

## PHARMACOLOGY, TOXICOLOGY AND PHARMACEUTICAL SCIENCE.

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### PHARMACOLOGICAL EFFECTS ON OXIDATIVE STRESS NO- DEPENDENT MECHANISMS IN THE BRAIN UNDER CHRONIC ALCOHOL INTOXICATION: ANGIOLIN ANTIOXIDANT EFFECTS

The aim of this work was to study antioxidant effect of the new original neuro- and cardioprotector with endothelium protective properties – "Angiolin" ((S)-2,6- diaminohexanoic acid 3-methyl-1,2,4- thiazolyl -5-thioacetate) in modeling of chronic alcohol intoxication on the effect on the basic indicators of the NO system and oxidative stress. Under forming chronic alcohol intoxication in rats within 30 days in the control group we registered the disorders in the nitric brain system (increase in the NOS activity, hyper-NO, L-arginine deficiency), that led to the activation of oxidative stress, as evidenced by the increase in oxidative protein modifications products and nitrotyrosine. 14-day therapy with Angiolin (100 mg/kg, intragastrically) after the 30-day alcoholization had a normalizing effect on the studied indicators of the NO system – the level of stable NO metabolites was decreased, the NOS activity was decreased in the setting of increasing concentrations of L-arginine. These positive changes under the influence of Angiolin in nitric brain system of animals with chronic alcohol intoxication proceeded in the setting of significant decrease of nitrotyrosine, aldehydfenihydrazones and carboxylfenihydrazones, indicating significant inhibition of oxidative stress. Angiolin authentically exceeds the reference medication Mildronate (250 mg/kg, intragastrically) by virtue of the antioxidant action for all studied indicators. The results are experimental justification for the inclusion of Angiolin in the traditional treatment of alcoholism.

**Keywords:** *chronic alcohol intoxication, the NO system, oxidative stress, Angiolin, Mildronate*

Since 1990, in the post-Soviet countries there is a new wave of alcohol abuse incidents along with the increase of population alcoholization indicators which are significantly higher than the average European level. Official data shows that more than 3% of the citizens of Russia, Ukraine, Moldova and other countries of the former Soviet Union chronically abuse alcohol [8, c.128-130]. In recent years, the growth of prevalence rate associated with the use of alcohol among young people, steady decline in the age of initiation to alcohol and the increase in the number of alcoholic teenagers become apparent [9, c. 5-7, 12, c. 115 ]. Despite the rather extensive coverage of the affected issues in the special literature, approaches to medicament remodeling of target organ damage, and especially the brain, remain poorly developed and are not always reasonable [4, c.16-30, 10, c. 368, 14]. Features of the alcohol neurodegeneration mechanisms, in particular NO-dependent as well as search and evaluation of the most promising target components for pharmacologic effects on this the process, require more detailed consideration. Therefore, disclosure of molecular and biochemical mechanisms of neuron death and in terms of alcohol abuse and the development of ways of pharmacological remodeling is one of the urgent problems of modern neurology, narcology, psychiatry and pharmacology. Neurometabolic cerebroprotector (thioacetam), which includes pyracetamum and thiotriazolol, showed

promising results in the treatment of alcoholism [1-3 c. 124]. The members of SPA "Farmatron" under the direction of professor Mazur I.A. worked out new original neuro- and cardio- protector with endotheliotropic properties – Angiolin ((S)-2,6- diaminohexanoic acid 3-methyl-1,2,4- thiazolyl -5-thioacetate) by chemical modification of 3-methyl-1,2,4- thiazolyl -5- thioacetate (thiotriazolol) molecule [6, 7]. Based on the above, the purpose of this paper is to investigate the Angiolin antioxidant effect under chronic alcohol intoxication on the effect on the basic parameters of the NO system and of oxidative stress.

