PharmacologyOnLine

Archives • 2021 • vol.3 • 1398-1406

DEVELOPMENT OF NITROOXIDATIVE STRESS IN RATS UNDER TOBACCO-NITRITE INTOXICATION AFTER APPLICATION OF MILDRONATE

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Abstract

The widespread prevalence of smoking is a global problem of mankind, and the efforts of many scientists and specialists are aimed at solving it. The danger of smoking is not only a negative impact on the health of smokers, but also on the well-being of non-smokers, but exposed to harmful pollutants that enter the air with tobacco smoke.

Due to the widespread use of nitrate fertilizers in agriculture and their entry into groundwater and, as a consequence, accumulation in food, the prevalence of nitrate poisoning has become threatening. The toxicity of nitrites is that they block hemin iron-containing respiratory enzymes. As a result, the oxygen capacity of the blood decreases and tissue hypoxia develops.

Today, more and more attention of researchers paid to the combined pathologies that may be due to the action of several toxic factors on the body.

Given the prevalence of smoking, as well as the active use of nitrites in industry (as food additives, nitrate and nitrite fertilizers), it is advisable to study the mechanisms of simultaneous effects on the body of these toxicants, taking into account the age aspect

The aim of this study was to investigate the content of various forms of hemoglobin and the development of nitrooxidative stress in rats under conditions of nitrite-tobacco toxicosis after the use of the antihypoxant mildronate (meldonium).

In an experiment on rats of different ages (immature, mature and senile) after poisoning for 24 hours and 72 hours sodium nitrite on the background of 45 days of tobacco smoke intoxication found a progressive increase in blood met- and carboxyhemoglobin, which is most pronounced in animals.

An increase in the content of nitrite ion and the activity of inducible NO-synthase against the background of a decrease in its endothelial isoform detected in the blood serum and liver of animals of all ages, which indicates the development of nitrooxidative stress in the body. Immature rats were the most sensitive to nitrite and tobacco poisoning. The antihypoxant mildronate (meldonium) used to reduce the content of both forms of hemoglobin in the blood of rats of different ages, as well as to restore the functioning of the NO system, which allows its further research to include in treatment regimens of pathological conditions accompanied by activation of free radicals and hypoxia.

Key words: nitrite-tobacco intoxication, rats, nitrooxidative stress, methemoglobin, carboxyhemoglobin, hypoxia antihypoxant mildronate (meldonium)

Introduction

The problem of the impact of chemical pollution on the human body is one of the priority and not fully studied [1,2]. Among the pollutants a significant threat is heavy metals, nitrate fertilizers, industrial waste. Oxygen-containing compounds of nitrogen (NO_3), nitrites (NO_2), nitrogen oxide (NO) and its various forms (N_2O, N_2O_3), nitrogen anhydride (N_2O_5) are currently a significant danger to humans among chemical pollutants. The intake of nitrates and nitrites causes the formation of excessive amounts of nitrogen oxide, which is able to initiate free radical chain reactions. This creates the preconditions for the formation of other active forms of nitrogen (peroxynitrite, NO_2, N_2O_3, etc.), which can cause hypoxic and free radical necrobiosis [3,4]. Under the influence of sodium nitrite, the first link in the pathogenesis is hemoglobin. The action of sodium nitrite manifested primarily in the oxidation of oxyhemoglobin to methemoglobin (MetHb) and a sharp increase in the intensity of free radical reactions.

One of the sources of nitrates in the body is smoking [5]. Tobacco smoke is currently one of the most common ecoanthropogenic agents, which has a wide range of effects on the morphofunctional state of various body systems [6,7]. Cigarette smoke contains a large number of oxidants, including hydroxyl radical, NO and hydrogen peroxide, peroxynitrite, which can enter the bloodstream and cause an increase in the formation of $ONOO^-$ in cells. The development of most pathological conditions occurs by a free radical mechanism, which at the cellular level is characterized by increased production of free radicals, among which a special place belongs to the active forms of oxygen and nitrogen [8,9]. Activation of free radical oxidation processes and the development of hypoxia is one of the most important causes of metabolic disorders under conditions of poisoning by toxicants of various origins. Cigarette smoke can block the formation of endogenous NO by reducing the expression of eNOS [10]. Under the conditions of passive smoking, the formation of endogenous NO blocked, as the gas phase of tobacco smoke contains nitrogen oxides and other nitrogen-containing compounds that act on the principle of feedback.

