutyric acid was carried out by chromatographic methods [10]. The method is based on the separation of adenosine triphosphate (ATP), adenosine diphosphate (ADP) and adenosine monophosphate (AMP) in the dioxane-isopropanol-water-ammonia system on a thin sorbent layer followed by quantitation by direct spectrophotometry at 260 nm. Isopropanol, dioxane, ammonia (Sigma, U.S.A.) and Merck sheets were used in experiments. 0.2 ml of protein-free tissue extract is applied to the starting line of sheet and chromatographed in a dioxane-isopropanol-water-ammonia (4:2:4:1) system. ATP, ADP, AMP are identified in the ultraviolet in a 365-nm UVS chromatographic layer. The samples are eluted in 4.0 ml of 0.1 N HCl and measured in spectrophotometer at 260 nm (Libra spectrophotometer, U.K.). The ATP, ADP and AMP (μmol/g of tissue) content is calculated from the calibration curve, corrected to a tissue weight. The obtained results were processed using the MS Excell computer program; probability of reliability was determined using the Student's T-test.

RESULT AND DISCUSSION

ACVD modeling leads to persistent disorders of energy metabolism. Reduction of energy resources of the brain occurred against the background of discortization of Krebs cycle reactions, as evidenced by a decrease in the level of malate, isocitrate, inhibitory activity of succinate dehydrogenase and cytochrome-c-oxidase (CHO). Compensatory activation of glycolysis was observed, as evidenced by an increase in lactate in brain tissues. These changes occurred against the background of the detected activation of the GABA system, which was expressed in the increase of glutamine decarboxylase and GABA-T, and a decrease in the content of glutamate and GABA in the brain tissues of experimental animals. In parallel, a decrease in the level of glycine was registered. In addition, there was an inhibition of energy transport and utilization, as shown by the decrease in ATP activity and mitochondrial creatine phosphokinase activity (m-CPK). Similar changes in the state of the GABA-ergic system in the creatine phosphokinase activity occur as a compensatory activation of additional shunt of energy creation under inhibition of Krebs cycle. Thus, the inhibition of the oxidation of α-ketoglutarate results in the activation of the gamma-aminobutyric acid and the conversion of glutamate to GABA, and then when the GABA-T is activated to amber semialdehyde, which, being converted into succinate, is oxidized in the Krebs cycle. However, the inhibition of the Krebs cycle on the site of isocyte succinate and the suppression of succinate reductase show inhibition of the succinate oxidase pathway of supply of protons to the respiratory chain and the inability to use succinate, which is additionally formed in the Roberts shunt. It is likely that the GABA amber semialdehyde turns into γ-hydroxy-butyric acid, which has a stronger

inhibitory effect than GABA and glycine, the deficit of which we have discovered, and is able to limit the harmful effects of harmful effect of excitatory aminoacids in cerebral ischemia. Thus, inhibition of oxidative production of energy, its transport and utilization, activation of compensatory ways of formation of ATP-glycolysis and Roberts shunt, which, however, do not fully satisfy the brain's need for energy and cause development of lactic acidosis and deficiency of inhibitory amino acids (GABA and glycine), is observed in the process of ACVD.

Glycine had a positive effect on the oxidative metabolism of the brain in ACVD, which was shown in the increase of the level of ATP and ADP. Administration of glycine contributed to the utilization of energy (increased ATP activity in the brain of animals receiving glycine). It reduced the activity of anaerobic glycolysis and limited the development of lactic acidosis. Glycine increased the oxide production of energy by means of normalization on the site of isocitrate-succinate in the Krebs cycle.

The use of a fixed combination of glycine with thiotriazoline in animals with ACVD resulted in significant activation of oxidative energy production in the dicarboxylic region of the Krebs cycle, as evidenced by an increase in malate and an increase in the activity of succinate dehydrogenase. At the same time there was an increase in the activity of cytochrome-C-oxidase and the level of isocitrate, which ensured the increase of ATP production. In parallel, there was an increase in the level of ADP and a decrease in the level of AMF (Table 1and Table 2).

