Research Article

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Study of the Stress-Protective Effect of Sodium 2-((4-amino-5-(thiophen-2-ylmethyl)-4H-1,2,4-triazol-3-yl)thio)acetate

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ABSTRACT

The aim was to study the stress-protective effect of sodium 2-((4-amino-5-(thiophen-2-ylmethyl)-4H-1,2,4-triazol-3-yl)thio)acetate (ASP). The analysis of the effect of the sample on the behavior and psycho-emotional state of animals, physiological parameters, antioxidant-prooxidant system was studied. Studies of stress-protective properties were performed on white outbred rats. As a comparison drug was used a Mebicar (Adaptol®) (reference sample).

One hour after the simulation of immobilization 6-hour stress, the animals were subjected to a series of tests: "Open Field", "Light and Dark Chamber", "Morris Water Labyrinth". Behavioral tests were performed according to generally accepted methods in simplified modifications. Serum and liver homogenate were used to determine the content of markers of the antioxidant-prooxidant system: quantitative content of diene conjugates (DC), thiobarbituric acid reactants (TBA reactants), as well as catalase and superoxide dismutase (SOD) activity.

Based on complex behavioral tests data, Catalase, TBA reactants, DC, SOD data in the serum and in the liver homogenate it can be concluded the presence of stress-protective properties of ASP in the model of acute immobilization stress.

Keywords: 1,2,4-triazole, immobilization stress, heterocyclic compounds.

1. Introduction

Stress is an integral part of everyone's life. Increasing the amount and intensity of nervous tension can lead to irreparable consequences, serious illness or even death. There are data in various studies that stress can induce inflammatory changes in the brain and peripheral immune system [1, 2]. Permanent neuroimmune disorders can lead to complex disorders of the central nervous system [3]. Stress prompts inflammation not only peripherally but also centrally through the dysregulation of the immune system. This leads to the development of stress-related diseases, particularly depression [4].

There are many medical methods of treating stress. For example, herbal (medicines based on valerian, hawthorn, motherwort, etc.) and synthetic (Adaptol®) sedatives (for mild forms of stress) or stronger tranquilizers (Diazepam®) are used. Medicines that increase mental capacity (Bifren®) are also used to treat stress [5].

Most of drugs also suppress efficiency and can affect the patient's reaction. Thus, modern society needs drugs [6, 7] that reduce stress and do not affect or increase efficiency. To find such substances, it is best to use systems that have already proven themselves as active bases for creating effective drugs [8-10]. One of these is the 1,2,4-triazole system and substances with different types of activity have already been found (hepatoprotective, antimicrobial, antifungal, antiradical, antipyretic and actoprotective activities) [11-14].

In addition, among this class of compounds, most are low toxic and exhibit antiradical, antipyretic activity [15-17]. 4-Amino-1,2,4-triazol-3-ylthio acetic acids with various substituents at the 5th position of the triazole ring are amino acids in their structure. It is well known that natural amino acids reduce the level of stress in the body and increase performance (tyrosine and methionine).

That is why the aim was to study the stress-protective effect of sodium 2-((4-amino-5-(thiophen-2-ylmethyl)-4*H*-1,2,4-triazol-3-yl)thio)acetate. This compound was synthesized earlier [18].

2. Material and Methods

The initial compound sodium 2-((4-amino-5-(thiophen-2-ylmethyl)-4*H*-1,2,4-triazol-3-yl)thio) acetate (ASP) was synthesized at the Department Of Natural Sciences For Foreign Students And Toxicological Chemistry of the Zaporizhzhia State Medical University (Ukraine) (Figure 1). All stages of synthesis and confirmation of ASP structure are described in a previously published work [18].

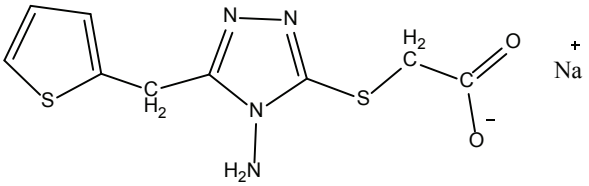

Figure 1. The initial compound sodium 2-((4-amino-5-(thiophen-2-ylmethyl)-4*H*-1,2,4-triazol-3-yl)thio)acetate (ASP)

The analysis of the effect of the sample on the behavior and psycho-emotional state of animals, physiological parameters, antioxidant-prooxidant system was studied.