The combination of this, effects of nitrites on the human body, and smoking give rise to the formation of combined pathological conditions and the emergence of multiorgan pathology.

Recently, for the correction of metabolic disorders in hypoxic conditions have been used drugs, which include mildronate, which is an antihypoxant and a drug of metabolic action [11,12]. Mildronate is considered a second-generation cytoprotector, the protection mechanism of which is based on the optimization of O_2 utilization, which determines the survival of cells under conditions of O_2 deficiency, regardless of the reasons for its occurrence.

Thus, given the prevalence of smoking, as well as the active use of nitrites in the industry (as food additives, nitrate, and nitrite fertilizers), it is advisable to study the mechanisms of simultaneous effects on the body of these toxicants, taking into account age, and search for new antihypoxants eliminate the identified violations.

The aim of this experimental work was to study the content of various forms of hemoglobin and the development of nitrooxidative stress in rats under conditions of nitrite-tobacco toxicosis after the use of the antihypoxant mildronate.

Methods

The experiments performed on white male rats kept on the standard diet of the TNMU vivarium. Rats divided into three age groups: immature with a body weight of 60-80 g (3 months of age), mature with a body weight of 180-200 g (12 months of age), and senile - with a body weight of 300-350 g (18 months of age).

The model of chronic exposure to tobacco smoke created using a sealed chamber with a volume of 30 liters, which allowed the animals exposed to the toxicant on a daily basis. Tobacco smoke formed from the burning of 6 Prima silver (blue) cigarettes containing 0.6 mg of nicotine and 8 mg of tar; manufacturer JSC "Imperial Tobacco Production Ukraine", through the holes in the chamber was fed inside it. There were 6 animals in the cell at the same time for 6 minutes. Animals in the control group also

kept in an airtight chamber for 6 minutes but not exposed to tobacco smoke [13].

Sodium nitrite of the animal obtained once intragastrically using a probe in the form of an aqueous solution at a dose of 45 mg/kg body weight, which is 1/4 of the LD50 [14]. The studies were performed 24 and 72 hours after ingestion of this toxicant.

Immature, mature, and senile rats after the defeat of both toxicants administered intragastrically after the defeat of the metabolic drug mildronate (Meldonium), manufacturer "Grindex" Latvia, at a dose of 120 mg/kg body weight, starting from the 15th day of tobacco intoxication and daily experiment [11].

The animals removed from the experiment on the 45th day of tobacco intoxication (and after 24 and 72 hours of sodium nitrite poisoning) by euthanasia under thiopental anesthesia.

The study material was liver homogenate, blood, and serum. Blood taken from the heart of the animals, which s centrifuged at a speed of 1100 g for 30 minutes. Selected organs (250 mg) used to obtain a homogenate using a magnetic homogenizer Silent Crusher S after pre-perfusion with 2.5 ml of saline. The content of carboxy- [15] and methemoglobin was determined in blood [16], the content of nitrite ion in blood serum and liver homogenate [17], and the activity of inducible [18] and endothelial NO synthase [19].

Animal keeping and experiments carried out in accordance with the provisions of the "European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes" [20].

Results

It is known that the intake of sodium nitrite was accompanied by the development of hemic hypoxia, as indicated by an increase in methemoglobin in the blood of rats after the lesion. The process of formation of methemoglobin in the body is free radical.

