ORIGINAL ARTICLE

# The effect of non-steroidal anti-inflammatory drugs with different mechanisms of action on the body temperature and cyclooxygenase pathway of the arachidonic acid cascade on the model of acute general cooling (air hypothermia) in rats

# Vliv nesteroidních protizánětlivých léčiv s různými mechanismy účinku na tělesnou teplotu a dráhu cyklooxygenázy kaskády kyseliny arachidonové na modelu akutního celkového ochlazení (vzduchová hypotermie) u potkanů

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**Summary**

NSAIDs are promising agents for preventing cold injury (frigoprotectors). The influence of prophylactic administration of the non-selective COX inhibitor diclofenac sodium (7 mg/kg) and the highly selective COX-2 inhibitor etoricoxib (5 mg/kg) on cyclooxygenase pathway biomarkers was studied on the model of acute general cooling (air hypothermia at –18 °C for 2 hours). Diclofenac completely prevented a decrease in body temperature, surpassing etoricoxib. In the liver of the rats immediately after cold exposure, the content of COX-1 was increased moderately and the content of COX-2 highly significantly. Very significantly, the level of PGE2 decreased, and the levels of PGF2α, especially PGI2 and TXB2, were elevated. In the blood serum, the level of COX-1 was decreased, and the changes in COX-2 and prostaglandins levels were similar to those in the liver. Diclofenac exerted a moderate effect towards the normalization of both COX isoforms in the liver, moderately increased the content of PGE2, and decreased – PGF2α and TXB2 without changing the level of PGI2. In serum, diclofenac reduced COX-1 level to subnormal values, and its effect on other biomarkers was similar to that in the liver, except for a moderate decrease in PGI2. Thus, diclofenac was inferior to etoricoxib, which normalized COX-1, COX-2, PGE2, and PGI2 in the liver and reduced the content of PGF2α and TXB2 in the liver to subnormal values. At the same time, in the blood serum, it decreased COX-1, COX-2, and PGE2 to subnormal values, normalized PGF2α, and PGI2, and significantly reduced TXB2. The opposite degree of intensity of the influence of diclofenac and etoricoxib on the cyclooxygenase pathway and body temperature indicates a dissociation of anti-inflammatory and frigoprotective activity. Inhibition of oxidative stress is not determinative for the frigoprotective activity of NSAIDs since diclofenac, despite the weaker influence on the content of 8-isoprostane in the liver, still exerts the maximum frigoprotective activity.

**Key words:** cold injury prevention • body temperature • cyclooxygenase • prostaglandins • diclofenac sodium • etoricoxib • frigoprotective activity

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**Souhrn**

NSAID jsou slibné látky pro prevenci poškození chladem (frigoprotektiva). Vliv profylaktického podávání neselektivního inhibitoru COX diklofenaku sodného (7 mg/kg) a vysoce selektivního inhibitoru COX-2 etorikoxibu (5 mg/kg) na biomarkery cyklooxygenázové dráhy byl studován na modelu akutního celkového ochlazení (vzduchová hypotermie při –18 °C po dobu 2 hodin). Diklofenak zcela zabránil poklesu tělesné teploty a předčil etorikoxib. V játrech potkanů byl bezprostředně po expozici chladu mírně zvýšen obsah COX-1, velmi výrazně obsah COX-2, snížena hladina PGE2 a zvýšeny hladiny PGF2α a zejména PGI2 a TXB2. V krevním séru byla hladina COX-1 snížena a změny hladin COX-2 a prostaglandinů byly podobné jako v játrech. Diklofenak působil mírně směrem k normalizaci obou izoforem COX v játrech, mírně zvyšoval obsah PGE2 a snižoval – PGF2α a TXB2, aniž by měnil hladinu PGI2. V séru diklofenak snížil hladinu COX-1 na podnormální hodnoty a jeho účinek na ostatní biomarkery byl podobný jako v játrech, s výjimkou mírného snížení PGI2. Diklofenak byl tedy horší než etorikoxib, který normalizoval COX-1, COX-2, PGE2, PGI2 v játrech, snížil obsah PGF2α a TXB2 v játrech na podnormální hodnoty, zatímco v krevním séru snížil COX-1, COX-2, PGE2 na podnormální hodnoty, normalizoval PGF2α a PGI2, významně snížil TXB2. Opačný stupeň intenzity vlivu diklofenaku a etorikoxibu na cyklooxygenázovou dráhu a tělesnou teplotu svědčí o disociaci protizánětlivé a frigoprotektivní aktivity. Inhibice oxidačního stresu není pro frigoprotektivní aktivitu NSAID rozhodující, protože diklofenak i přes slabší vliv na obsah 8-isoprostanu v játrech stále vykazuje maximální frigoprotektivní aktivitu.