Studies of stress-protective properties were performed on 28 white outbred rats aged 12 months, which were kept in standard conditions for this species on the basis of the vivarium of the Educational and Scientific Institute of Applied Pharmacy of the National University of Pharmacy [19, 20]. All stages of the study were conducted in accordance with the Directive of the European Parliament and of the Council 2010/63 / EU of 22 September 2010 "On the protection of animals used for scientific purposes" (Approval of the ethics committee session of NUPh Ukraine № 6 of 08.06.2021) [21].

Animals were randomized in 4 experimental groups (7 animals each) after the period of adaptation: Negative control (NC) (without immobilization stress), Positive control (PC) (animals that have been subjected to immobilization stress), Reference group (RG) (animals that received a reference sample (mebicar (Adaptol®) is a heterocyclic compound and contains nitrogen atoms in its structure) on the background of immobilization stress), Experimental group (EG) (animals that received a test sample (ASP) on the background of immobilization stress).

A new synthesized substance ASP (test sample) was used as the object of research (a dose is 100 mg/kg). In this dose test sample has already demonstrated the presence of actoprotective properties [18]. As a comparison drug was used a Mebicar (Adaptol®) (reference sample). It is a commercial drug with

stress-protective, nootropic, anxiolytic and antioxidant activity. It was chosen because it has a moderate sedative and stress-protective effect. Unlike benzodiazepines, it does not have a hypnotic effect. The dose of the reference sample was selected 100 mg/kg (taking into account daily therapeutic doses for clinical use). According to its structure, the comparison drug is also a cyclic substance containing nitrogen atoms. As for pharmacological properties, mebikar also increases performance as ASP [18].

The substances were administered intragastrically in the form of a suspension with purified water (20 mg/mL) for 5 days in appropriate doses on an empty stomach using a special metal probe. It is this period of introduction that is standard according to the research methodology. One hour after the simulation of immobilization 6-hour stress [22-24], the animals were studied using "Open Field", "Light and Dark Chamber", "Morris Water Labyrinth" tests. Behavioral tests were performed according to generally accepted methods in simplified modifications.

The "Open field" is the standard test for determining the functioning of the CNS of laboratory animals [22]. After pre-keeping without light, the animals were placed in the middle of the equipment "Open field" (size - 75x75 cm, height of the side wall - 30 cm, the number of squares of the field - 25, the diameter of the holes at the intersections - 3 cm). The rat was observed for 3 minutes. During the test indicators of motor research activity and emotional reactivity were recorded, namely: motor horizontal and vertical activity, orientation research activity, number of fecal boluses, urination, grooming acts, calculated total amount of activities.

The "Light-dark chamber" test (size - 56x43x62) was performed to assess the behavior of rats under conditions of variable stress with the ability to freely choose comfortable conditions. This test is used to identify the anxiolytic properties of the studied drugs [22]. Animals were placed in a dark chamber and observed behavior and research activity for 3 minutes, during the test recorded the number of transitions between chambers and the total time spent in each cell.

The "Morris Water Labyrinth" test was used to assess hippocampal-associated processes under stress. Two series of training sessions were conducted the day before the administration of the substances. The animals were placed in a round pool (d = 120 cm) filled with water (27 ° C), on the sides of which there are marks of different shapes and colors so that the animal could see them while swimming. There were no other spatial landmarks, than labels in the pool or test room. In the center of one of the quarters of the pool was a pedestal, the platform of which was level with the water, covering it by 0.5 cm (while swimming in training tests, the animals saw the top of the platform). During the training tests, the animal was alternately placed in 3 opposite quarters of the pool. It was allowed to get on the platform for 120 s. If the animal did not get on the pedestal during this time, it was moved there by hand. After 30 seconds on the platform, the animal was removed from the installation. During the control test, the water in the pool was colored with food coloring and covered the platform by 0.5 cm, so that it was not visible to animals during swimming. During the control test, the time when animal found the platform was recorded, which was limited to two minutes per attempt [25].