A study of the content of MetHb in the blood of rats affected by sodium nitrite on the background of tobacco intoxication showed its increase in all age groups during the experiment. After the defeat of both toxicants, the most sensitive were immature rats, in which this indicator increased sharply and by the end of the experiment was the highest - 3.3 times compared with control animals (Fig. 1). During this period of the study, the content of MetHb in the blood of mature rats increased 2.7 times, in senile - 2.6 times relative to the level of control animals. Mildronate used by us for correction led to a decrease in this indicator in the blood of immature rats by 53%. Mildronate had a similar effect on methemoglobin formation in mature and senile rats.

Another indicator that characterizes pathological changes in the body of animals after exposure to tobacco smoke and contributes to the deepening of hypoxia is carboxyhemoglobin. Carbon monoxide, or carbon monoxide contained in tobacco smoke, has the ability to bind the respiratory pigment of blood - hemoglobin. The carboxyhemoglobin formed at the same time is not capable to transfer oxygen; as a result, the processes of tissue respiration disrupted.

By the end of the experiment in the blood of immature rats (under the action of both toxicants) HbCO content increased the most - 3 times higher than normal, in mature - 1.5 times and in the senile - 2.3 times higher than the level of animals in the control group (table 1).

At all times of the study, mildronate had a positive effect on this indicator in the blood of rats of all experimental groups. The content of carboxyhemoglobin in immature rats decreased 1.7 times after 45 days of TD poisoning and 72 hours after ingestion of sodium nitrite. At the same time, this figure in the blood of mature rats decreased 1.4 times, in the blood of senile rats - 1.6 times. The effectiveness of mildronate was observed throughout the experiment and was manifested by a probable ($p < 0.05$) decrease in the content of carboxyhemoglobin in the blood of animals of all experimental groups.

The obtained results allow to note that the most sensitive to the simultaneous defeat of sodium nitrite and tobacco smoke are immature rats, in which the content of carboxyhemoglobin after the defeat is the highest.

We have previously shown that sodium nitrite poisoning leads to nitric oxidative stress, which caused by the formation of a significant amount of nitrogen oxide. In turn, tobacco smoke contains nitrogen dioxide and nitrogen oxide, which when toxic enter the body due to the formation of peroxynitrite, nitrite, and nitration, which initiate the LPS reaction. Thus, in the body, along with oxidative disorders, nitrooxidative stress develops.

NO-synthase activity, as well as enzymatic and non-enzymatic reactions of reduction of nitrate and nitrite ions, considered the main ways of nitrogen oxide formation. The presence of NO-synthase mechanism provides endogenous NO synthesis, which is ultimately oxidized to nitrite and nitrate ions [22,23].

As shown by the results of our studies, the simultaneous defeat of sodium by nitrite and tobacco smoke, led to a significant activation of the formation of nitrite ion in the body of rats of different ages. In the serum of immature rats after the defeat of both toxicants in the last period of the study, the content of nitrite ion increased 2.3 times (Fig. 2), which can lead to the significant formation of endogenous nitrogen oxide and the development of nitrooxidative stress. At the same time, this figure exceeded the norm by 2 times in the serum of mature and senile animals. Mildronate used by us proved to be effective, probably reducing this figure during the experiment.

After exposure to toxicants, an increase in the content of nitrite ion in the liver of rats of all ages observed. Probable changes ($p < 0,05$) noted in all terms of research. This figure markedly reduced under the influence of mildronate.

Excessive accumulation of nitrite ion in the organs of rats after the lesion may lead to increased formation of nitrogen oxide. It previously believed that NOS-dependent synthesis of physiologically necessary NO ("basal NO") carried out with the participation of eNOS and nNOS, and NOS-dependent synthesis of additional NO in the cell with the development of various pathological conditions realized with the participation of iNOS. This leads to its excessive activation and dysfunction of the arginase metabolic pathway, which competes with NO-synthase for the substrate - L-arginine [24].

In our experiments, it noted that after the defeat of sodium rats with nitrite on the background of tobacco intoxication in the serum progressively increased activity of iNOS in all age groups (Table 2).