**Klíčová slova:** prevence poškození chladem • tělesná teplota • cyklooxygenáza • prostaglandiny • diklofenak sodná sůl • etorikoxib • frigoprotektivní aktivita

## Introduction

Cold trauma (CT) remains an urgent medical and social problem. An accurate assessment of the actual prevalence of CT is limited by the fact that official statistics mainly include the most severe cases[1]. According to data[2] in 2019, in the United States, the number of deaths from hypothermia in people aged 15 years and older ranged from 0.2 to 8.6 cases per 100,000 population, with the highest rates in rural areas and in older people. A high frequency of CT is inherent in military personnel, athletes, and the homeless. In particular, among the USA military in 2019, the frequency of CT was 36.5 cases per 100,000 people[3, 4]. The number of frostbites per 1,000 climbers is 366, and the frequency of CT in skiers reaches 20%. So, the topical task is to find effective frigoprotectors, which are the agents that protect the organism from the effects of low temperatures.

Eicosanoids, which are formed in the cyclooxygenase (COX) pathway of the arachidonic acid cascade, are involved in the pathogenesis of CT. In particular, the role of prostaglandins F2α (PGF2α) and thromboxane A2 (TXA2) is known, and these mediators are responsible for the development of an inflammatory response, tissue ischemia as a result of increased platelet aggregation, and vasoconstriction[5, 6]. Prostaglandin I2 (PGI2) contributes to increased heat transfer due to the dilation of peripheral vessels[7].

Given the role of eicosanoids in the pathogenesis of CT, non-steroidal anti-inflammatory drugs (NSAIDs) attract attention as promising frigoprotectors. Early studies have shown that prophylactic administration of indomethacin and other COX inhibitors blocks PGD2 production and prevents the development of a hypothermic reaction[10]. According to the results of clinical studies, acetylsalicylic acid and ibuprofen improved the prognosis of frostbite[11]. Body temperature under CT is affected by a disturbance of the balance between individual eicosanoids. To a certain extent, this effect is explained by the impact of hypothermia on the formation of endothelin, PGI2, and TXA2. These substances play an important role in regulating local blood circulation, and an imbalance between them contributes to vasoconstriction and thrombosis, disrupting blood circulation and leading to ischemic damage[8]. Under sufficient metabolic energy supply, a hyperpyretic effect of prostaglandin E2 (PGE2) occurs, but when energy resources are depleted during cooling, hypothermic reactions mediated by prostaglandin D2 (PGD2) may increase[9]. For example, in response to the administration of certain pyrogens, the rectal temperature decreased in rats, which is associated with the induction of PGD2 synthesis in the brain, especially in the preoptic region[9].

In an experiment on a model of acute general cooling (AGC, air hypothermia) in mice, among 11 COX inhibitors of various mechanisms and selectivity of action, significant frigoprotective properties of acetylsalicylic acid, diclofenac sodium, ibuprofen, mefenamic acid, meloxicam, celecoxib, etoricoxib, darbufelone mesylate were established. Diclofenac sodium was the undoubted leader among non-selective COX inhibitors and etoricoxib – among highly selective COX-2 inhibitors. Under the influence of these drugs, the life expectancy of mice increased by an average of 30–55%[12, 13]. In experiments on rats, diclofenac sodium provided the maximum frigoprotective activity in the antihypothermic effect, which is an integral indicator[14, 15]. In addition, the anti-inflammatory and frigoprotective effects of NSAIDs appeared to dissociate: in AGC in mice, diclofenac sodium reduced the degree of hypothermia but was almost devoid of the anti-inflammatory (antiexudative) effect on the model of carrageenan edema (when it was induced during cold exposure of animals)[16]. In addition, the severity of the inflammatory response in hypothermia, regardless of its cause (animals exposed to low ambient temperatures or after administration of chlorpromazine), is decreased, which is shown in the

model of carrageenan edema both in mice[16] and rats[17].

Notably, the nephrotoxic effect (typical of the non-selective COX inhibitors) was not manifested under the conditions of cold injury. On the contrary, diclofenac sodium (as well as etoricoxib) contributed to the maintenance of the renal excretory function, supporting the ability to excrete water loading[15]. It is a very significant advantage. Otherwise, the investigation of NSAIDs as potential frigoprotectors would hardly be expedient. Specific mechanisms of this phenomenon – qualitative change of COX inhibitors renal effects under the influence of cold – have not been studied in detail. Nevertheless, it can be supposed that the primary maintenance of body temperature and stress-protective effect (also proven in the study)[15] could also provide the conditions for the absence of the abrupt haemodynamics changes with further limitations in the renal blood flow. On the other hand, the lack of the nephrotoxic effect and abrupt changes in the intravascular volume allows determining the relatively unaffected levels of serum metabolites.