#### Materials and methods

The study was conducted on 70 white outbred male rats weighing 160 to 180 g, obtained from the vivarium of the Institute of Pharmacology and Toxicology, Academy of Medical Sciences of Ukraine. All manipulations were carried out according to the regulations on the use of animals in biomedical experiments (Strasbourg, 1986, as amended in 1998). Chronic alcohol intoxication was induced by daily intragastric administration of 15% solution of ethanol in a dose of 4 g/kg on the first 10 day, 15% ethanol in dose of 6 g/kg on the next 10 days, and 25% ethanol in dose of 4 g/kg on subsequent 10 days. After 30 days alcoholization was stopped, experimental treatment by studied drug was conducted and the monitoring was continued within 14 days [3, c. 512, 4, c. 16-30]. Studied drugs

were administered intragastrically 1 time per day for 14 days after 30-day alcoholization in a suspension stabilized with Tween-80 using a metal probe: 100 mg/kg of Angiolin, 250 mg/kg of Mildronate [3]. In this series of experiments there were four groups of animals:

- 1) intact (received normal saline with Tween-80 (10 rats);
- 2) control (untreated chronic alcohol intoxication (CAI), received normal saline with Tween-80 (20 rats);
- 3) animals with CAI receiving "Angiolin" (20 rats);
- 4) animals with CAI receiving Mildronate (20 rats).

We used: 200 mg of Angiolin tablets of PJSC "Kyivmedpreparat" production and 250 mg of Mildronate capsules of JSC "Grindex" (Latvia) production. At the end of the experiment the animals were narcotized by aethaminalum-natrium (40 mg/kg), the brain and the blood from the abdominal aorta were ejected. The brain was rapidly stripped from blood, separated from the meninges and the studied pieces in liquid nitrogen were grounded to powder and then stirred into 10-fold volume of medium at (2°C) containing (in mmol): sucrose - 250, Tris -HCl -buffer - 20, EDTA - 1 (pH 7,4) [5]. The mitochondrial and cytosolic fractions were isolated at a temperature (+4°C) by differential centrifugation (17000g) on refrigerating centrifuge Sigma 3-30k (Germany). Nitrogenic system was estimated by the activity of NO-synthase (NOS), the content of stable NO metabolites, the L-arginine level. The activity of oxidative stress was assessed by the level of the products of oxidative protein modification (OPM) - adelhydphenihydrazones (AFH), carboxylphenihydrazones (CFH) and nitrotyrosine in the cytosolic fraction of the brain. The stable NO metabolites were determined in rat's brain cytosol using BCM Diagnostics kits at 540 nm detection. The total NOS activity was determined in brain cytosol from the difference between the rates of NADPH oxidation recorded by fluorometric method in two parallel samples both containing and not containing NOS inhibitor [5, c. 81]. L-arginine was determined in brain cytosol spectrophotometrically after separation by thin-layer chromatography [5, c. 81]. L-arginine of the Sigma-Aldrich company (Cat.№ A 5006) was used as standard. The number of AFH and CFH was determined by spectrophotometry on oxidated amino acid residues interaction with 2,4-dinitrophenylhydrazine [5, c. 81]. Nitrotyrosine was determined by immuno sorbent solid-phase sandwich method (ELISA) (ELISA Kit (Cat. № HK 501-02) of Hycult Biotech production). The protein was determined by the Lowry method. The spectrophotometer Libra S70 PS (Biochrom Ltd. production, United Kingdom) was used in the work.

The results of the investigation were calculated using the standard analysis package of computer program «STATISTICA® for Windows 6.0» (StatSoft-Inc., №AXXR712D833214FAN5), as well as «SPSS

16.0», «Microsoft Office Excell 2003». Verification of normality was performed using the Shapiro-Wilk test. Data are presented as the sample mean. Accuracy of differences between sample means was assessed using Student t-test under normal distribution. The Mann-Whitney U test was used in the case of nonnormal distribution or analysis of ordinal variables. The analyses of variance (ANOVA) under normal distribution or Kruskal-Wallis test for nonnormal distribution were used for comparison of the independent variables in more than two samples. The difference  $p < 0.05$  (95%) was considered statistical significant for all analyses.