39 hours after the immobilization was completed, the animals were euthanized humanely in a CO_2 chamber. The period before euthanasia was due to the period of change manifestation at the stage of stress response [22]. Whole blood was taken with a syringe from the inferior vena cava. Serum was obtained by centrifugation at 1500 g for 10 minutes on a refrigerated centrifuge Eppendorf 5702R (Eppendorf, Germany). All obtained serum samples were stored at -20 °C [26].

Serum and liver homogenate were used to determine the content of markers of the antioxidant-prooxidant system: quantitative content of diene conjugates (DC), thiobarbituric acid reactants (TBA reactants), as well as catalase and superoxide dismutase (SOD) activity. Verification of the results of biochemical studies (absorption) was performed by photometric methods on the photoelectrocolorimeter KFK-3 and spectrophotometer SF-46.

Quantitative content of DC in samples of biological material was determined photometrically by routine method after extraction with heptane at a wavelength of 233 nm [27].

Analysis of TBA reactants was performed by standard reaction with thiobarbituric acid, after precipitation of proteins with trichloroacetic acid, the measurement of the optical density of the sample is performed against the control sample at 532 nm [28].

Determination of catalase activity was performed by a conventional method based on the reaction of for-

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mation of colored complexes of hydrogen peroxide and ammonium molybdate with an optimal absorption length of 410 nm [28].

Determination of SOD activity of biological material in samples was measured by the dynamics of autoxidation of adrenaline at a wavelength of 347 nm every minute for 3 minutes; enzyme activity was expressed in conventional units, which corresponded to the percentage of inhibition.

The results were expressed as the arithmetic mean (M) and the standard error of the mean (SEM) or the minimum and maximum values (min ÷ max) and as the median (Me) with the lower and upper quartiles (Q25; Q75) depending on the nature of the distribution samples and aspects of data representation. Kruskal-Wallis test and Mann-Whitney U-test methods were used to compare the samples. The probability of differences was determined by the significance level $P<0.05$. Statistical processing was performed using the basic software package MS Exel 2007 and IBM SPSS Statistics 22.

3. Results and Discussion

The Open Field Test is a standard test to determine the effect of the drug on the central nervous system. Usually, a significant change in locomotor, orientation and psycho-emotional behavior of animals in the experiment requires chronic stressors, but in some cases and after acute stress can be observed manifestations of stressful behavior of animals, which is primarily associated with decreased motor activity, which can be interpreted as the fear of getting into a situation that provokes a recurrence of stress.

Thus, in this study, acute immobilization stress for 6 hours only contributed to a decrease in locomotor activity, which was expressed in a decrease in the average number of squares of the field by 32.6% ($p<0.05$) against NC). All other indicators, including the sum of activities did not show differences from the intact norm (Table 1).

When the reference drug Mebicar was used, there was an even greater decrease in the parameter of the squares passed than in untreated animals from the PC group. The decrease in this locomotor index was 44.2% compared with a similar parameter of animals in the negative control group ($p<0.05$). At the same time, there was also a probable decrease in the sum of all activities in the NC group by 45.5%, which was not observed even in animals of the PC group. This effect of the reference agent on stressed animals may be due to its tranquilizing properties and ability to depress the central nervous system. Such features of pharmacodynamics are confirmed by information from the instructions for use and the results of subsequent behavioral tests.

ASP did not show an additional inhibitory effect and normalized all the tested parameters to the level of animals in the negative control group ($p>0.05$), thereby correcting the effects of stress. It shows the pronounced stress-protective properties (Figure 2).

Immobilization stress for 6 hours contributed to the classic manifestations of animal's stressful behavior in the test "Light-dark chamber". The results obtained during the test are presented in table 2.

In the Positive control group, the number of camera transition attempts probably decreased in contrast to intact ones. The time of arrival in the illuminated chamber also decreased, and the advantage of staying in the dark chamber increased accordingly ($p<0.05$), which indicates a significant effect of model stress.