Taking into account these features of NSAIDs' action in cold trauma, **the aim** of the study is to find out the effects of the most effective among them (diclofenac sodium, etoricoxib) on the indicators of the cyclooxygenase pathway of the arachidonic acid cascade on the model of AGC in rats and to collate these effects with the changes in body temperature.

## Experimental part

### *Materials*

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.

### *Methods*

*Study design*

For hypothermia modelling, the rats were placed in separate transparent plastic containers of a volume of 5000 $cm^3$, which underwent exposure at –18 °C for 2 hours in a "Nord Inter-300" freezer. Rectal temperature was measured using a Panlab TMP812 RSMT-1931 thermometer (with a resolution of 0.1 degrees) 5 minutes before and 5 minutes after the air hypothermia exposure[18].

Ten minutes after AGC, the rats were decapitated under thiopental sodium anesthesia (40 mg/kg intraperitoneally). After 30 min of clotting at 25 °C, the blood was centrifuged (10 min at 3000 rpm), and the blood serum was immediately collected. The liver was removed and washed from the blood with a cooled 0.9% sodium chloride solution, immediately frozen with liquid nitrogen. The liver and blood serum were stored in a freezer at –70 °C until immunochemical studies were performed. Using standard species-specific kits (produced by MyBioSource, USA), the content of COX-1 (Cyclooxygenase-1 (COX-1), ELISA Kit), COX-2 (Cyclooxegenase-2 (COX-2), ELISA Kit), prostaglandins: PGE2 (Prostaglandin E2 (PG-E2) ELISA Kit), PGF2α (Prostaglandin F2alpha (PGF2alpha) ELISA Kit), PGI2 (Prostacyclin (PGI2) ELISA Kit), TXB2 (Thromboxanes B2 (TXB2), ELISA Kit) were determined in liver homogenate and blood serum. Also, the content of 8-isoprostane (8-isoprostane ELISA, Enzyme immunoassay for the quantitative determination of 8-isoprostane) was determined in the liver. An enzyme-immunoassay analyser STAT FAX 303+ (USA) was used.

*Experimental animals and grouping*

Twenty-four Wistar albino male rats weighing 250–260 g were used in the experiment, which was conducted in compliance with bioethical principles following "Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes" (Brussels, 2010). The study protocol was reviewed and approved by the bioethics committee of the National University of Pharmacy, Kharkiv, Ukraine (protocol No. 5 from 25.03.2021).

Wistar rats bred in the vivarium of the Educational and Scientific Institute of Applied Pharmacy of the National Pharmaceutical University (Kharkiv, Ukraine) were used. During the experiment, they were kept in standard conditions in the mentioned vivarium, at an air temperature of 22–24 °C, relative humidity of 50%, 12-hour day/night cycle) with free access to water and food.

The rats were randomly divided into 4 groups of 6 animals each:

- group 1 – intact control;
- group 2 – rats that underwent AGC (the untreated control);
- group 3 – rats treated with diclofenac sodium at a dose of 7 mg/kg prior to AGC;
- group 4 – rats treated with etoricoxib at a dose of 5 mg/kg before AGC.

The doses of diclofenac sodium and etoricoxib were calculated for rats based on the most effective doses increasing life expectancy in mice on the AGC model in screening experiments[12, 13], and these doses were also used in previous studies on rats[14, 15]. Diclofenac sodium and etoricoxib tablets were suspended in water with the addition of Tween-80. The obtained suspensions (in a volume of 0.5 ml per 100 g of rat body weight) were administered to rats once intragastrically using a prophylactic regimen 30 minutes before AGC[18]. Rats of the intact control and untreated control groups received intragastrically drinking water with the addition of Tween-80 in a similar regimen.

*Statistical analysis*

Statistical processing of the results was performed using the program "Statistica 10.0". The normality hypothesis was rejected by the Shapiro-Wilk test.

A comparison of the central tendencies of independent samples was made using the Kruskal-Wallis H-test and the Mann-Whitney-U-test. The differences between dependent samples were evaluated using the paired samples Wilcoxon test. The differences were considered statistically significant at $p < 0.05$. Quantitative data are given as arithmetic means with standard errors of the mean (M ± m) and medians with 25% and 75% percentiles (Me [Q25; Q75]).

