



N-acetylcysteine relieves neurologic signs of acute ethanol hangover in rats

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Abstract

Introduction: Alcohol abuse is one of the grave social and medical problems in many countries, including Russia. Alcohol not only negatively affects health, social and family relationships, but also a person's performance. Hangover, which is a one of the negative consequences of alcohol intake, is a complex of neurological and somatic symptoms that occur when ethanol is almost completely metabolized to acetaldehyde. This condition, despite the severity and potential economic damage, remains poorly understood, and there are no effective medicines to treat it.

Aim: to provide an experimental basis for the possibility of using N-acetylcysteine (NAC), a precursor of glutathione, as a medicine for prevention of the neurological and cognitive impairments due to alcohol intoxication.

Materials and Methods: The study used male Wistar rats, which were intraperitoneally injected with ethanol at a dose of 3 g/kg to simulate acute ethanol intoxication. Sixty minutes before the injection, the animals from the experimental groups were gavaged with NAC (1 g/kg) or with an equivalent volume of saline. Immediately after awakening and 3 h after it, the animals were assessed for neurological deficits, motor skills, spontaneous motor activity, and cognitive functions. After the completion of the behavioral tests, the animals were euthanized to assess the level of glutathione, triglycerides (TGs), and malonic dialdehyde (MDA) in liver homogenates, and to determine the activity of enzymatic antioxidant systems and serum aminotransferases.

Results and Discussion: The ethanol intoxication in the animals from the control group was associated with pronounced signs of neurological and cognitive impairments, including low spontaneous motor and exploratory activity, impaired fine motor skills in the adhesive test, and cognitive function decline in the Morris water maze test. The rats which had received NAC before ethanol injection demonstrated better fine motor skills in the adhesive test, a higher level of spontaneous motor activity and better performance in the Morris water maze test (in comparison to the animals treated with saline before alcohol intoxication). In the animals which had received NAC, the levels of glutathione, MDA, and TGs, as well as the activity of liver antioxidant enzymes, were closer to the values of the intact rats to a greater extent than in the animals that had been injected with ethanol and received saline.

Conclusion: Orally administered NAC before acute ethanol intoxication led to a decrease in the severity of neurological deficiency in rats and reduced the amnesic effect of ethanol. This could be due to an improvement of ethanol metabolism and a decrease in the severity of disorders associated with oxidative stress and liver dysfunction.

Keywords

acetylcysteine, ethanol, hangover, pre-clinical study, post-intoxication, rat.

Introduction

The abuse of alcoholic beverages is a grave medical and social problem. Alcohol affects almost all systems and organs, increases the risk of liver cirrhosis, gastrointestinal bleeding, pancreatitis, cardiomyopathy, trauma, mental disorders (anxiety, depression, loss of consciousness, dementia), various cancers, and shortens life expectancy. Severe psycho-emotional and physical disturbances often occur after taking **ethanol** and can provoke its repeated use, leading to binge drinking. Mental and physical addictions to alcohol are formed over a certain period, the duration of which depends on many factors, but the patient almost always denies the addiction. Reducing the total amount of alcohol consumed or facilitating its metabolism reduces the negative impact on the body, improving physical condition and improving the quality of human life (Verster and Penning 2010, Witkiewitz et al. 2018). Alcohol abuse is the second leading cause of cirrhosis in the USA after hepatitis C (Setiawan et al. 2016). As a result of a long (over several years) period of alcohol consumption, scar tissue is formed, leading to impaired blood circulation. First, the liver parenchyma is damaged, further leading to nodal fibrosis. Further, resistance to portal blood flow increases, which can lead to hypertension, splenomegaly, transudative ascites, or gastrointestinal varicose veins (Boll and Merkle 2009). Daily intake of more than 60 g of alcohol causes morphological changes in the liver. An increase in the content of glycerol-3-phosphate increases the content of fatty acids and provokes the development of steatosis (Eaton et al. 1997). Liver steatosis is reversible upon cessation of alcohol consumption. Otherwise, the likelihood of developing alcoholic hepatitis with subsequent cirrhosis is high (Eaton et al. 1997, Heuman et al. 2019).

