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## COMPARATIVE ANALYSIS OF THE EFFECT OF DICLOFENAC SODIUM AND ETORICOXIB ON ENERGY METABOLISM IN RAT LIVER IN THE ACUTE GENERAL COOLING MODEL

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**Abstract.** Comparative analysis of the effect of diclofenac sodium and etoricoxib on energy metabolism in rat liver in the acute general cooling model. Shtrygol' S.Yu., Koiro O.O., Kudina O.V., Yudkevych T.K., Gorbach T.V. When the ambient temperature decreases, physiological mechanisms that prevent heat loss are activated. However, under cold stress hypothermia develops, which significantly disrupts the functioning of the body and can be transformed into life-threatening. Preventive use of nonsteroidal anti-inflammatory drugs, especially diclofenac sodium and etoricoxib, has been found to reduce the severity of cold trauma. Given that their frigoprotective effect can accompany the impact on the synthesis of eicosanoids when exposed to low temperatures, it is advisable to study mechanisms for preventing hypothermia independent of cyclooxygenase, particularly the influence on energy metabolism. The aim of the study was to figure out the effect of diclofenac sodium and etoricoxib on the indicators of energy metabolism in the liver of rats after acute general cooling. Experiments were carried out on 28 sexually mature male rats, which were given diclofenac sodium (7 mg/kg), etoricoxib (5 mg/kg), or solvent 30 minutes before cold trauma intragastrically (in the intact control and pathology control groups). Acute hypothermia was caused by exposure of animals for 2 hours at a temperature of -18°C. Rectal temperature was measured before and after acute general cooling. The content of lactate, pyruvate and adenosine triphosphate in the liver was measured and the lactate/pyruvate ratio was calculated. Diclofenac sodium, unlike etoricoxib, was found to significantly reduce the severity of hypothermia. Both nonsteroidal anti-inflammatory drugs prevent energy metabolism disorders caused by exposure to cold, namely, reducing the concentration of lactic acid and the ratio of lactate/pyruvate, increasing the content of pyruvate and adenosine triphosphate in the liver of animals. Etoricoxib normalizes the content of energy metabolism intermediates to their levels in intact animals. Diclofenac sodium has a similar effect, the expression of which is inferior to the selective cyclooxygenase-2 inhibitor. Therefore, when administered prophylactically before acute general cooling, diclofenac sodium effectively prevents hypothermia in rats, surpassing etoricoxib. Etoricoxib completely prevents a decrease in the content of pyruvate and adenosine triphosphate, as well as the accumulation of lactic acid in the liver. Diclofenac sodium is inferior to etoricoxib in its effect on energy metabolism, which indicates other mechanisms of frigoprotective action of a non-selective cyclooxygenase inhibitor. The frigoprotective and ergotropic properties of nonsteroidal anti-inflammatory drugs dissociate.

**Реферат.** Порівняльний аналіз впливу диклофенаку натрію та еторикоксибу на показники енергетичного обміну в печінці щурів на моделі гострого загального охолодження. Штриголь С.Ю., Койро О.О., Кудіна О.В., Юдкевич Т.К., Горбач Т.В. При зниженні температури доводяться активуються фізіологічні механізми, що запобігають втратам тепла. Однак в умовах холодового стресу розвивається гіпотермія, яка значно порушує функціонування організму та може становити загрозу для життя. Установлено, що профілактичне застосування нестероїдних протизапальних препаратів, особливо диклофенаку натрію та еторикоксибу, дозволяє зменшити ступінь тяжкості холодової травми. Зважаючи на те, що за впливу низьких температур їх фригопротекторна дія може дисоціювати із впливом на синтез ейкозаноїдів, доцільно дослідити