## Results

### *Changes in body temperature after a cold injury*

A two-hour exposure of rats at –18 °C caused an expectable decrease in body temperature. In animals with AGC, rectal temperature significantly ($p < 0.05$) decreased by an average of 1.7 °C, or by 4.7% compared to the initial value (Table 1).

Under etoricoxib, the temperature decrease was slightly less pronounced (–1.5 °C or –1.4% $p < 0.05$ compared to the basal level). However, under diclofenac sodium, the body temperature did not undergo statistically significant changes (+0.4 ± 0.3 °C compared to the basal level).

Thus, the frigoprotective effect of diclofenac sodium surpasses that of etoricoxib.

### *Changes in the level of COX isoforms and different prostaglandins after cold trauma*

The results of the determination of COX-1, COX-2, and prostaglandins of different classes are shown in Table 2. In the liver of rats exposed to AGC, the content of COX-1 was increased by an average of 9% compared to the intact control group ($p < 0.01$), and COX-2 content was increased drastically (by 206%, $p < 0.01$). At the same time, the content of PGE2 was decreased by 51% ($p < 0.01$), and the level of prostaglandins of other classes was elevated: PGF2α – by 40% ($p < 0.01$), PGI2 – by 76% ($p < 0.01$), TXB2 – by 76% ($p < 0.01$).

Under diclofenac sodium, the increment of COX-1 and COX-2 was less pronounced (2%, $p < 0.05$, and 41%, $p < 0.01$ compared to the intact control value, respectively), and the level of both COX isoforms was statistically significantly lower than in the liver of rats of the untreated control group ($p < 0.01$). PGE2 under diclofenac sodium was decreased by 28% ($p < 0.01$) as against the intact control value, and the increment in PGF2α and TXB2 was less significant – by 13% ($p < 0.01$) and by 24% ($p < 0.01$) in comparison with the untreated control group. It should be noted that all the mentioned differences were directed towards normalization and reached the level of statistical significance with the values of the untreated control group ($p < 0.01$). Diclofenac sodium did not change the increased PGI2 content.

Etoricoxib contributed to the normalization of COX-1 and COX-2, PGE2, and PGI2 in the liver (these indicators did not differ practically from those of the animals). At the same time, the content of PGF2α and TXB2 was changed by etoricoxib in the opposite to diclofenac sodium way: PGF2α decreased by 13% ($p < 0.01$), TXB2 – by 37% ($p < 0.01$) compared to the intact control value. All the markers changed by etoricoxib statistically significantly differ from the corresponding values of the untreated control group as well as the from the group receiving diclofenac sodium ($p < 0.01$).

In the blood serum, slightly different changes in the cyclooxygenase pathway of the arachidonic acid cascade were observed (Table 2).

In rats of the untreated control group, the serum level of COX-1, in contrast to hepatic one, was decreased by 8% compared to the value of the intact control ($p < 0.01$), and the content of COX-2 increased

*Table 1. The effects of diclofenac sodium and etoricoxib on rectal temperature (°C) on a model of acute general cooling (air hypothermia) in rats (M ± m, Me[Q25; Q75])*

| Group, number of animals | Rectal temperature, °C | | |
|---|---|---|---|
| | Basal level | 2 hours after cold exposure at –18 °C | Difference |
| Intact control (n = 6) | 36.2 ± 0.1<br>36.2 [36.0; 36.3] | | |
| **Acute general cooling (air hypothermia)** | | | |
| Untreated control (AGC, n = ) | 36.2 ± 0.1<br>36.3 [36.0; 36.4] | 34.5 ± 0.2[&]<br>34.6 [34.0; 34.9][&] | –1.7 ± 0.2<br>–1.7 [–2.3; –1.2] |
| AGC + diclofenac sodium (n = 6) | 36.2 ± 0.1<br>36.2 [36.2; 36.3] | 36.6 ± 0.3<br>36.5 [36.4; 37.0] | 0.4 ± 0.3[##$]<br>0.25 [0.0; 0.8][##$] |
| AGC + etoricoxib (n = 6) | 36.5 ± 0.1<br>36.3 [36.2; 36.9] | 35.0 ± 0.5[&]<br>35.2 [33.8; 35.4][&] | –1.5 ± 0.5[^]<br>–1.75 [–2.4; –0.8][^] |

AGC – acute general cooling, n – number of animals in the group

[##] statistically significant difference compared to the untreated control group ($p < 0.01$)

[^] statistically significant difference compared to the group treated with diclofenac sodium ($p < 0.05$)

[$] statistically significant difference compared to the group treated with etoricoxib ($p < 0.05$)

[&] statistically significant difference compared to the basal level value ($p < 0.05$; Wilcoxon criteria)

to a lesser extent – by 111% ($p < 0.01$). The direction of changes in blood serum prostaglandin levels coincided with that in the liver: PGE2 was reduced by 31%, while PGI2, PGF2α, and TXB2 were increased by 100%, 30%, and 74%, respectively, relative to intact control values (all the changes are statistically significant, $p < 0.01$).