After 10 years of chronic alcohol use, the risk of hepatocellular carcinoma increases fivefold, with life expectancy after diagnosis usually in the range of 6–20 months. In about 30% of cases, this disease occurs due to excessive alcohol consumption. The metabolism of **ethanol** and its products occurs in hepatocytes and is accompanied by a gradual depletion of glutathione, the main antioxidant agent in mitochondria, which leads to an increase in the concentration of reactive oxygen species damaging nucleic acids (Mansoori and Jain 2015). The onset of oncological diseases in chronic alcoholism may be a consequence of the toxic effect of the products of its metabolism, mainly **acetaldehyde**. Some variations of the gene for alcohol dehydrogenase (an enzyme that promotes the metabolism of alcohol) have a protective effect, which is obviously associated with the accelerated metabolism of not only **ethanol**, but also its products (Hashibe et al. 2008). Alcohol use is one of the leading causes of esophageal cancer in the USA (the overall 5-year survival rate for all stages of esophageal cancer is approximately 20%, while survival for late stages is approximately 5%) (Prabhu et al. 2014). The risk ratio for squamous cell carcinoma among patients consuming more than 30 g of **ethanol** per day is 4.61. Pancreatic cancer often develops asymptotically for a long time and is

detected at advanced stages. Chronic inflammation associated with the toxic effects of alcohol and its metabolites is one of the main causes of this disease (Barone et al. 2016), which accounts for 3.2% of all cancers registered in the USA and 7.5% of all cancer-related deaths. With surgery (the highest chance of cure), relapses are common.

According to the Centers for Disease Control and Prevention stroke is the fifth leading cause of death in the USA, affecting up to 800,000 people annually and the main source of disability among adults. Intake of alcohol in small to moderate amounts is accompanied by anticoagulant and secondary protective effects on the cardiovascular system. The risk of ischemic and hemorrhagic strokes increases when alcohol is consumed in large doses, regardless of the regularity (Rehm and Roerecke 2017, Tadi and Lui 2020), which is obviously associated with its negative effect on hemodynamics. Hypertension, ischemic heart disease, stroke, angio- and cardiomyopathies often occur on the background of prolonged use of large doses of alcohol. The toxic effect of alcohol and its metabolites directly weakens the myocardium, increases oxidative stress, and increases the risk of thrombus formation, negatively affects the cardiac conduction system, causing arrhythmias. The classic consequence of long-term alcohol consumption is the development of dilated cardiomyopathy, an irreversible process in which the ventricular chambers enlarge, whereas the muscle layer does not change, which is accompanied by systolic dysfunction, arrhythmias, risk of thromboembolism, holiday heart syndrome, Brugada syndrome (Achaiah and Andrews 2016), and sudden death (Carey et al. 2014). Chronic consumption of high doses of alcohol causes a decrease in immunity (Ratna and Mandrekar 2017). Brain damage during chronic alcohol use is associated with the direct neurotoxic effect of **ethanol** and its metabolites, as well as with the resulting thiamine deficiency, impaired cerebral hemodynamics, the development of hepatic encephalopathy, and head injuries.

Thus, the direct toxic effect of ethyl alcohol and/or its metabolites causes severe diseases, which are linked pathogenically with the depletion of intracellular antioxidant enzymes, an increase of reactive oxygen species, nucleic acid damage, and other signs of oxidative stress. Hangover is the most obvious consequence of the dysfunction of the **ethanol** metabolic pathway resulting from the use of high doses of **ethanol**. **Acetaldehyde** is a product of alcohol metabolism and causes most of the symptoms of hangover. An increase in the concentration of **acetaldehyde** is observed as a result of a slowdown in its biodegradation under the action of **acetaldehyde** dehydrogenase, which is largely associated with the depletion of reduced **glutathione** in hepatocytes (Mackus et al. 2020). **Glutathione** is the most important regulator of redox reactions in cells; the depletion of its reserves causes a slowdown in metabolism, including the processes associated with the conversion of **acetaldehyde** into less toxic acetic acid. Restoring **glutathione** content or preventing a significant decrease in its level can become a promising approach to facilitate