The most active was iNOS in the serum of immature rats, which by the end of the experiment increased 3.3 times after exposure to toxicants. Mature rats were the most stable - the activity of the enzyme in them increased 1.7 times relative to the group of intact control at the end of the study, in the elderly, this indicator in the serum increased 2.7 times. The use of mildronate resulted in a probable decrease in iNOS activity during the experiment in the serum of rats of all ages. Thus, at the end of the experiment in immature rats, this figure decreased 1.8 times relative to the group of affected animals, in mature and senile - 1.5 times ($p <0.05$).

The activity of iNOS in the liver of rats of different ages after exposure to toxicants studied, and it noted that it shows the greatest activity in immature animals. By the end of the experiment, the activity of the enzyme was 4.3 times higher than normal. In mature animals, this figure increased 1.8 times by the end of the experiment. Increased iNOS activity observed in the liver of senile rats - 3.5 times by the end of the study (Fig. 3). Mildronate was effective, after the introduction of which into the body of rats, this figure probably decreased at all times of the study. In immature rats at the end of the study, it was 2.2 times lower than the level of affected animals. A similar decrease in iNOS activity under the influence of mildronate was in the liver of mature and senile rats.

Obviously, the activation of iNO-synthase that can explain the results obtained by us, which indicate a significant increase in the level of nitrogen oxide metabolites, in particular nitrite ion, in the serum and organs of rats after infection. It is known that iNOS synthesizes high concentrations of NO (> 300 nm) [25, 26], while eNOS produces low concentrations of NO. iNOS is a calcium-independent isoform of NOS and, unlike eNOS, is not constantly (constitutively) expressed.

Based on this, we investigated the activity of eNOS in the serum and liver of rats of different ages after exposure to experimental toxicants. The activity of the enzyme in the serum of immature rats at all times of the study was almost the same decrease and at the end of the experiment was below control by 56%. At the same time, in elderly rats, this figure also decreased by 56%, in adults - by 50% (Fig. 4). Mildronate effectively increased the serum activity of eNOS in rats of different ages throughout the experiment.

We studied the activity of eNOS in the liver of affected rats and the influence of corrective factors on it. The defeat of rats by both toxicants simultaneously led to a decrease in the activity of eNOS in the liver of animals of all ages (Table 3).

The lowest eNOS activity was recorded in the liver of immature rats at the end of the experiment, it decreased after injury by 5.1 times relative to the intact control group of the same age. In adult rats, this figure decreased 3 times, in elderly rats 3.8 times relative to the control in the last period of the study. The antihypoxant used by us significantly increased ($p < 0.05$) the activity of the enzyme in all terms of the study. The closest to the control were the indicators in the liver of adult animals with the use of mildronate.

Conclusions

The defeat of rats with sodium nitrite on the background of tobacco intoxication leads to the development of hemic and circulatory hypoxia, as indicated by elevated blood levels of meth and carboxyhemoglobin, the most pronounced changes of which were in immature animals.

When sodium nitrite poisoning on the background of tobacco intoxication in the body of animals develops nitrooxidative stress, which, along with oxidative stress, causes the severity of the pathological process. The most pronounced changes in the functioning of the NO system observed in the body of immature rats after simultaneous exposure to sodium nitrite and tobacco smoke.

The antihypoxant mildronate used reduced the content of meth and carboxyhemoglobin in the blood of rats of all ages, as well as inhibited the activity of inducible and restored the activity of endothelial NO synthase in the serum and liver of animals of different ages. This makes it appropriate to further study this tool in order to apply it in various pathological conditions, accompanied by increased oxidative processes and hypoxia.