Under diclofenac sodium, serum COX-1 level, in contrast to hepatic one, was decreased by 10% ($p < 0.05$), and COX-2 level was augmented similarly to its hepatic level by 46% ($p < 0.01$). The content of PGE2 was reduced by 16%, PGI2, PGF2α, and TXB2 – were increased by 32% ($p < 0.05$), 14%, and 23% ($p < 0.01$), respectively. With the exception of COX-1, all the studied indicator changes were directed towards normalization, and the differences reached statistical significance with the values of the untreated control group ($p < 0.01$).

In animals treated with etoricoxib, the maximum decrease in serum COX-1 was observed by 21% compared to the control ($p < 0.01$), and the level of COX-2 was not increased, as in other groups, but was reduced by 30% ($p < 0.01$). The content of serum PGE2 was reduced compared to the intact control to a lesser extent than under the influence of diclofenac sodium – only by 8% ($p < 0.01$). PGF2α and PGI2 levels tended to be lower (8% and 3%, respectively), and TXB2 was 12% higher than in the intact control group ($p < 0.05$). All these differences reached the level of statistical significance with the values of the untreated control group (Table 1).

***Changes in the level of 8-isoprostane in the rat liver after cold injury***

As can be seen from Table 3, the content of 8-isoprostane in the liver of rats exposed to AGC

*Table 2. The effect of diclofenac sodium and etoricoxib on COX-1, COX-2, and levels of prostaglandins of various classes in rat liver and blood serum on the model of acute general cooling (air hypothermia, M ± m, Me[Q25; Q75])*