незалежні від циклооксигенази механізми запобігання гіпотермії, зокрема вплив на енергетичний обмін. Мета дослідження – з'ясувати вплив диклофенаку натрію та еторикоксибу на показники енергетичного обміну в печінці щурів після гострого загального охолодження. Досліди проведено на 28 статевозрілих щурах-самцях, яким за 30 хв. до моделювання холодової травми вводили одноразово внутрішньошлунково диклофенак натрію (7 мг/кг), еторикоксиб (5 мг/кг) або розчинник воду питну (у групах інтактного контролю та контрольної патології). Гостру гіпотермію викликали шляхом експозиції тварин протягом 2 год. при температурі  $-18^{\circ}\text{C}$ . До та після гострого загального охолодження оцінювали ректальну температуру. У гомогенаті печінки вимірювали вміст лактату, пірувату та аденозинтрифосфату, розраховували співвідношення лактат/піруват. Установлено, що диклофенак натрію, на відміну від еторикоксибу, достовірно знижує тяжкість гіпотермії. Обидва нестероїдні протизапальні препарати запобігають порушенням енергетичного обміну, спричиненим впливом холоду, а саме: зменшують концентрацію молочної кислоти та співвідношення лактат/піруват, збільшують вміст пірувату та аденозинтрифосфату в печінці тварин. Еторикоксиб нормалізує вміст інтермедіатів енергетичного обміну до їх рівнів в інтактних тварин. Диклофенак натрію чинить подібний вплив, за вираженістю якого поступається селективному інгібітору циклооксигенази-2. Отже, за профілактичного введення перед гострим загальним охолодженням диклофенак натрію ефективно запобігає гіпотермії в щурів, перевершуючи еторикоксиб. Еторикоксиб повністю запобігає зниженню вмісту пірувату та аденозинтрифосфату, а також накопиченню молочної кислоти в печінці. Диклофенак натрію поступається еторикоксибу за впливом на енергетичний обмін, що вказує на інші механізми фригопротекторної дії неселективного інгібітора циклооксигенази. Фригопротекторні та енерготропні властивості нестероїдних протизапальних препаратів дисоціюють.

Cold trauma (CT) significantly threatens human health and life. It is hardly possible to accurately estimate the real prevalence of CT because in publications data are limited to the most severe cases [1]. In the United States, in 2019, the prevalence of hypothermia-related deaths among people aged 15 and older ranged from 0.2 to 8.6 cases per 100,000 populations, with the highest rates recorded in rural areas, as well as in older people [2]. CT is often found in military personnel, athletes, and homeless people. Thus, in USA 366 cases of frostbite are registered per 1,000 climbers, and the frequency of lesions in skiers is 20% [3]. This determines the relevance of the search for effective frigoprotectors – medicines that protect the organism from the effects of low temperatures.

The relative stability of human and other mammalian body temperatures is provided by a number of physiological mechanisms. In acute general cooling (AGC), heat loss is counteracted by narrowing of peripheral vessels and centralization of blood circulation [4], contraction of skeletal muscles (contractile thermogenesis) [5], intensification of heat production due to activation of basal metabolic processes, including oxidation of fatty acids in brown adipose tissue (non-contractile thermogenesis) [6]. In CT, when heat transfer exceeds heat production, hypothermia occurs. The body is not able to generate enough heat needed to maintain homeostasis [7]. The prognosis depends on the severity of hypothermia. If the body temperature is too low, deep disorders of the nervous, cardiovascular and respiratory systems develop, which can cause death [7].

Eicosanoids, primarily prostaglandin  $F_{2\alpha}$  ( $\text{PGF}_{2\alpha}$ ) and thromboxane  $A_2$  ( $\text{TXA}_2$ ), play a significant role in the pathogenesis of CT. They are involved in the