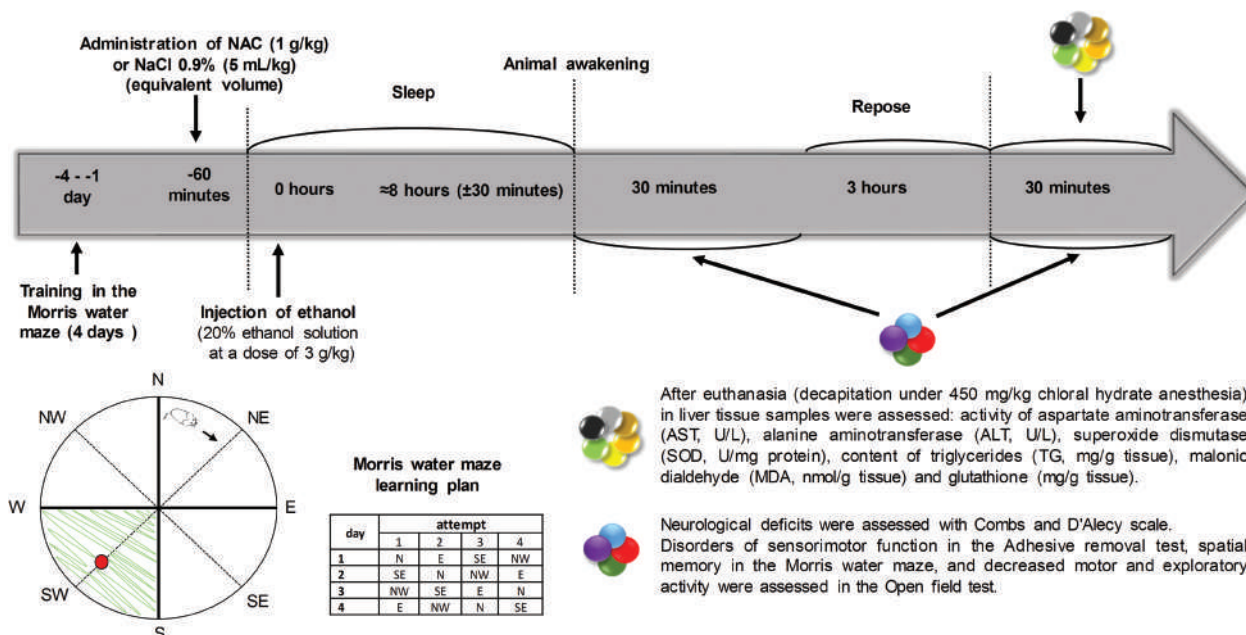


Figure 1. Study design.

the metabolism of ethyl alcohol and reduce the toxic effect of its metabolic products, which will not only prevent hangover, but also possibly prevent the development of the above diseases. The use of **N-acetylcysteine (NAC)**, which increases the content of reduced glutathione, may be promising in this regard (Green et al. 2013). The aim of this study was to provide an experimental basis for the possibility of use of **NAC**, a precursor of **glutathione**, as a medicine for prevention of the neurological and cognitive impairments, occurring after alcohol intoxication.

Materials and Methods

Animals

The study used male Wistar rats (body weight 300–350 g), obtained from Rappolovo Breeding Nursery. The animals were acclimated at 20 ± 2 °C and 40–60% humidity in a standard 12/12-h light-dark cycle with food and tap water ad libitum for 14 days before the experiments. All manipulations were carried out in compliance with the Russian laws and the EAEU technical standards for Good laboratory practice (GOST R 53434-2009 and GOST R 51000.4-2011). The study design and the protocol were reviewed and approved by the Department of the Ethical, Legal, and Sociological Expertise in Medicine of Volgograd Medical Research Center [registration number: IRB 00005839 IORG 0004900 (OHRP)] on May 20, 2019 (protocol number 132).

Study design

A scheme of the study design is presented on Figure 1. Before alcohol intoxication was simulated, all the rats were trained to find a platform under the water in the

Morris water maze for 4 days. The rats were randomly assigned to one of 3 study groups ($n = 10$ for each group):

1. Rats from the intact group (negative control) were gavaged with **saline** (5 mL/kg, Gematec, Russian Federation), and, 60 min after, were injected with **saline** intraperitoneally (15 mL/kg);
2. Rats from the positive control group were gavaged with **saline** (5 mL/kg), and, 60 min after, were injected with **ethanol** (3 g/kg, as 20% solution);
3. Rats from the **NAC** group were gavaged with **NAC** (1 g/kg, Sandoz, Russian Federation), and, 60 min after, were injected with **ethanol** (3 g/kg).
4. After the injection of ethanol, the latent time of losing the righting reflex (the ability to return to an upright position after staying on its back) (Morkovin et al. 2018), the time of onset and the duration of sleep were recorded in the animals.

Thirty minutes and 3 h after the animals woke up, the neurological and cognitive deficits were assessed using the Combs and D'Alecy scale (Combs and D'Alecy 1987), and the following tests were performed: adhesive test (a test with adhesive tape applied on the volar surface of the forelimbs) (Bouet et al. 2009), open field test (Prut and Belzung 2003), and Morris water maze test (Vorhees and Williams 2006). The rats that did not wake up 8 h 30 min after the **ethanol** injection were excluded from the experiment.