| Indicators | Intact control (n = 6) | Acute general cooling (air hypothermia) | | |
|---|---|---|---|---|
| | | Untreated control (AGC, n = 6) | AGC + diclofenac sodium (n = 6) | AGC + etoricoxib (n = 6) |
| **Liver** | | | | |
| COX-1, pg/g | 1187.1 ± 7.4<br>1187.3 [1175.5; 1200.1] | 1302.6 ± 12.5$^{**}$<br>1299.2 [1283.1; 1312.1]$^{**}$ | 1214.9 ± 6.1$^{*\#\#\$\$}$<br>1214.5 [1200.1; 1228.4]$^{*\#\#\$\$}$ | 1170.7 ± 8.3$^{\#\#\wedge\wedge}$<br>1171.3 [1156.8; 1182.5]$^{\#\#\wedge\wedge}$ |
| COX-2, ng/g | 97.1 ± 3.7<br>96.4 [89.8;106.1] | 297.1 ± 3.3$^{**}$<br>296.3 [290.3; 305.6]$^{**}$ | 136.5 ± 6.6$^{**\#\#\$\$}$<br>133.8 [129.0; 146.5]$^{**\#\#\$\$}$ | 98.7 ± 2.6$^{\#\#\wedge\wedge}$<br>97.9 [95.2; 103.7]$^{\#\#\wedge\wedge}$ |
| PGE2, pg/g | 440.2 ± 16.1<br>453.8 [439.9; 461.1] | 216.9 ± 3.8$^{**}$<br>216.3 [209.1; 224.7]$^{**}$ | 316.6 ± 4.4$^{**\#\#\$\$}$<br>317.8 [311.3; 323.8]$^{**\#\#\$\$}$ | 407.6 ± 2.8$^{\#\#\wedge\wedge}$<br>408.3 [402.4; 411.1]$^{\#\#\wedge\wedge}$ |
| PGF2α, pg/g | 1601.9 ± 11.3<br>1604.6 [1589.3; 1622.2] | 2239.5 ± 79.9$^{**}$<br>2275.3 [2148.7; 2326.4]$^{**}$ | 1805.2 ± 5.6$^{**\#\#\$\$}$<br>1804.9 [1793.2; 1815.4]$^{**\#\#\$\$}$ | 1397.9 ± 18.4$^{**\#\#\wedge\wedge}$<br>1402.6 [1359.7; 1431.5]$^{**\#\#\wedge\wedge}$ |
| PGI2, ng/g | 3.3 ± 0.1<br>3.2 [3.1;3.3] | 5.8 ± 0.1$^{**}$<br>5.8 [5.6;6.0]$^{**}$ | 5.8 ± 0.1$^{**\$\$}$<br>5.6 [5.7;6.0]$^{**\$\$}$ | 3.3 ± 0.1$^{\#\#\wedge\wedge}$<br>3.3 [3.2;3.4] $^{\#\#\wedge\wedge}$ |
| TXB2, pg/g | 412.0 ± 4.8<br>410.3 [402.1; 421.3] | 803.8 ± 4.5$^{**}$<br>804.9 [794.6; 811.4]$^{**}$ | 509.7 ± 6.8$^{**\#\#\$\$}$<br>508.7 [497.2; 520.0]$^{**\#\#\$\$}$ | 261.2 ± 3.5$^{**\#\#\wedge\wedge}$<br>259.9 [255.5; 270.1]$^{**\#\#\wedge\wedge}$ |
| **Blood serum** | | | | |
| COX-1, pg/ml | 68.5 ± 0.8<br>68.2 [66.8; 70.3] | 62.7 ± 1.6$^{**}$<br>64.4 [59.9; 65.5]** | 61.5 ± 2.5$^{*}$<br>63.2 [61.0; 64.5]$^{*}$ | 54.3 ± 1.0$^{**\#}$<br>54.62 [53.1; 55.1]$^{**\#}$ |
| COX-2, ng/ml | 12.2 ± 0.7<br>11.7 [10.9; 13.9] | 25.7 ± 0.9$^{**}$<br>26.0 [23.9; 27.2]$^{**}$ | 17.9 ± 0.7$^{**\#\#\$\$}$<br>17.7 [16.3; 19.8]$^{**\#\#\$\$}$ | 8.5 ± 0.3$^{**\#\#\wedge\wedge}$<br>8.3 [8.0; 8.65]$^{**\#\#\wedge\wedge}$ |
| PGE2, pg/ml | 80.2 ± 0.9<br>79.8 [79.0; 82.33] | 55.1 ± 1.3$^{**}$<br>54.7 [53.9; 56.2]$^{**}$ | 67.2 ± 3.5$^{*\#\#}$<br>64.4 [62.1; 66.4]$^{*\#\#}$ | 73.5 ± 1.0$^{**\#\#}$<br>73.2 [72.1; 75.3]$^{**\#\#}$ |
| PGF2α, pg/ml | 131.7 ± 2.1<br>130.6 [127.6; 135.0] | 263.8 ± 8.$^{**}$<br>260.2 [247.5; 275.2]$^{**}$ | 174.1 ± 10.0$^{*\#\#\$}$<br>181.0 [175.3; 188.9]$^{*\#\#\$}$ | 127.1 ± 3.5$^{\#\#\wedge}$<br>129.8 [119.7; 133.1]$^{\#\#\wedge}$ |
| PGI2, ng/ml | 75.0 ± 1.4<br>74.6 [71.9;77.4] | 97.2 ± 2.3$^{**}$<br>97.0 [92.3;102.3]$^{**}$ | 85.4 ± 1.3$^{**\#\#\$}$<br>85.7 [84.8;87.2]$^{**\#\#\$}$ | 72.3 ± 3.6$^{\#\#\wedge}$<br>70.3 [69.5;71.1]$^{\#\#\wedge}$ |
| TXB2, pg/ml | 49.0 ± 1.0<br>48.7 [46.8; 51.1] | 85.3 ± 2.0$^{**}$<br>87.3 [80.2; 88.7]$^{**}$ | 60.1 ± 1.1$^{**\#\#\$\$}$<br>59.8 [58.0; 61.0]$^{**\#\#\$\$}$ | 54.8 ± 1.0$^{*\#\#\wedge\wedge}$<br>54.9 [53.4; 56.4]$^{*\#\#\wedge\wedge}$ |

AGC – acute general cooling, n – number of animals in the group

$^{*}$/$^{**}$ statistically significant difference compared to the intact control group ($p < 0.05/0.01$ respectively)

$^{\#}$/$^{\#\#}$ statistically significant difference compared to the untreated control group ($p < 0.05/0.01$ respectively)

$^{\wedge}$/$^{\wedge\wedge}$ statistically significant difference compared to the group treated with diclofenac sodium ($p < 0.05/0.01$ respectively)

$^{\$}$/$^{\$\$}$ statistically significant difference compared to the group treated with etoricoxib ($p < 0.05/0.01$)

increased 2.6 times ($p < 0.005$ compared to the intact control value).

Diclofenac sodium contributed to a decrease in this indicator: it exceeded the value of intact control by 1.4 times ($p < 0.005$), which is significantly lower than in the untreated control group ($p < 0.005$). Etoricoxib contributed to the complete normalization of the content of 8-isoprostane, which was statistically significantly ($p < 0.005$) lower than in the untreated control group and in the group receiving diclofenac sodium. It indicates a decrease in oxidative stress under the influence of both NSAIDs, especially etoricoxib.