In the Reference group the animals almost ignored being in a dark cell, and were always in the light. This behavior on the one hand indicates significant anxiolytic properties of the comparison drug. However, the obtained effect cannot be called corrective, as the changes were too drastic and not only offset the depressive effect of immobilization stress, but also led to changes in the preference of the place even in comparison with healthy animals ($p<0.05$).

The test sample at a dose of 100 mg/kg also showed a marked anxiolytic effect, with all changes occurring only in animals of the positive control group ($p<0.05$), which indicates a pronounced stress-protective effect (Figure 3).

Taking into account previous stress training cycles in the "Morris Labyrinth" test, after 6 hours of immobilization there was a statistically significant increase in the median time spent by animals searching for the platform, almost 2.5 times compared to the same value in the negative control group (Table 3).

The use of the reference drug did not cause significant changes in the duration of the search for the platform compared to the same indicator in the group of positive control.

At the same time, the use of test sample significantly reduced the latency of the platform search in stressed

Table 1. The effect of ASP 34 on the behavior of rats in the "Open Field" test under acute immobilization stress (n = 7, M (Min ÷ Max))

Indexes	Negative control	Positive control	Reference group	Experimental group
Latent period, s	6,43 ±4,61	6,29±2,77	31,00 ±42,57	7,14 ±3,63
Number of squares intersections	19,71±3,95	13,29±6,81[a]	11,00 ±5,71[a]	14,57 ±7,63
Number of vertical racks	6,29±4,04	2,71±1,38	2,57 ±2,65	4,29 ±2,04
The number of peeks in the burrows	1,29±1,18	1,14 ±0,81	0,29±0,41	1,00 ±1,14
Number of defecations	1,71±1,67	1,57±1,51	0,43±0,49	1,29 ±1,18
Number of urinations	1,29±1,55	1,86±1,55	1,00 ±0,85	1,14 ±0,77
Number of washes	1,43±1,06	1,00 ±1,14	2,00 ±1,14	0,86 ±0,73
The amount of emotional activity	4,43±3,75	4,43±2,89	3,43 ±0,65	3,29 ±1,55
Total amount of activities	31,71±8,81	21,57 ±6,77	17,29 ±8,24[a]	23,14 ±10,73

[a] Differences are statistically significant for the values of the NC group, $p<0.05$ (Mann-Whitney U-test).

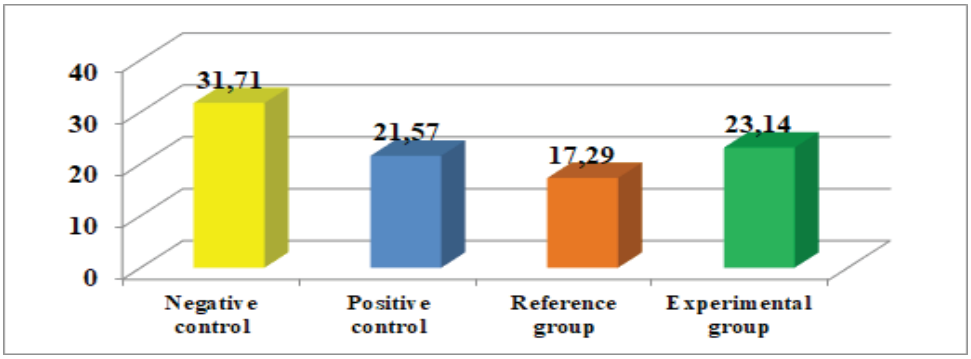

Figure 2. Total amount of rats' activities in the "Open Field" test under acute immobilization stress

Table 2. The effect of ASP 34 on the behavior of rats in the "Light-dark chamber" test under conditions of acute immobilization stress (n = 7, Me (Q25; Q75))

Indexes	**Negative control**	**Positive control**	**Reference group**	**Experimental group**
Peering into the hole	3,71±1,10	1,86±1,59[a]	1,00±0,57[a]	1,86±1,30[a]
Transitions completely	1,00	0,57±0,49	0,29±0,41[a]	0,57±0,49
Light camera, s	44,00±12,57	18,71±13,95[a]	141,57±49,18[a/b]	104,00±74,28[b]
Dark chamber, s	136,00±12,57	161,29±13,95[a]	38,43±49,18[a/b]	76,00±74,28[b]

[a] Differences are statistically significant relative to the values of the NC group, $p<0.05$ (Mann-Whitney U-test)
[b] Differences are statistically significant for the values of the PC group, $p<0.05$ (Mann-Whitney U-test).