### Results and its discussion

As a result of studies, it was found that chronic alcohol intoxication in rats leads to the activation of oxidative and nitrosating stress, as evidenced by the increase of AFH and CFH, as well as protein nitrosylating products - nitrotyrosine in the brain and plasma. The free radicals attack the proteins of the entire length of the polypeptide chain, breaking not only primary but also secondary and tertiary protein structure, leading to protein molecule aggregation or fragmentation. Many enzymes containing SH-groups, such as adenosine triphosphatases, or dehydrogenases are easily oxidized by free radical attack. Thus, in the control group AFH and CFH increased by 54.35% ( $P \leq 0.05$ ) and 71.43% ( $p \leq 0, 05$ ), respectively, compared with the intact group and nitrotyrosine in plasma and brain of rats increased by 212.74% ( $p \leq 0.05$ ) and 737.32% ( $p \leq 0,05$ ) respectively, compared with the intact group (Table. 1).

Oxidative stress results in damage of the most important polymers - nucleic acids, proteins and lipids, AOS causing DNA damage (bases oxidation and their modification, chain breaks, chromosomal damage). Consequently their diverse functional activity (enzymatic, regulatory, participation in matrix synthesis, transport of ions and lipids) is reduced or eliminated, and as a result of all this, the normal functioning of the neuronal brain activity is changed, memory and cognitive-mnemonic functions are impaired [1-3, 12, c. 115-127].

Angiolin demonstrated strong antioxidant effect under chronic alcohol intoxication, reducing AFH and CFH at 32,39 ( $p \leq 0,05$ ) and 36.66% ( $p \leq 0,05$ ), as well as reducing nitrotyrosine in the blood plasma by 60% ( $p \leq 0,05$ ) and in the brain by 67.2% ( $p \leq 0,05$ ) comparing with the same parameters of control group (Table. 1). Antioxidant effects of Angiolin include AFH retardation in glutamate-calcium cascade and in mitochondrial bioenergetic systems, as well as inactivation of oxygen radicals [6,7]. Mildronate showed antioxidant effect which is less apparent than Angiolin. For example, the therapy with mildronate led to AFH decrease by 16% ( $p \leq 0,05$ ) and CFH by 15% ( $p \leq 0,05$ ), and also to plasma and brain nitrotyrosine reducing by 23.6% ( $p \leq 0,05$ ) and 39.20% ( $p \leq 0,05$ ) respectively, as related to the same parameters of control group.

Table 1

**Influence of Angiolin and Mildronate on the indicators of oxidative stress in the brain in a 30 day simulation of chronic alcoholism and subsequent 14-day treatment**

Animal groups	OPM products, c.u./g protein		Nitrotyrosine in blood plasma, nM/g of protein	Nitrotyrosine in brain, nM/g of tissue
	AFH	CFH		
Intact (n=10)	0,368±0,02	0,175±0,02	6,21±0,53	17,23±1,05
Control (CAI)	0,568±0,06*	0,3±0,02*	19,5±2,13*	144,27±18,5*
CAI+Mildronate, 250 mg/kg (n=10)	0,472±0,03** (-16%)	0,255±0,015** (-15%)	14,85±1,17** (-23%)	87,71±6,4** (-39%)
CAI+Angiolin, 100 mg/kg (n=10)	0,384±0,03** <sup>1</sup> (-32%)	0,19±0,02** <sup>1</sup> (-36%)	7,73±0,51** <sup>1</sup> (-60%)	47,26±2,63** <sup>1</sup> (-67,2%)

**Note:**\* –  $p \leq 0,05$  intact;\* –  $P \leq 0,05$  control;<sup>1</sup> –  $p \leq 0,05$  mildronate;

The NO system and its cytotoxic derivatives - peroxynitrite, nitrosonium ion, etc. play an important role in neuron's damage in the brain under chronic alcohol intoxication [2,3,11, c. 12-16, 12, c. 115-127]. We have found that the simulation of chronic alcohol

intoxication in rats led to nitrenergic brain system disorder and hyper-NO, that was testified with the increase of the level of nitrite in 3,5 times ( $p \leq 0,05$ ), of the level of NOS activity in 2,5 times ( $p \leq 0,05$ ) in the setting of simultaneous reduce of L-arginine content ( $p \leq 0,05$ ) by 80% (Table 2).