## Discussion

Changes in body temperature of rats exposed to a 2-hour temperature of – 18 °C are quite typical. In the previous studies[14, 15] on a similar model of AGC at n = 6–7, the rectal temperature in rats of the untreated control group decreased by 1.4 ± 0.3 °C and 1.8 ± 0.8 °C on average ($p < 0.05$ compared to the baseline values), diclofenac sodium enabled body temperature to remain almost stable (± 0.0 ± 0.1 °C i ± 0.0 ± 0.2 °C), on the background of etoricoxib the dispersion of the values was slightly higher: the temperature decreased by 1.0 ± 0.3 °C ($p < 0.05$ compared to the baseline value) and 0.5 ± 0.4 °C ($p > 0.05$). Thus, the results of this study confirmed the pronounced frigoprotective effect of the non-selective COX inhibitor diclofenac sodium, while the highly selective COX-2 inhibitor etoricoxib is inferior to diclofenac sodium.

The data in the literature concerning the systemic changes in inflammatory mediators under conditions of general cooling (in contrast to data on such changes in local skin injuries or hypothermia of certain organs and tissues, including under the influence of ischemia/reperfusion) are quite limited. For example, in the spleen of quails after acute cold stress, pro-inflammatory processes were activated, leading to inflammatory tissue damage with increasing levels of COX-2, PGE2-synthase, iNOS, NF-κB, TNF-α, Hsp70 mRNA[19].

At the same time, COX inhibitors have multifaceted actions, and the specificity of their realization can be expected depending on the selectivity/specificity and local microenvironment. Moreover, the local effects of different metabolites of the arachidonic cascade cold injuries are obviously different. Thus, PGF2α and TXA2 increase platelet aggregation and vasoconstriction, further exacerbating the course of ischemia[20]. PGI2 (prostacyclin) and its stable metabolite iloprost as well as PGE1, in contrast, are used in the treatment of frostbite[21, 22].

According to the results of our study, there is a dissociation of the frigoprotective activity of NSAIDs in general cooling and their effect on the cyclooxygenase pathway of arachidonic acid metabolism. The most active frigoprotector diclofenac sodium influences this inflammatory cascade to a lesser extent than etoricoxib, which is inferior to diclofenac sodium in frigoprotective activity. A similar dissociation, as already noted, also exists between the frigoprotective and anti-inflammatory action, which is significantly decreased in cold exposure[16]. It is noteworthy that, despite belonging to highly selective COX-2 inhibitors, in our model of AGC, etoricoxib significantly reduced the level of both COX-2 and COX-1. This fact is not unique: it is known that other highly selective COX-2 inhibitors (celecoxib) can also exhibit the properties of non-selective COX inhibitors, in particular in increased doses[23]. Such patterns, in particular, were observed for measurements in whole blood, in contrast to the data obtained *in vitro*, where the selectivity for COX-2 is clearly determined by the chemical structure of NSAIDs[24, 25]. In volunteers, even after taking the highly selective COX-2 inhibitor rofecoxib, a decrease in COX-1 activity (albeit a moderate one, by 7–8%) was found, measured as $TXB_2$ generation in clotting whole blood[26]. The importance of pharmacokinetic parameters determining the duration of contact of enzyme isoforms with the drug[24, 25] is also emphasized.

*Table 3. The effect of diclofenac sodium and etoricoxib on the level of 8-isoprostane in rat liver on the model of acute general cooling (air hypothermia, M ± m, Me[Q25; Q75])*

| Group | 8-isoprostane, ng/g of protein |
|---|---|
| Intact control (n = 6) | 11.67 ± 0.54<br>11.85 [11.08; 12.00] |
| Untreated control (AGC, n = 6) | 29.68 ± 2.13 ***$$$^^^<br>29.77 [28.79; 30.12] |
| AGC + diclofenac sodium (n = 6) | 16.86 ± 0.38 ***###^^^<br>16.97 [16.49; 17.08] |
| AGC + etoricoxib (n = 6) | 11.97 ± 0.55 ###$$$<br>12.14 [11.86; 12.34] |

AGC – acute general cooling, n – number of animals in the group
*** statistically significant difference compared to the intact control group ($p < 0.005$)
### statistically significant difference compared to the untreated control group ($p < 0.005$)
$$$ statistically significant difference compared to the group treated with diclofenac sodium ($p < 0.005$)
^^^ statistically significant difference compared to the group treated with etoricoxib ($p < 0.005$)