After euthanasia (decapitation under 450 mg/kg chloral hydrate anesthesia), liver tissue samples were taken from the animals for further analysis. All reactions were carried out in triplicates. The activity of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in blood plasma of the animals was determined using appropriate reagents (manufactured by Diacon-DS, Russian

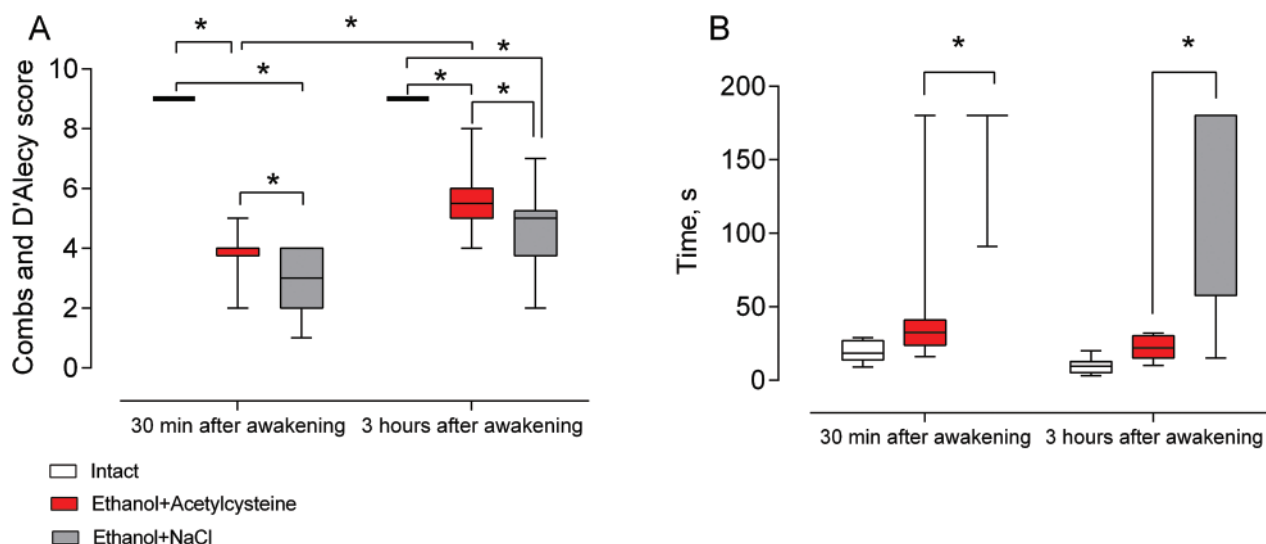


Figure 2. The effect of acetylcysteine on the neurological deficits assessed with *Combs and D'Alecy* scale (A) and on the time needed to remove an adhesive tape from volar surface of forelimbs (B) in rats after acute ethanol intoxication. **Note:** * – $p < 0.05$ (one-way ANOVA, Newman-Keuls *post-hoc*); the compared datasets are connected with horizontal lines; % – share of animals having removed an adhesive tape from any forelimb.

Federation). The content of triglycerides (TGs) in homogenates of liver tissue was determined after extraction with heptane and isopropanol followed by fractionation with sodium alcoholate photometrically (after incubation with 2,4-pentanedione at a wavelength of 410 nm) (Biggs et al. 1975, Lee et al. 2009). The concentration of malonic dialdehyde (MDA) in homogenates was determined using the reaction with thiobarbituric acid (Ohkawa et al. 1979, Shanmugam et al. 2011); the concentration of reduced glutathione was measured in the reaction of reduction of 5,5-dithiobis-(2-nitrobenzoic acid) (Shaik and Mehvar 2006). Superoxide dismutase (SOD) activity was determined by a photometric method based on the assessment of a degree of inhibition of the epinephrine oxidation reaction (Misra and Fridovich 1972, Shanmugam et al. 2011).

Statistical analysis

The results of the study were statistically processed by descriptive and analytical statistics. The distribution of the quantitative indicators was assessed using the Shapiro-Wilk test. The intergroup differences were assessed using one-way analysis of variance (ANOVA) with Newman-Keuls *post-hoc* test, and the numerical values were presented as the arithmetic mean and standard error of the arithmetic mean (SEM). To assess the differences in categorical data, the chi-square test (χ^2) was used.