inflammatory response and cause tissue ischemia due to increased platelet aggregation and vasoconstriction [8]. Thus, inhibition of the arachidonic acid cascade, in particular by nonsteroidal anti-inflammatory drugs (NSAIDs), is a promising direction of frigoprotection. The use of acetylsalicylic acid and ibuprofen improves the prognosis of treatment for frostbite [9]. It was proved experimentally the frigoprotective properties of acetylsalicylic acid, diclofenac sodium, ibuprofen, mefenamic acid, meloxicam, celecoxib, etoricoxib, darbufelone mesylate, but not the analgesic-antipyretic of paracetamol [10, 11], as well as inhibitors of the arachidonic acid cascade of another mechanism of action, such as the leukotriene receptor blocker montelukast [12]. When administered prophylactically, these NSAIDs increase the life expectancy of mice at a temperature of  $-18^{\circ}\text{C}$  of an average by 15-60% and reduced the severity of hypothermia, and among non-selective cyclooxygenase (COX) inhibitors, diclofenac sodium is the leader in frigoprotective action, among highly selective COX-2 inhibitors – etoricoxib [10, 11]. These NSAIDs have a stress-protective effect, favorably affect kidney function, prevent a decrease in contractile heart function and prolongation of the QT interval, and diclofenac sodium does not increase the effect of AGC on intraventricular conduction [13] and improves the cognitive functions of animals with CT [14].

However, the frigoprotective effect of NSAIDs dissociates with the anti-inflammatory effect, and therefore with the effect on the arachidonic acid cascade. At low temperature in a model of carrageenin edema in mice, the anti-inflammatory activity of diclofenac sodium almost disappears, and the degree of hypothermia of the body decreases [15].

Therefore, it is advisable to analyze possible COX-independent mechanisms of frigoprotective action of NSAIDs, in particular the effect on energy metabolism.

The aim of the study is to find out the effect of the most effective frigoprotective NSAIDs with different selectivity of COX inhibition (diclofenac sodium, etoricoxib) on the indicators of energy metabolism in the liver of rats after AGC.

## MATERIALS AND METHODS OF RESEARCH

**Studied drugs.** A non-selective COX inhibitor diclofenac sodium (Voltaren® tablets, Novartis, Switzerland) and highly selective COX-2 inhibitor etoricoxib (Arcoxia®, tablets, Merck Sharp&Dohme idea Inc, USA) were used in the study.

**Experimental animals and groups.** Experiments were carried out on 28 white random-bred male rats weighing 250-260 g in accordance with Principles of the Helsinki Declaration on the humane treatment of animals (2000) and Directive 2010/63/EU of the European Parliament and the Council of the EU "On the protection of animals used for scientific purposes". The experiment protocol was reviewed and approved by the bioethics committee of the National Pharmaceutical University, Kharkiv, Ukraine (Protocol No. 5 of 25 March 2021).

The animals were kept in standard vivarium conditions (air temperature 22-24°C, relative humidity 50%, 12-hour day/night cycle) of the Educational and Scientific Institute of Applied Pharmacy of the National Pharmaceutical University (Kharkiv, Ukraine) with free access to water and food. Rats were distributed into 4 groups of 7 animals each: group 1 – intact control (received intragastrically (IG) water); group 2 – control pathology (AGC, before which IG water was administered); group 3 – rats treated with diclofenac sodium at a dose of 7 mg/kg before AGC; group 4 – rats treated with etoricoxib at a dose of 5 mg/kg before AGC. Diclofenac sodium and etoricoxib doses were selected as the most effective for their frigoprotective effect [13, 14]. Diclofenac sodium and etoricoxib tablets were suspended in water with the addition of tween-80. The resulting suspensions were administered to rats once IG 30 minutes before CT modeling [16]. Rats of the intact control and pathology control groups received IG drinking water in a similar mode.

**Cold injury modeling, body temperature control.** For AGC modeling rats were placed in separate transparent plastic containers with a volume of 5 dm<sup>3</sup>, which ensure the mobility of animals, and were placed in the freezer "Nord Inter-300" for 2 hours at a

temperature of -18°C [16]. Rectal temperature was measured using a Microlife Mt-1931 thermometer 5 minutes before and 5 minutes after cold exposure.

**Extraction and storage of biological material.** 10 minutes after acute cold injury, rats were removed from the experiment by decapitation under thiopental sodium anesthesia (40 mg/kg intraperitoneally). The liver was removed and washed from the blood with a cooled 0.9% sodium chloride solution. The organ was frozen with liquid nitrogen and stored until biochemical studies at a temperature of -70°C.