Table 2

**Influence of Angiolin and Mildronate on the indicators of the NO system in the brain of rats in the setting of chronic 30-day alcohol intoxication and subsequent 14-day treatment**

Animal groups	NO metabolites (NO <sub>2</sub> ), µm/g	NOS, nM/mg/protein/min	L-arginine, mcM/g of tissue
Intact (n=10)	4,84±0,83	2,41±0,37	3,67±0,44
Control (chronic alcoholization) (n=10)	16,90±1,6*	6,02±0,97*	0,77±0,08*
Mildronate, 250 mg/kg (n=10)	15,86±1,5 (-6%)	5,76±0,75 (-4%)	0,79±0,06 (+2%)
Angiolin, 100 mg/kg (n=10)	7,67±0,68** <sup>1</sup> (-54%)	2,43±0,21** <sup>1</sup> (-59%)	3,08±0,4** <sup>1</sup> (+289%)

**Note:**\* –  $p \leq 0,05$  intact;\* –  $P \leq 0,05$  control;<sup>1</sup> –  $p \leq 0,05$  mildronate;

The 14-day course of treatment by Angiolin led to the decrease of the level of stable NO metabolites and NOS activity by 54% ( $p \leq 0,05$ ) and 59% ( $p \leq 0,05$ ) respectively, as related to the control group. At the same time the increase of L-arginine indicators is observed in the brain of animals treated with Angiolin by 289% ( $p \leq 0,05$ ) as related to the control group. Mildronate has not shown the effect on the indicators of the system of nitric oxide in the brain of rats with chronic alcoholism. Antioxidant effect of Mildronate unlike the similar Angiolin effect is preset in the final stages of free radical oxidation due to the decrease of fatty acids delivery in the cytosol and mitochondria and inhibition of Fe<sup>2+</sup>-induced reactions of lipoprotein and proteins oxidation.

Thus, as a result of the study it was found that the prescription of "Angiolin" to the animals after chronic alcohol abuse normalizes the NO system indicators and inhibits the reaction of oxidative stress. Our data do not contradict the results of other researchers who have shown that Angiolin in acute and chronic brain ischemia inhibits the formation of mitochondrial

dysfunction and AOS by bioenergy systems, reduces the glutamate excitotoxicity, thereby reducing the production of AOS of neuronal NOS by increasing the affinity of the GABA- benzodiazepine receptor complex, as well as raising the level of a restored intermediates of thiol-disulfide system.

**Conclusions:**

1. In the formation of chronic alcohol intoxication in rats within 30 days in the control group we registered the disorders in nitroxidergic brain system – increase in nitrite levels in 3,5 times ( $p \leq 0,05$ ), an increase of the NOS activity in 2,5 times ( $p \leq 0,05$ ), in the setting of simultaneous reducing of the L-arginine by 80% ( $p \leq 0,05$ ), resulting in the activation of oxidative stress, as evidenced by the increase in AFH to 54.35% ( $p \leq 0,05$ ) and CFH to 71,43% ( $p \leq 0,05$ ) and nitrotyrosine in blood plasma and brain to 212,74% ( $p \leq 0,05$ ) and 737.32% ( $p \leq 0,05$ ) respectively, compared with the intact group.

2. 14-day therapy after the 30-day alcoholization, with Angiolin (100 mg/kg, intragastrically) had a

normalizing effect on the studied indicators of NO system and significantly reduced the oxidative stress processes.

3. Angiolin authentically exceeds the reference medication Mildronate (250 mg/kg, intragastrically) by virtue of the antioxidant action for all studied indicators.

4. The results are experimental justification for the inclusion of Angiolin in the traditional treatment of alcoholism.

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