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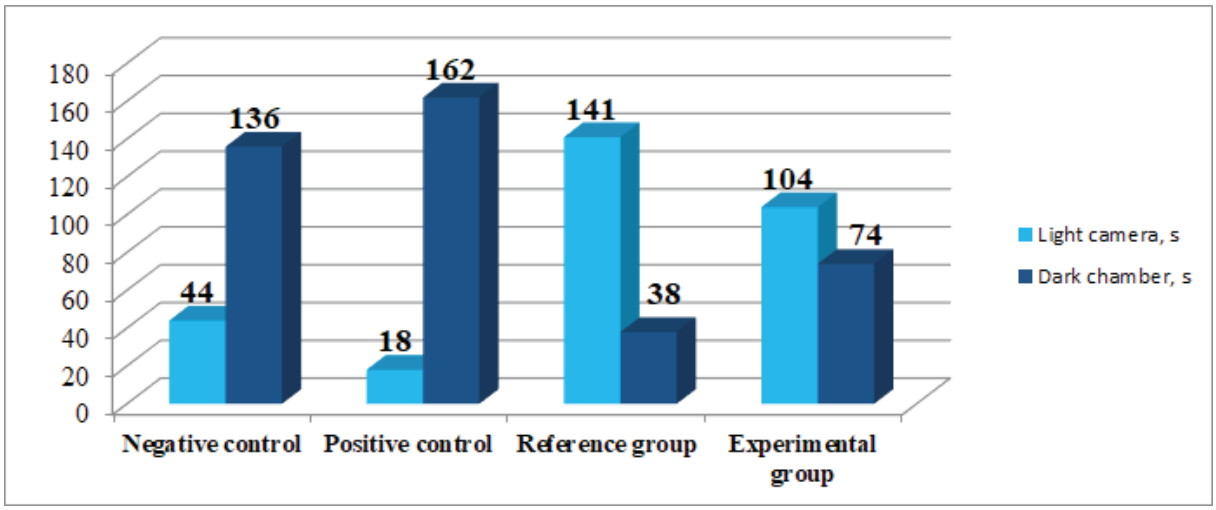

Figure 3. The effect of test sample on the rats' behavior in the "Light-dark chamber" test under conditions of acute immobilization stress

animals, which may indicate an improvement in the consolidation of spatial memory against the background of acute stress (Figure 4).

The results obtained in this test indicate the potential ability of the test sample to improve spatial memory and other cognitive functions under conditions of specific neurological pathologies, which determines the prospects for further study of this compound.

Removal of organs sensitive to stress is an important step in verifying the effects of the studied drugs. And although one episode of acute stress is not always enough to significantly change the mass of the relevant organs, this stage of the study still allows us to track the presence of the drug's susceptibility to them.

Against the background of a single episode of acute immobilization stress, no macroscopic changes were observed that could appear in such a short period. The internal organs of the animals of the PC group did not differ visually from those removed from conditionally healthy animals. The determination of mass coefficients also did not show differences in these parameters of both control groups (Table 4).

In the Reference group there was a significant increase in the mass coefficient of the liver both in comparison with NC (by 16.7%) and in comparison with PC (by 9.8%); probable increase in the mass ratio of the adrenal glands compared to NC by 23.3%; as well as a significant increase in the thymus - by 27.9% ($p<0.05$ against NC). Such an unpredictable change in the mass coefficients of the internal organs may indicate a high tropism of the drug to these organs and be one aspect of pharmacodynamics, and may indicate the peculiarities of the toxicodynamics of the drug.