References

1. Gunchak V.M. (2013). To the toxicology of nitrates and nitrites in animals. Scientific Bulletin of LNUVMBT named after SZ Gzycki, 57 (3), 62-70.
2. Krasovsky K.S. (2009). Consequences of the global tobacco epidemic. Preventive medicine, 4, 72-74.
3. Helmke S.M., Duncan M.W. (2007).Measurement of the NO metabolites, nitrite and nitrate, in human biological fluids by GC-MS. J Chromatography, 851(1-2), 83-92.
4. Lundberg J.O., Weitzberg E. (2009). NO generation from inorganic nitrate and nitrite: Role in physiology, nutrition and therapeutics. Arch Pharm Res., 32(8), 1119-26.
5. Cigremis Y., Turkoz Y., Tuzcu M., Ozen H., Kart A. & Gaffaroglu M. (2006). Effects of chronic exposure to ethanol and cigarette smoke on the formation of peroxynitrite, level of nitric oxide, xanthine oxidase and myeloperoxidase activities in rat kidney. Mol Cell Biochem., 291(1-2), 127-38.
6. Ilkovich M.M., Kuzubova N.A. & Kiseleva E.A. (2010). Tobacco control as the basis for the prevention of chronic obstructive pulmonary disease. Pulmonology, 2, 37-39.
7. Kvasha E.A. (2010). Medical aspects of smoking. Health of Ukraine, 20 (249), 40-41.
8. Powers S.K., Talbert E.E. & Adhihetty P.J. (2011). Reactive oxygen and nitrogen species as intracellular signals in skeletal muscle. J. Physiol., 589(9), 2129–2138.
9. Weidinger A., Kozlov A.V. (2015). Biological Activities of Reactive Oxygen and Nitrogen Species: Oxidative Stress versus Signal Transduction. Biomolecules, 5, 472–484.
10. Lykhatskyi P.H., Fira L.S. (2017). Use of mildronate under conditions of oxidative stress in

rats affected by sodium nitrite, against the background of intoxication with tobacco smoke. The world of medicine & biology, 3 (61), 128-134.

11. Jargin S.V. (2016). Meldonium (Mildronate): Primum non nocere. Pharmacological Research, 12, 114-294.

12. Churg, A., Wright, J. (2010). Animal models of cigarette smoke-induced chronic obstructive pulmonary disease. Expert Rev Respir Med., 4(6), 723-34.

13. Smolyar V.I., Tsyhanenko O.I. & Petrashenko H.I. (2007). Nitrates, nitrites and nitrosamines in food and diet. Problems of nutrition, 3, 7-8.

14. Lykhatsky P.G., Fira L.S., Fira D.B. & Kuzmak I.P. (2017). Molecular mechanisms of metabolic disorders in the organs of rats of different ages affected by sodium nitrite on the background of tobacco intoxication. Bulletin of Dnipropetrovsk University. Biology, medicine, 2 (8), 259-264.

15. Scherer G. (2006). Carboxyhemoglobin and thiocyanate as biomarkers of exposure to carbon monoxide and hydrogen cyanide in tobacco smoke. Exp Toxicol Pathol., 58(2-3), 101-24.

16. Ansari F.A., Mahmood R. (2016). Sodium nitrite enhances generation of reactive oxygen species that decrease antioxidant power and inhibit plasma membrane redox system of human erythrocytes. Cell Biol Int., 40(8), 887-94.

17. Bryan N.S., Grisham M.B. (2007). Nitric Oxide and its Metabolites in Biological Samples. Free Radic Biol Med., 43(5), 645–657.

18. Wu S.J., Ng L.T. (2007). Tetrandrine inhibits proinflammatory cytokines, iNOS and COX-2 expression in human monocytic cells. Biol Pharm Bull., 30(1), 59-62.

19. Webb A.J. (2008). Mechanisms underlying erythrocyte and endothelial nitrite reduction to nitric oxide in hypoxia. Role for xanthine oxidoreductase and endothelial nitric oxide synthase. Circ. Res., 108, 175-810.

20. Gross D., Tolba R.H. (2015). Ethics in Animal-Based Research. Eur. Surg. Res., 55(1-2), 43-57.

21. Jannot A.S., Agoritsas T. & Gayet-Ageron A. (2013). Citation bias favoring statistically significant studies was present in medical research. J. Clin. Epidemiol., 66(3), 296-301.

22. Mudra A.E. (2014). NO-synthase and pro-inflammatory cytokines in acute toxic hepatitis and under the influence of modulators of nitric oxide synthesis. Science. Not. Ternopil. ped. un-tu. Ser. Biol., 4 (61), 141-147.