Table 1. The content of adenine nucleotides in the cerebral cortex of rats on the 4th day of ischemia

Animal group	ATP μm/g of tissue	ADP μm/g of tissue	AMP μm/g of tissue	m-CPK μmol/mg protein/min	ATPase activity
Intact animals	2,85±0,05	0,47±0,01	0,13±0,02	1,876±0,021	21,47±0,78
Animals with ACVD (control)	1,00±0,08	0,27±0,01	0,21±0,01	0,621±0,012	16,44±0,65
Animals with ACVD + glycine	2,11±0,01*	0,33±0,01*	0,15±0,03*	0,724±0,022	19,22±0,23*
Animals with ACVD + glycine + thiotriazoline	2,79±0,01*[1]	0,44±0,02*[1]+	0,13±0,01*[1]	2,132±0,011*[1+]	25,07±0,12*[1+]
Animals with ACVD+ piracetam	1,67±0,04	0,3±0,01	0,18±0,03	0,685±0,02	18,55±0,2

Hereinafter: * $p < 0,05$ as related to control; *[1] $p < 0,05$ as related to piracetam group; *[+] $p < 0,05$ as related to glycine group.

Glycine in combination with thiotriazoline suppresses anaerobic activity of glycolysis (lowered lactate levels), reduced the "flow" of inhibitory amino acids in the compensatory and energy-less beneficial Roberts shunt (Table 2). Also, the level of glutamate, GABA and glycine was increased against the background of decreasing the activity of glutamate carboxylase and GABA-T (GABA-transferase). An increase in the level of inhibitory amino acids under the action of the combination is

likely to limit the action of the excitatory aminoacids of the brain and, thus, aggravating the total neuroprotective effect of the drug. A fixed combination of glycine with thiotriazoline had a positive effect on the oxidative energy production in the brain of rats with ACVD, and intensified transport and energy utilization, as evidenced by the corresponding increase in m-CPK activity and ATP activity in a series of animals which were administered glycine with thiotriazoline (Table 3).

Table 2. The content of carbohydrate-energy metabolism parameters in the cerebral cortex of rats on the 4th day of ischemia

Animal group	Pyruvate, μm/g of tissue	Lactic acid, μm/g of tissue	Malate, μm/g of tissue	Isocitrate, μm/g of tissue	Succinate dehydrogenase, μm/mg/min	Cytochrome-c-oxidase, μm/mg/min
Intact animals	0,46±0,01	2,32±0,06	0,31±0,02	0,52±0,07	6,44±0,10	3,44±0,11
Animals with ACVD (control)	0,22±0,01	8,52±0,11	0,11±0,05	0,20±0,03	2,88±0,17	1,00±0,07
Animals with ACVD + glycine	0,34±0,02*	5,22±0,21*	0,18±0,06*	0,33±0,01*	5,22±0,12*	2,77±0,10^{*+}
Animals with ACVD + glycine + thiotriazoline	0,44±0,01^{*1+}	3,85±0,12^{*1+}	0,47±0,03^{*1+}	0,57±0,03^{*1+}	7,89±0,33^{*1}	3,95±0,22^{*1}
Animals with ACVD+ piracetam	0,3±0,02	5,8±0,15	0,16±0,05	0,28±0,03	4,85±0,15	2,2±0,15

Hereinafter: * p <0,05 as related to control; ^{*1} p <0,05 as related to piracetam group; $^{*+}$ p <0,05 as related to glycine group.

Table 3. Content of indicators of GABA-ergic system in the cerebral cortex of rats on the 4th day of ischemia

Animal group	GBA, μm/g of tissue	Glycine, μm/g of tissue	Glutamate, μm/g of tissue	Glutamic acid decarboxylase, μm/ mg /h	GABA-T μm/ mg /h
Intact animals	3,87 ± 0,12	6,42 ± 0,21	14,72 ± 0,3	14,16±0,7	12,7±0,1
Animals with ACVD (control)	1,12 ± 0,04	2,33 ± 0,22	5,02 ± 0,05	18,05±0,1	24,1±0,3
Animals with ACVD + glycine	3,00 ± 0,07*	6,51 ± 0,34*	11,00 ± 0,10*	15,22±0,5*	16,1±0,4*
Animals with ACVD + glycine + thiotriazoline	3,85 ±0,15*	7,78± 0,33^{*+1}	14,21 ± 0,11^{*1+}	15,10±0,7*	15,2±0,7^{*1}
Animals with ACVD+ piracetam	2,65±0,06	5,1±0,2	9,7 ± 0,11	16,8±0,35	20,5±0,55

Hereinafter: * p <0,05 as related to control; ^{*1} p <0,05 as related to piracetam group; $^{*+}$ p <0,05 as related to glycine group.