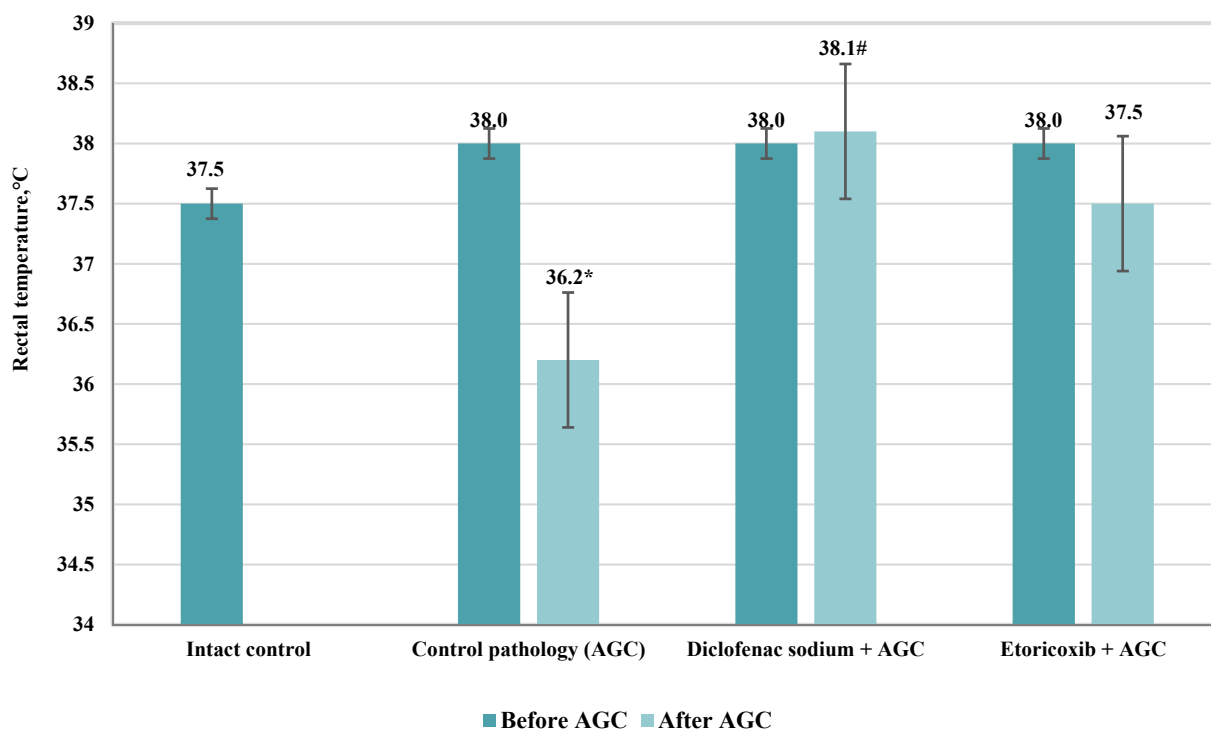
**Biochemical analysis.** The content of lactate [17], pyruvate [18], and adenosine triphosphoric acid (ATP) [19] was measured by standard methods in liver. A spectrophotometer Scolari PV 1252 was used. Lactate and pyruvate levels were expressed in nmol/1 g of tissue, and ATP in mmol/1 g of tissue. The lactate/pyruvate ratio was calculated, an increase in which is a marker of glycolysis activation and may be a precursor of an unfavorable functional consequence.

**Statistical analysis** was conducted using the program Statistica 10.0 (StatSoftInc., serial No. STA999K347156-W). Quantitative data were presented as medians, 25% and 75% percentiles (upper and lower quartiles), which were calculated in accordance with the recommendations for Biomedical Research (Me [Q<sub>25</sub>;Q<sub>75</sub>]). In addition, the data was traditionally presented as an arithmetic mean with a standard error of the mean (M±m), percent. The central trends of independent samples were compared using the Mann-Whitney criterion U. The differences were considered significant at a p<0.05.