The analysis test sample did not lead to significant changes in the mass coefficients of individual internal organs, which in the general pool of data can be interpreted as a positive aspect of the safety profile of this substance.

Under conditions of pathology after 39 hours in animals, disorders of the pro- / antioxidant system were predicted, which was reflected in the imbalance in the formation of products of lipid peroxidation and the activity of enzymes that regulate redox reactions. The results of biochemical measurements are shown in Table 5.

Thus, animals of the positive control group showed a significant increase in serum DC by 54.7% compared with intact animals, while there was also a tendency to increase TBA-active substances by 37.3%. In the liver homogenate of animals in the PC group, catalase activity was statistically reduced by 20.3% and the content of diene conjugates increased by 48.2% ($p<0.05$ vs. NC).

The use of the reference sample mebicar did not lead to significant changes in the markers of the pro- / antioxidant system in the liver tissues of stressed animals, but in the serum, there was a significant decrease in DC content by 24.5% compared to PC group.

Table 3. The effect of ASP 34 on the rats' behavior in the "Morris Labyrinth" test under conditions of acute immobilization stress (n = 7, Me (Q25; Q75))

Indexes	Negative control	Positive control	Reference group	Experimental group
Latent search time of the platform, s	22,71±10,53	41,14±5,55[a]	32,00±9,71	31,71±7,67[b]

[a] Differences are statistically significant relative to the values of the NC group, p<0.05 (Mann-Whitney U-test)
[b] Differences are statistically significant for the values of the PC group, p<0.05 (Mann-Whitney U-test).

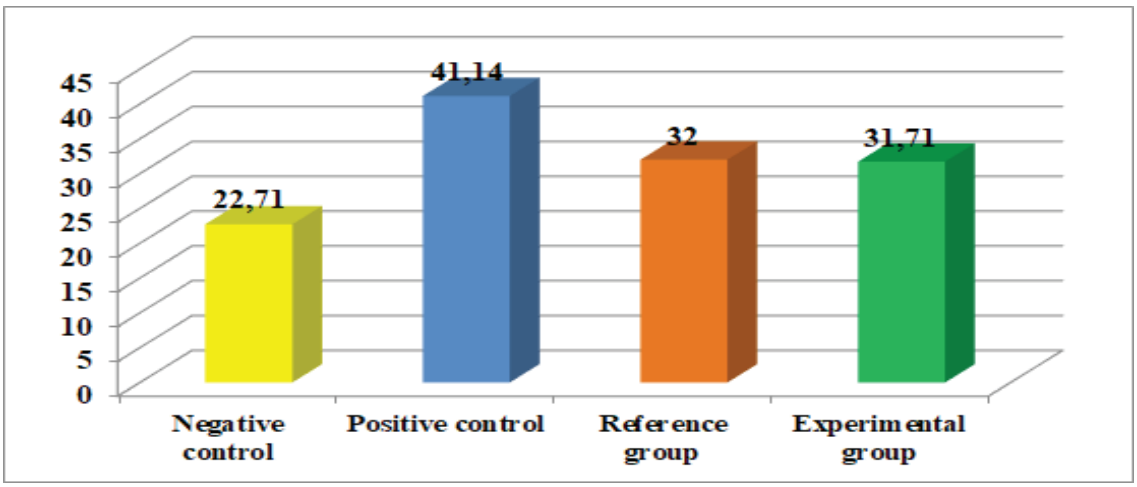

Figure 4. The effect of test sample on the rats' behavior in the "Morris Labyrinth" test under conditions of acute immobilization stress

Table 4. The effect of ASP on the coefficients of rats' internal organs under conditions of acute immobilization stress

Indexes	Negative control	Positive control	Reference group	Experimental group
Liver, %	2,32±3,13	2,66±3,37	3,03±4,01 [a]/b	2,62±3,58[a]
Adrenal glands, %	0,028±0,032	0,026±0,049	0,029±0,048[a]	0,021±0,042
Thymus, %	0,049±0,083	0,058±0,080	0,068±0,144[b]	0,055±0,088[c]

[a] Differences are statistically significant relative to the values of the NC group, p<0.05 (Mann-Whitney U-test)
[b] Statistically significant differences in the values of the PC group, p<0.05 (Mann-Whitney U-test);
[c] Differences are statistically significant for the values of the RG, p<0.05 (U-Mann-Whitney test).