Results and discussion

After the intraperitoneal injection of ethanol, the rats fell asleep within several minutes, and their sleep lasted on average 8 ± 30 min. All the animals selected for the experiment were comparable in terms of the onset and duration of sleep.

The post-intoxication state of the animals was characterized by depressed behavior: they were lethargic and moved slowly, with evident signs of severe drowsiness (eyes closing, lying down, sluggish reaction to touch). In the open field test, the animals showed low exploratory and motor activities. Whereas the control group retained low indices in the open field test 3 hours after the therapy, the rats which had received NAC demonstrated higher activity (Fig. 3B, C). Thus, NAC provided a rapid recovery of locomotor and exploratory activities after alcohol intoxication.

When assessing neurological deficit according to the Combs and D'Alecy scale, the animals from the positive control group demonstrated symptoms of severe neurological deficits at wake-up, but the severity of the deficits slightly decreased 3 hours later. In the rats which had received NAC, the severity of neurological deficit 30 min after awakening was significantly lower than in the positive control group, and decreased even further 3 hours after awakening (Fig. 2A).

Thirty minutes after awakening, the animals from the control group did not notice the adhesive tape on the volar surface of forelimbs, and 3 hours later, the sensorimotor function of these animals was restored only partially: only 4 out of 10 animals were able to detect and remove an adhesive tape, and the animals spent more time removing the adhesive tape than the rats from the other groups (Fig. 2B). Nine out of 10 rats which had received NAC before the injection of ethanol found and removed an adhesive tape from at least one paw ($p < 0.05$), and upon repeated testing, all the animals of this group did this significantly ($p < 0.05$) faster (Fig. 2B).

All the animals for 4 days before the intraperitoneal ethanol injection were trained to find the hidden platform in the Morris water maze. The rats from the positive control group performed poorly in the test performance,