## RESULTS AND DISCUSSION

Rats can withstand a two-hour exposure to a temperature of -18°C. The effect of the studied NSAIDs on the dynamics of body temperature according to AGC is shown in the Figure.

In the control pathology group, a decrease in rectal temperature was observed by 1.8±0.8°C (p<0.05) compared to the initial state. Diclofenac sodium significantly reduced the severity of hypothermia, as evidenced by the absence of changes in rat body temperature relative to the initial state (p>0.05), as well as higher indicators compared to the control pathology group (body temperature after AGC 38.1±0.1°C and 36.2±0.9°C, respectively, p<0.05). Etoricoxib also prevented a decrease in body temperature compared to the initial level, but there were no statistically significant differences compared to the control pathology.



AGC – acute general cooling; statistically significant differences: \* –  $p < 0.05$  relative to initial state; # –  $p < 0.05$  – relative to the control pathology group.

**Changes in rat rectal temperature after acute general cooling with diclofenac sodium and etoricoxib**

The results of energy metabolism indicators determining are shown in the Table.

AGC caused significant disturbances in energy metabolism in the rat liver (Table). The lactate content increased by an average of 145.6%, and the pyruvate content decreased by 44.7% ( $p < 0.005$ ) in the

control pathology group. At the same time, the lactate/pyruvate ratio increased by 4.25 times ( $p < 0.005$ ), and the ATP content decreased by 12.8% ( $p < 0.005$ ), which reflects a decrease in the intensity of the aerobic pathway of energy metabolism.

**Effect of diclofenac sodium and etoricoxib on lactate, pyruvate and ATP content in rat liver after acute general cooling ( $M \pm m$ ;  $M[Q_{25}; Q_{75}]$ )**

Indicator	Intact control (n=6)	Acute general cooling		
		control pathology (n=10)	diclofenac sodium, 7 mg/kg (n=10)	etoricoxib, 5 mg/kg (n=10)
Lactate, nmol/g	4.30±0.49 4.28 [3.96; 4.81]	10.56±0.98** <sup>s</sup> 10.48 [9.83; 10.00]	7.06±0.63** <sup>s</sup> 7.03 [6.45; 7.64]	4.49±0.08# <sup>^</sup> 4.29 [4.19; 5.00]
Pyruvate, nmol/g	372.4±11.5 372.9 [361.8; 380.4]	206.1±4.8** <sup>s</sup> 207.9 [202.1; 209.3]	285.4±7.1** <sup>s</sup> 287.8 [277.9; 290.1]	365.4±0.1# <sup>^</sup> 367.6 [361.8; 370.0]
Lactate / pyruvate	0.012±0.002 0.012 [0.011; 0.013]	0.051±0.004** <sup>s</sup> 0.051 [0.047; 0.028]	0.025±0.003** <sup>s</sup> 0.024 [0.022; 0.028]	0.012±0.082# <sup>^</sup> 0.012 [0.011; 0.014]
ATP, mmol/g	1.95±0.05 1.94 [1.90; 2.00]	1.70±0.02** <sup>s</sup> 1.70 [1.68; 1.71]	1.80±0.03** <sup>s</sup> 1.80 [1.77; 1.81]	1.96±0.08# <sup>^</sup> 1.94 [1.90; 2.00]

Notes: ATP – adenosine triphosphoric acid; n – number of animals in the group; statistically significant differences: \* –  $p < 0.005$  compared to the intact contact group; # –  $p < 0.005$  compared to the control pathology group; ^ –  $p < 0.005$  compared to the diclofenac sodium group; <sup>s</sup> –  $p < 0.005$  compared to the etoricoxib group.



Etoricoxib completely normalized the content of pyruvate, lactate, lactate/pyruvate ratio, and ATP content in the liver of animals exposed to cold. There were no significant differences with the intact control group in all indicators.