The apparent neuroprotective effect of the combination of glycine and thiotriazoline, in our opinion, is explained by the mutually intensifying effect of these drugs. Thus, thiotriazoline, which is an effective scavenger of active forms of oxygen, limits the oxidative modification of protein structures of receptors including NMDA Red/Oxi-dependent way; prevent the formation of energy deficiency, oxidative stress [6]. Glycine, due to its connection with the glycine sites of the NMDA receptors, ensures the normal functioning of the entire receptor- ionform complex, preventing its excessive activation and thereby limiting glutamate excitotoxicity and possibly increasing the action of magnesium ions [4]. It was found that the administration of a fixed combination of glycine with thiotriazoline to animals with ACVD resulted in a significant activation of the oxidative energy production in the dicarboxylic region of the Krebs cycle. It was found that the administration of the combination of glycine with thiotriazoline inhibited the activity of anaerobic glycolysis, which leads to a decrease in lactic acidosis. The combination had a positive effect on oxidative energy production in the brain of rats with ACVD, and intensified transport and energy utilization. It was found that the administration of fixed combination of glycine and thiotriazoline to animals with ACVD resulted in the normalization of GABA-shunt and restored the concentration of inhibitory transmembrane amino acids, which increases the total neuroprotective effect of the drug. The combination of glycine with thiotriazoline was better than such reference drugs like piracetam and glycine by degree of influence on the parameters of energy metabolism of the brain, indicating the prospect of further research of the proposed combination indicating the relevance of further study of the proposed combination.

REFERENCES

1. Gusev, E.I., Skvortsova, V.I. (2001). Brain ischemia, Medicine, Moscow, p.328.
2. Mazur, I.A., Chekman, I.S., Belenichev, I.F. (2007). Metabolitotropic drugs, Pechatnyi Dom, Zaporozhye, p.309.
3. Galenko-Yaroshevskiy, P.A., Chekman, I.S., Gorchakova, N.A. (2001). Essays on the pharmacology of metabolic therapy, Medicine, Moscow, p.240.
4. Khizhnyak, A.A., Kursov, S.V. (2003). Participation of excitatory amino acid transmitters in the mechanisms of neurodestruction and perspective methods of pathogenetic correction. Pain Anesthetization and Acute treatment, 1, 43 – 46.
5. Astakhov, A. (2004). Glycised-KMP: just amino acid or multipurpose anti stress medication? Ukraine Drugs, 1, 35 – 36.
6. Gorchakova, N.A., Belenichev, I.F., Mazur, I.A. (2007). Mechanism of antioxidant and anti-ischemic action of thiotriazoline. Medicine Pharmacy News, 2(206), 11-21.
7. Belenichev, I.F., Gorbachova, S.V., Golovkin, V.V., Bukhtiyarova, N.V. (2006). Influence of the composition “Magnelong”, glycine, emoxipine and piracetam on the development of oxidative

stress in the brain of rats with acute cerebrovascular disorders (ischemic stroke). Medical Chemistry, 3, 107-110.

8. Mazur, I.A., Chekman, I. S., Belenichev, I.F., Gorchakova, N.A. (2011). Development of drugs based on fixed combinations with antioxidants - a promising area of modern pharmacology. Pharmacology and Drug Toxicology, 5, 199–200.
9. Kozhemyakin, Y.M., Khromov, O.S., Filonenko, M.A., Sayftedinovna, G.A. (2002). Scientific and methodical recommendations for the maintenance of laboratory animals and work with them. Avitsena Kiev, p.156.
10. Voronina, T.A., Seredinin, S.B. (2002). Manual on experimental (preclinical) study of new pharmacological substances, Information and analytical portal of the Ministry of Health of the Russian Federation ZAO "Remedium", p.320.