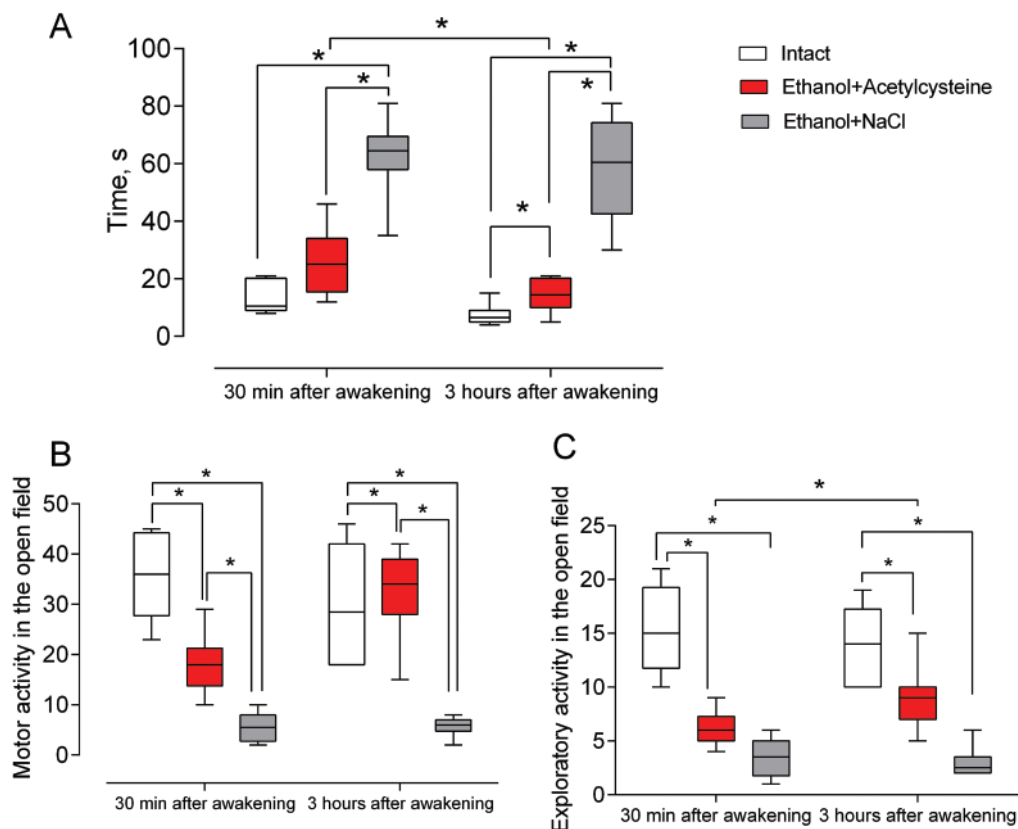


Figure 3. The effect of acetylcysteine on latent time of platform-finding in Morris water maze test (A), on motor activity (B) and exploratory activity (C) in open field test in rats after acute ethanol intoxication. **Note:** * – $p < 0.05$ (one-way ANOVA, Newman-Keuls post-hoc); the compared datasets are connected with horizontal lines; motor activity was counted as the total number of lines crossed; exploratory activity was defined as the total number of verticalizations and nose-pokes.

whereas the rats which had received NAC demonstrated a higher rate of platform-finding with a shorter latent time (Fig. 3A). Thus, the preliminary administration of NAC reduced the severity of amnesia caused by ethanol in the animals.

At the end of the study, liver tissue samples were taken to determine the activities of SOD, TG and MDA content, and blood was sampled to assess the activities of AST and ALT. In the rats from the positive control group, the glutathione content in liver homogenates reached 88.6 ± 5.05 mg/g tissue (versus 116.6 ± 3.13 mg/g tissue in intact animals; $p < 0.05$). In the rats which had received NAC, the glutathione content (110.8 ± 7.34 mg/g tissue) was significantly higher than in the animals from the positive control group ($p < 0.05$; Fig. 4D), but the values were comparable to those observed in the intact animals. Thus, the prophylactic administration of NAC increased the content of reduced glutathione in the liver and, probably, contributed to a decrease in the severity of alcohol intoxication and the recovery of animals, apparently due to the facilitation of alcohol metabolism.

The levels of AST and ALT activities in blood plasma of the rats from the negative control group were 133.4 ± 5.12 and 26.7 ± 2.17 U/L, respectively. In the animals from the positive control group, these indicators statistically significantly ($p < 0.05$) increased, reaching 154.8 ± 9.13 and 41.9 ± 5.17 U/L, respectively, which indi-

cates an impaired metabolic function of hepatocytes as a result of the hepatotoxic effect of high doses of ethanol. In the rats which had received NAC, the levels of AST and ALT activities were not significantly increased (133.2 ± 4.05 and 30.9 ± 1.17 U/L, respectively; $p > 0.05$) when compared with the values recorded in the animals from the negative control group; Fig. 4A, B).

In the rats from the positive control group, the SOD activity in liver homogenates was 39.5 ± 3.14 U/mg protein (versus 54.6 ± 1.52 U/mg protein in intact animals; $p < 0.05$), and the MDA content was 29.8 ± 1.13 nmol/g of tissue (versus 22.7 ± 1.15 nmol/g of tissue in intact animals; $p < 0.05$). In the rats that had been gavaged with NAC the listed parameters did not differ significantly from those recorded in the intact animals ($p > 0.05$ for all variables): SOD activity and MDA content were 40.5 ± 1.18 U/mg protein and 27.8 ± 1.15 nmol/g tissue, respectively (Fig. 4E, F). Thus, a single administration of NAC prevented the development of signs of oxidative stress in rats caused by alcohol intoxication.

An increase in the content of TGs in hepatocytes is a characteristic sign of degenerative changes due to toxic liver damage (Boll and Merkle 2009). In the rats which had received saline and ethanol, the triglyceride content in liver homogenates was statistically significantly higher than in the animals from the negative control group (30.7 ± 0.51 versus 20.5 ± 0.91 mg/g tissue; $p < 0.05$). In the