23. Treumova S.I. (2013). The role of nitric oxide, prostacyclin in the pathogenesis of bronchopulmonary and cardiovascular pathology. Visn.probl.biol. and honey, 103 (2), 47-50.

24. Khmil K., Mishchenko A.V. & Kostenko V.O. (2017). The role of NO-synthase and arginase in the mechanisms of oxidative-nitrate stress in the skin of rats under conditions of excessive intake of sodium nitrate. Ukrainian Journal of Medicine, Biology & Sports, 2 (11), 54-59.

25. Powers S.K., Talbert E.E. & Adhihetty P.J. (2011). Reactive oxygen and nitrogen species as intracellular signals in skeletal muscle. J. Physiol., 589(9), 2129–2138.

26. Shiva S. (2013). Nitrite: A physiological store of nitric oxide and modulator of mitochondrial function. Redox Biology, 1(1), 40-44.

Table 1. The content of carboxyhemoglobin (g/l) in rats of different ages affected by sodium nitrite on the background of tobacco intoxication, and after the use of mildronate (M±m; n=6)

Study period, day/hous	Groups of experimental animals		
	immature rats	mature rats	senile rats
Control rats	0,0148±0,0012	0,0207±0,0016	0,0188±0,0017
45 day TS+24 h SN	0,0428±0,0040*	0,0328±0,0028*	0,0408±0,0026*
45 day TS+24 h SN +mildronate	0,0262±0,0020**	0,0211±0,0020**	0,0283±0,0025**
45 day TS+72 h SN	0,0448±0,0038*	0,0311±0,0022*	0,0437±0,0035*
45 day TS+72 h SN + mildronate	0,0257±0,0018**	0,0222±0,0021**	0,0273±0,0019**

Note: here and in the next tables * - probable changes between the rates of intact rats and rats with nitrite tobacco toxicosis; ** - probable changes between rats with nitrite-tobacco toxicosis and affected toxicants and treated with mildronate

Table 2. Activity iNOS (ng/ml) in the serum of rats of different ages affected by sodium nitrite on the background of tobacco intoxication, and after the use of mildronate (M±m; n=6)

Study period, day/hous	Groups of experimental animals		
	immature rats	mature rats	senile rats
Control rats	13,16±0,95	18,84±1,93	14,86±1,27
45 day TS+24 h SN	40,13±3,15*	29,10±2,54*	37,73±2,49*
45 day TS+24 h SN +mildronate	20,03±1,73**	21,10±1,67**	25,73±2,53**
45 day TS+72 h SN	42,91±4,24*	31,36±2,86*	40,31±2,76*
45 day TS+72 h SN + mildronate	23,74±1,90**	20,75±1,88**	27,12±2,25**

Table 3. Activity eNOS (ng/ml; 1 ml - 10^6 liver cells) in the liver of rats of different ages affected by sodium nitrite on the background of tobacco intoxication, and after the use of mildronate (M±m; n=6)

Study period, day/hous	Groups of experimental animals		
	immature rats	mature rats	senile rats
Control rats	3,26±0,26	4,11±0,19	3,42±0,23
45 day TS+24 h SN	0,73±0,06*	1,45±0,12*	1,02±0,08*
45 day TS+24 h SN +mildronate	2,02±0,15**	3,88±0,32**	3,07±0,17**
45 day TS+72 h SN	0,64±0,05*	1,39±0,10*	0,91±0,09*
45 day TS+72 h SN + mildronate	2,11±0,17**	3,98±0,30**	3,11±0,19**