Similar changes in the content of energy metabolism intermediates were observed in the group receiving diclofenac, but it was significantly inferior in effectiveness to etoricoxib ( $p < 0.005$  in all indicators). As can be seen from the table, diclofenac sodium reduced the content of lactate in the liver by 33.9% ( $p < 0.005$ ) and increased the content of pyruvate ( $p < 0.005$ ) by 38.5% ( $p < 0.005$ ) compared to similar indicators of the control pathology group. The lactate/pyruvate ratio decreased by 2 times ( $p < 0.005$ ), and the ATP content increased by 5%. Despite the positive effect on energy metabolism, diclofenac sodium did not restore its indicators to the values of intact animals: the lactate content remained increased by 64.2%, and the level of pyruvic acid was reduced by 23.4%, and the lactate/pyruvate ratio was 2.1 times higher. The ATP content in the liver remained reduced by 7.7%.

Thus, according to AGC in rats of the control pathology group, the efficiency of energy metabolism decreases, which is associated with the development of lactate acidosis. The result is a decrease in the content of ATP in the liver. Etoricoxib eliminates lactate acidosis and normalizes the ATP content. Diclofenac sodium has a similar but less pronounced effect.

Heat losses when the ambient temperature decreases are prevented by reducing heat transfer and increasing heat production. The main site of contractile thermogenesis is superficially located muscles, which contract to increase the hydrolysis of ATP and release energy that goes to warm the body. Non-contractile thermogenesis occurs in brown adipose tissue. Brown adipocytes, through the separating protein thermogenin (UCP1), disrupt ATP synthesis in mitochondria, as a result of which the energy of biological oxidation, not spent on phosphorylation, is dissipated in the form of heat [20, 21]. Other tissues, in particular the liver, are also involved in adapting to low temperatures [21]. AGC can increase liver glucose production (glycogenolysis, gluconeogenesis), increase systemic energy expenditure and its utilization in peripheral tissues [22]. These metabolic changes are mainly aimed at enhancing thermogenesis and are necessary for maintaining body temperature [23].

A significant decrease in rectal temperature by an average of 1.8°C in rats of the control pathology group after a 2-hour exposure at -18°C and prevention of hypothermia with diclofenac sodium and, to a lesser extent, etoricoxib corresponds to the data of

previous studies of the frigoprotective properties of COX inhibitors [13, 14], which indicates a high reproducibility of these results. The frigoprotective effect of NSAIDs may be associated with inhibition of prostaglandin synthesis, which contributes to increased heat transfer due to dilation of peripheral vessels, in particular PGI<sub>2</sub> [24]. Also, the preservation of body temperature against the background of CT may be due to an imbalance of individual eicosanoids. It was found that hyperpyretic PGE<sub>2</sub> effects occur under sufficient energy however, with the depletion of energy resources, which we observed in this experiment (a decrease in ATP levels), PGD<sub>2</sub>-mediated hypothermic reactions may increase [25].

It is possible that NSAIDs affect thermogenesis in brown adipocytes, separating the processes of oxidation and phosphorylation. Non-selective COX inhibitor indomethacin *in vitro* stimulated differentiation of brown preadipocytes in mice and enhanced the expression of mRNA and thermogenin protein dose-dependently, which breaks oxidative phosphorylation. In addition, indomethacin increased the expression of the gamma-coactivator of the 1 $\alpha$  receptor which is activated by peroxisome proliferators (PPARGC1A) and is involved in muscle tissue metabolism, fat and carbohydrate metabolism [20].

Under the influence of low temperatures, the role of the liver in non-contractile thermogenesis increases, since when the release of fatty acids from white adipose tissue is activated, it provides brown adipose tissue with acylcarnitines-intermediates of redox reactions occurring in mitochondria accompanied by ATP synthesis [21]. A decrease in the efficiency of energy metabolism in animals of the control pathology group, which was shown in our experiment, is associated with the development of lactate acidosis. The cause of the lactate acidosis is obviously tissue hypoxia caused by circulatory insufficiency in peripheral tissues, which leads to activation of anaerobic metabolism and excessive lactate production. A decrease in the content of ATP in the liver can be associated not only with a decrease in synthesis but also with increased utilization in order to increase heat production when exposed to cold.