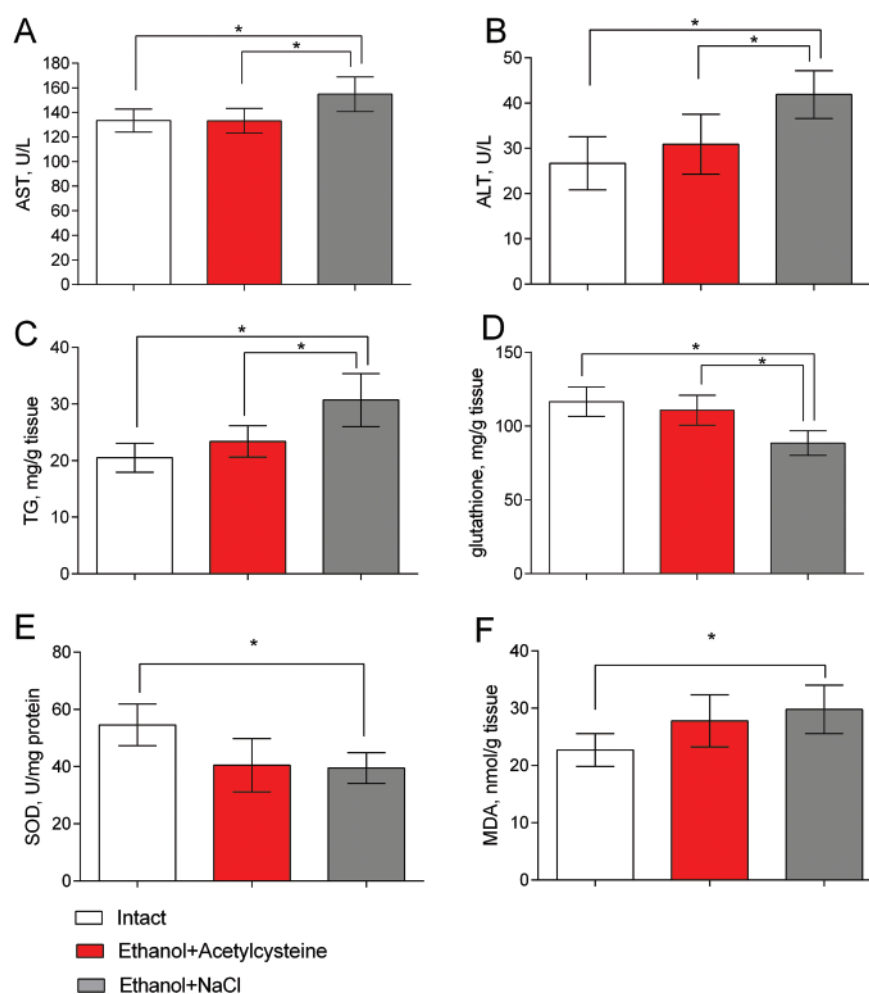


Figure 4. The effect of acetylcysteine on the liver [glutathione](#) levels in rats after acute [ethanol](#) intoxication. **Note:** **A.** AST – aspartate aminotransferase (U/L); **B.** ALT – alanine aminotransferase (U/L); **C.** TG – [triglycerides](#) (mg/g tissue); **D.** [glutathione](#) (mg/g tissue); **E.** SOD – superoxide dismutase (U/mg protein); **F.** MDA – [malonic dialdehyde](#) (nmol/g tissue); data shown as the mean \pm SEM (n = 8–10 as a grand mean of triplicates); * – $p < 0.05$ (one-way ANOVA, Newman-Keuls *post-hoc*); compared datasets are connected with horizontal lines.

animals that had been gavaged with [NAC](#) before [ethanol](#) injection, the content of TGs in liver homogenates reached 23.4 ± 1.35 mg/g of tissue, which was comparable to that in the intact animals ($p > 0.05$; Fig. 4C). The changes in the activity of transaminases in blood plasma and in liver TG levels make it possible to conclude that [NAC](#) is able to prevent the development of toxic liver damage caused in rats by a single intraperitoneal injection of ethanol.

The prevalence of alcoholism is one of the central public health problems in medical, social and economic terms. According to the latest WHO data, alcoholism is one of the leading causes of health deterioration worldwide, and the European Region has the highest premature mortality attributed to [ethanol](#) consumption (due to an increase in the number of somatic pathologies and death due to alcohol intoxication) (Kupchik et al. 2012, Kurkin et al. 2019, Morkovin et al. 2019a, b).

Alcohol abuse leads to the development of a hangover syndrome, which is determined by pronounced neurological and somatic symptoms, occurring when alcohol is almost completely metabolized to [acetaldehyde](#). In some cases, especially when alcohol is used to relieve hangover,

this condition can be a critical factor in the formation of alcohol addiction. Since the severity of [ethanol](#) hangover can be exacerbated and prolonged by the depletion of liver [glutathione](#) stores; [NAC](#), which increases [glutathione](#) production, may be a promising and affordable treatment for hangover, which is consistent with several previous studies. At the same time, prophylactic administration of this drug may be promising (Green et al. 2013, Mackus et al. 2020).