Instead, the use of the test sample contributed to certain corrective therapeutic changes in both serum and liver tissue homogenate. In the rats' serum the content of TBA reactants probably decreased by 31.6% and the content of DC by 23.2% compared to similar indicators in the PC group (p<0.05). At the same time in the liver parenchyma there was also a moderate decrease in the content of diene conjugates by 14.2% (p<0.05 vs. NC)

4. Conclusions

In the Open Field, Light Dark Camera, and Morris Labyrinth tests, the test sample at a dose of 100 mg/kg was able to correct the effects of immobilization stress on the central nervous system under prophylactic medication for 5 days before the creation of control pathology (Approval of the ethics committee session of NUPh Ukraine № 6 of 08.06.2021). Since almost all the studied parameters under the influence of this drug returned to normal and did not differ

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Table 5. The effect of ASP 34 on the biochemical parameters of the antioxidant system of blood serum and rats' liver tissue under conditions of acute immobilization stress (n = 7, M ± SEM)

Indexes	Negative control	Positive control	Reference group	Experimental group
		In the serum		
Catalase, μmol / (min * l)	11,97±0,40	13,29±1,18	13,00±1,05	14,70±1,72
TBA reactants, μmol / l	0,244±0,027	0,335±0,034[a]	0,293±0,047	0,229±0,020[b]
DC, μmol/l	0,245±0,024	0,379±0,037[a]	0,283±0,035[b]	0,291±0,032[b]
SOD, standard units (% inhibition)	59,24±5,61	48,69±5,96	54,34±4,75	52,93±8,12
		In the liver homogenate		
Catalase, μmol / (min * g)	2,46±0,07	1,96±0,08[a]	2,06±0,10[a]	2,07±0,10[a]
TBA reactants, μmol / l	43,77±7,29	61,54±9,83	55,31±4,95	54,76±4,73
DC, μmol/l	65,94±5,51	97,73±6,93[a]	82,47±6,10[a]	83,83±3,38[a/b]
SOD, standard units (% inhibition)	40,45±4,40	34,11±4,30	37,09±3,03	38,34±4,37

[a] Differences are statistically significant relative to the values of the NC group, $p<0.05$ (Mann-Whitney U-test)
[b] Differences are statistically significant for the values of the PC group, $p<0.05$ (Mann-Whitney U-test).

from similar parameters of animals of the negative control group, in this case the effect can be considered significantly stress-protective, in contrast to the comparison drug (RG), which stress-protective effects were caused by tranquilizing activity and were the consequences of suppression of the central nervous system.

The results of the study demonstrate the presence of sodium 2-((4-amino-5-(thiophen-2-ylmethyl)-4*H*-1,2,4-triazol-3-yl)thio)acetate stress-protective properties in the model of acute immobilization stress, which was reproduced for 6 hours. The stress-protective properties of ASP were also previously described on the basis of histological studies [29]. On the basis of complex studies (behavioral tests and histological studies made by us earlier [29]), it is possible to conclude about the presence of stress-protective properties of the compound.

In addition, against the background of acute stress under the influence of the test sample was determined by a probable decrease in markers of peroxidation of lipids in the serum of animals, which indicates the presence of indirect antioxidant and membrane-protective properties.

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Conflict of Interest

The author has no conflicts of interest.

Statement of Contribution of Researchers

Concept: A. S., D.D., Y.V.; Design: D.L., O.P., A.K., L.M.; Control: A. S, Y.V., D.L.; Sources: O.P., A.K., L.M.; Materials: A. S., D.D.; Data Collection and Processing: A. S; Analysis and Interpretation: A. S., Y.V.; Literature Review: O.P., A.K., L.M.; Manuscript Writing: A. S., D.D., Y.V., D.L.; Critical Review: Y.V., D.L.

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