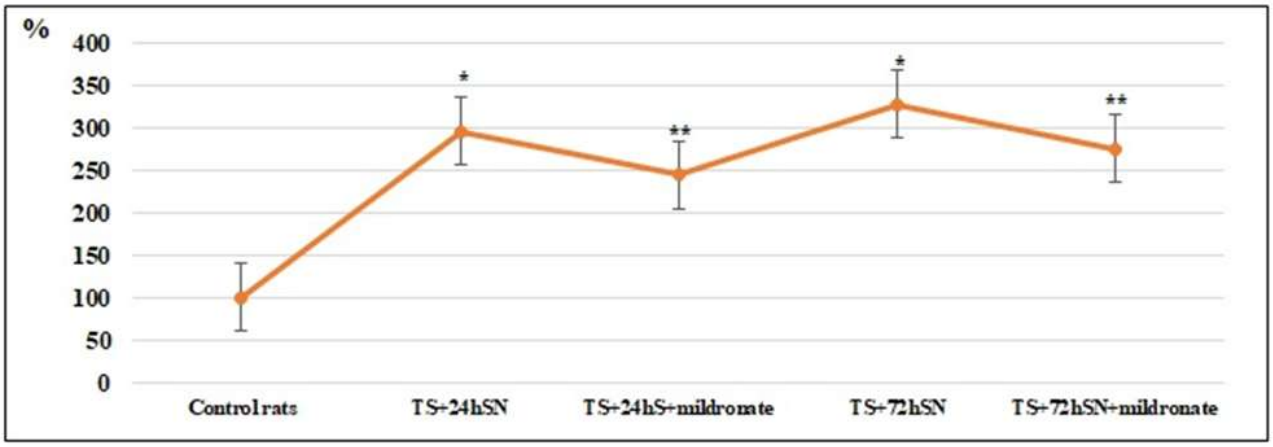

Figure 1. The content of MetHb in the blood of immature rats poisoned with sodium nitrite on the background of tobacco smoke and after the use of mildronate, %

Note: here and in the next figures * - probable changes between indicators of intact rats and rats with nitrite-tobacco toxicosis; ** - probable changes between rats with nitrite-tobacco toxicosis and affected toxicants and treated with mildronate

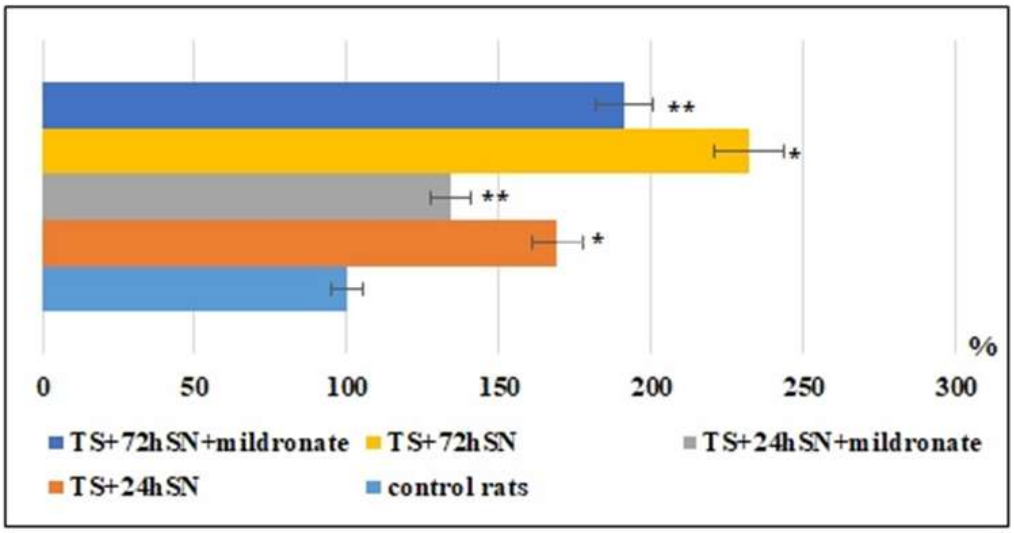

Figure 2. The content of nitrite ion in the serum of immature rats poisoned with sodium nitrite on the background of tobacco smoke and after the use of mildronate, %