The results obtained are consistent with the literature data. After cold exposure for 2 hours phosphorylation of fructose-2,6-diphosphate continued in the liver and accumulation of glycolysis intermediates, such as fructose-1,6-diphosphate and pyruvate, decreased [26]. Acute cold stress (4°C for 0, 2, 4 and 6 hours) in male mice of the C57BL/6 line was accompanied by a short-term increase, and then a sharp decrease in the content of glycolysis products in the liver-fructose-1,6-diphosphate and pyruvate,



however, in contrast to our results, the content of ATP increased [22].

Etoricoxib and, to a lesser extent, diclofenac sodium contributed to the normalization of the content of pyruvate, lactate, ATP, and the lactate/pyruvate ratio in the rat liver under AGC. There were no significant differences with the intact control group in all these indicators using selective COX-2 inhibitor. It draws attention to the fact that after AGC, the direction of the effect of NSAIDs on energy processes in the liver changes. As can be seen from the table, under a decrease in the severity of hypothermia when using diclofenac sodium and etoricoxib, the ATP pool is restored and the content of lactate and pyruvate is normalized (see table.). In contrast, spheroidal cultures of rat hepatocytes research *in vitro* showed that diclofenac significantly reduced glucose and lactate release, as well as pyruvate uptake [27]. Diclofenac promotes intracellular accumulation of lactate by disrupting its secretion, which is associated with the ability to inhibit glycolysis [27].

The results indicate a positive effect of etoricoxib and diclofenac sodium on energy metabolism in the liver of rats exposed to acute cold. Thus, this effect may be involved in the frigoprotective effect of NSAIDs. However, it is not decisive, since diclofenac sodium eliminates metabolic disorders to a lesser extent than etoricoxib, but, unlike etoricoxib, completely prevents a decrease in body temperature. Consequently, there is dissociation of the frigoprotective and energotropic effects of NSAIDs. A similar dissociation, as already noted, also exists between the frigoprotective and anti-inflammatory effects. Indeed, the anti-inflammatory effect of diclofenac sodium in AGC is significantly reduced [15]. It is possible that the maximum frigoprotective efficacy of diclofenac sodium among 8 other NSAIDs with different selectivity of action on COX is associated with a complex of factors, including the antiplatelet effect inherent in non-selective COX inhibitors, which can improve microcirculation impaired in CT,

although the frigoprotective activity of diclofenac sodium exceeds the known antiplatelet agent acetylsalicylic acid [10, 11]. The presence of other specific pharmacological properties of NSAIDs, in particular diclofenac sodium, which determine the frigoprotective activity, is also possible. This requires further clarification.

#### CONCLUSIONS

1. In the acute general cooling model, the non-selective COX inhibitor diclofenac sodium (7 mg/kg), when administered prophylactically, effectively prevents hypothermia in rats, surpassing the highly selective COX-2 inhibitor etoricoxib (5 mg/kg) as a frigoprotector.

2. Etoricoxib prevents disruption of energy metabolism in the liver of rats exposed to cold. It normalizes the content of pyruvate, lactic acid, and ATP in the liver.

3. Diclofenac sodium also has a beneficial effect on energy metabolism in the rat liver but is inferior in effectiveness to etoricoxib. This indicates dissociation between energotropic and frigoprotective effects, which substantiates the feasibility of further elucidation of the mechanisms of the latter.

#### Contributors:

Shtrygol' S.Yu. – conceptualization, methodology, review and revision, supervision;

Koiron O.O. – data analysis, writing;

Kudina O.V. – data collection, data analysis, writing;

Yudkevych T. K. – data collection;

Gorbach T.V. – data collection, data analysis.

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