In the framework of the present work, it was found that in the rats that had received [NAC](#) before acute ethanol intoxication a decrease in [glutathione](#) level in hepatocytes was less pronounced than in the rats that had not received [NAC](#) on the background of [ethanol](#) intoxication. This is consistent with the results of our previous studies (Kupchik et al. 2012, Kurkin et al. 2019, Morkovin et al. 2019a, b), in which the therapeutic effects of [NAC](#) were evaluated (with a single oral administration after alcohol intoxication). In both cases, the animals were injected intraperitoneally with [ethanol](#), which excluded the pharmacokinetic interaction of [NAC](#) with [ethanol](#), which could have contributed to a lower systemic absorption of the

latter. Thus, the observed effects should be considered as the primary pharmacodynamic effects of the study drug.

Oxidation of **ethanol** under the influence of alcohol dehydrogenase leads to the formation of **acetaldehyde**, which disrupts some of the functions of cells and may contribute to their death. This also occurs due to the depletion of the reserves of reduced **glutathione**, which leads to a decrease in the ability to utilize reactive oxygen species, to damage to mitochondria and other cell organelles. Furthermore, **ethanol** itself is also capable of inhibiting **glutathione** synthesis (Cederbaum 2012, Rushworth and Megson 2014). The ratio of reduced and oxidized forms of **glutathione** in the cell is one of the most important parameters that determines the level of oxidative stress, which increases during alcohol consumption and hangover development (Green et al. 2013, Rushworth and Megson 2014).

NAC is a medicine with a well-established medical use, which has been used for a long time in clinical practice. This amino acid can not only act as a precursor in the synthesis of **glutathione**, which is involved in the detoxification of reactive oxygen species and in the conjugation of xenobiotics metabolites of various classes, but also has its own antioxidant properties due to the presence of a sulfhydryl group in its structure. In addition, according to recent publications, **NAC** can act as an endogenous neuromodulator due to the ability to bind to γ -glutamyl fragments of NMDA- and AMPA-receptors (Aldini et al. 2018). Thus, **NAC** should be considered not only as an antioxidant and a source of cysteine for the synthesis of **glutathione**, but also as a medication capable to simulate neurotransmission through the glutamate system.

The described mechanisms can explain a decrease in neurological deficit noted in the rats that were gavaged with **NAC** before **ethanol** injection: the animals from this group (compared with the rats that had not received **NAC** before **ethanol** administration) demonstrated higher motor activity, less pronounced motor function impairment against better preserved cognitive functions. In particular, the rats from **NAC** group found a platform in the Morris water maze test much faster and in higher numbers than the animals from the positive control group, which indicated the suppression of the amnesic effects of **ethanol**. This effect is possibly due to the modulation of glutamatergic transmission. Glutamate, an excitatory amino acid, acts as an antagonist of γ -aminobutyric acid, an inhibitory neurotransmitter of the nervous system, and the action

on the GABA-receptors explains the amnesic effects of ethanol, barbiturates, and benzodiazepines. Furthermore, there is limited data on the ability of **NAC** to reduce the reinforcing properties of **ethanol**, which could be also considered as a beneficial effect (Morkovin et al. 2020).

Thus, as follows from the presented results, the prophylactic administration of **NAC** reduces the severity of the consequences of acute **ethanol** intoxication in rats due to a number of beneficial metabolic and neuro-metabolic effects. This makes the further development of drugs containing **NAC** and/or other sulfur-containing amino acids promising for both correction and prevention of post-intoxication conditions caused by high dose **ethanol** intake.

Conclusion

Preventive single oral administration of **NAC** at a dose of 1 g/kg before an acute alcohol intoxication facilitates alcohol metabolism, restores **glutathione** content in the liver and leads to a decrease in the severity of neurological and cognitive deficits in experimental animals, and a decrease in oxidative stress. The results of the study indicate the prospects of using **NAC** for the prevention and reduction of the severity of hangover syndrome.

In the rats which had been given a single oral dose of **NAC** before **ethanol** intoxication, the severity of neurological deficit, manifesting in a decrease in motor activity and motor disorders, was lower than in the animals gavaged with **saline** before alcoholization. **NAC** also improved the reproduction of the skill of platform-finding in the Morris water maze, which could be explained by the ability of **NAC** to suppress the amnesic effect of **ethanol**. This could be due to an improvement in **ethanol** metabolism and a decrease in the severity of disorders caused by either oxidative stress, or degenerative changes in the liver. Thus, the prophylactic administration of **NAC** before alcohol intoxication can reduce the severity of the post-intoxication state, which in rats is close to the hangover syndrome observed in humans.

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