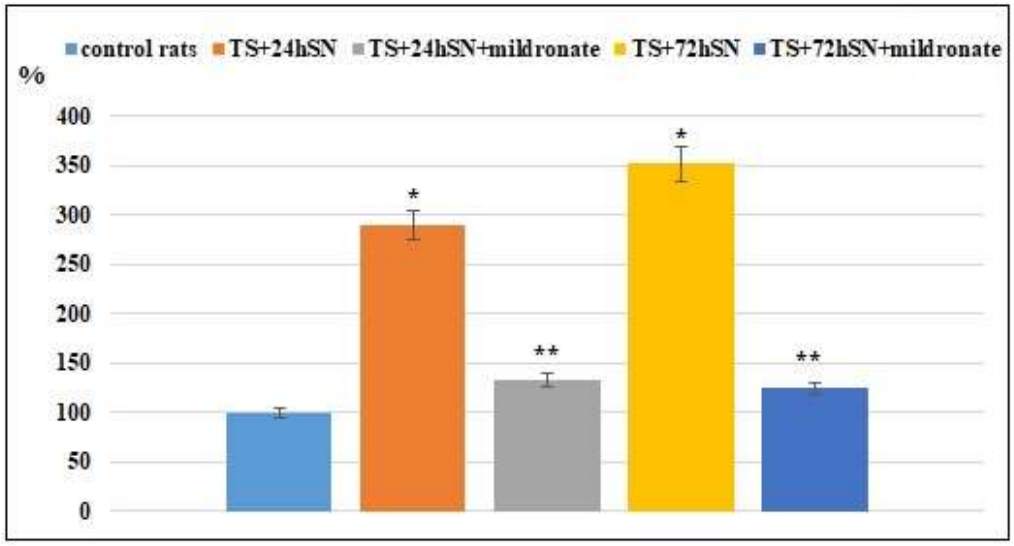

Figure 3. Activity of iNOS in the serum of senile rats affected by sodium nitrite on the background of tobacco intoxication, and after the use of mildronate, %

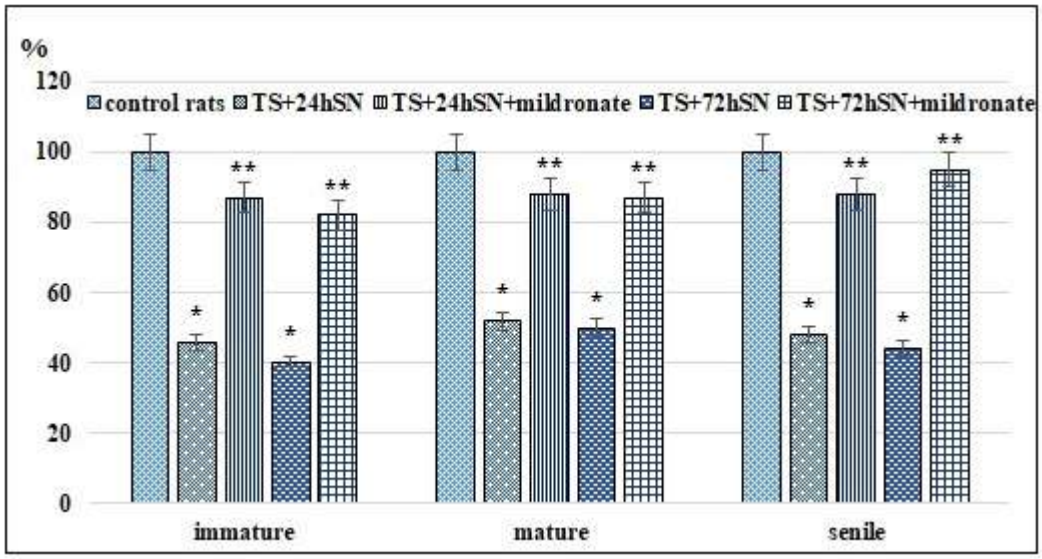

Figure 4. The activity of eNOS in the serum of rats of different ages affected by sodium nitrite on the background of tobacco intoxication, and after the use of mildronate, %