It is hardly possible to comprehensively explain this phenomenon proceeding from the results of experimental or clinical studies. There are quite a few studies within the field of the discussed problem. In particular, it has long been known that the level of TXA2 and PGI2 is elevated in wound exudate after CT[27]. At the same time, it was shown that the role of TXA2 and PGI2 in vascular disorders after CT is not significant, and similar patterns were observed after thermal injury. The ability of ibuprofen to promote vascular patency after cold and thermal injuries is associated by the authors of the cited study[27] not with the effect on PG synthesis but with the activation of fibrinolysis. When the fibrinolytic activity of wound exudate was measured with the addition of ibuprofen or indomethacin, the latter did not show the ability to activate fibrinolysis. In contrast, its activation by ibuprofen could help restore vascular patency; still, detailed mechanisms were not discussed in the articles[27, 28]. In an even earlier study in the model of local hypothermia of an extremity in rabbits, the efficacy of the NSAID oxyfenbutazone was shown. This efficacy has been associated with the impedance of the action of plasmin inhibitors. In contrast, the drug had no effect on capillary permeability proceeding from the absence of the differences in the extent of edema[29].

The effect on platelets is considered to be of high importance in overcoming the negative changes in microcirculation induced by temperature factors. Thus, in volunteers, acetylsalicylic acid in low doses and clopidogrel counteract vasodilation under high temperatures, with a certain involvement of COX--dependent and NO-dependent mechanisms[30, 31]. Moreover, in the study of genetically determined hypersensitivity of individuals of African descent to local cooling of the upper extremities, it was found that it is not caused by the differences in the cyclooxygenase pathway and is not modulated by acetylsalicylic acid at a dose of 600 mg[32].

Another target of the studied drugs can be considered among the processes of direct vascular tone regulation within the prostacyclin-thromboxane system as well as among other mechanisms. Thus, it was found that in healthy volunteers, acetylsalicylic acid at a single dose of 325 mg facilitated vasoconstriction under conditions of cold influence, reducing the effects of vasodilatory prostaglandins and increasing vascular resistance under stress conditions, which lead to a more significant increase in blood pressure[33]. At the same time, celecoxib, in contrast to diclofenac sodium and rofecoxib, contributed to the decrease in vascular tone by enhancing potassium currents and suppressing L-type calcium currents[34]. According to the opinion of the authors of the cited study, these results can explain the differential risk of cardiovascular events in patients taking NSAIDs of different selectivity/specificity. It is noteworthy to mention that the dissimilar type of influence of NSAIDs on ion channels is not interrelated with their selectivity in relation to the COX isoforms. Unfortunately, there are no data available about the etoricoxib influence on the ion channels, and just this drug has proven to be the most effective frigoprotector among highly selective COX-2 inhibitors.

It is necessary to re-emphasize that in our study, the level of PGI2 was increased significantly after AGC both in the liver and in the blood serum of animals in the untreated control group. It might be supposed that this increase reflects a compensatory mechanism aimed at microcirculation improvement. Interestingly, diclofenac sodium did not change hepatic PGI2 level and moderately decreased this value in the blood serum. At the same time, etoricoxib significantly reduced this biomarker, which may indicate excessive suppression of the compensatory response. The positive role of PGI2 in CT has been clinically proven. As already mentioned, its stable metabolite iloprost is used to treat severe frostbite in combination with alteplase and heparin[21]. Thus, the results of our studies allow considering the lesser influence of diclofenac sodium on PGI2 in CT as one of the keys to understanding its higher efficacy as a frigoprotector.

The effects of diclofenac sodium mentioned above within certain vascular beds may appear to be favourable in hypothermia, in contrast to vasodilation. Obviously, it is only a theoretically substantiated mechanism that needs further experimental confirmation, especially in the context of the lack of explicit data on etoricoxib and given the ability of the selective COX-2 inhibitors to increase the vasoconstrictor effect of norepinephrine in acute inflammation directly[35].

The distinct aspect of COX inhibitors' biological activity is the change in the formation of 15-(R)-epilipoxin A(4): it has been shown that some NSAIDs (including diclofenac sodium) also inhibit 15-hydroxyprostaglandin dehydrogenase and 15-oxoprostaglandin 13-reductase and thus decelerate the metabolism of certain eicosanoids, including 15-(R)-epilipoxin A(4). It has even been proposed to classify these NSAIDs as a separate subgroup of dual COX–EOR inhibitors[36]. Increased synthesis of epilipoxin A(4), e.g., caused by acetylsalicylic acid, is not indifferent to vascular tone, including its local changes in areas of inflammation and its involvement in systemic blood pressure modulation[37]. These data might be appealing for the explanation of the frigoprotective efficacy of diclofenac sodium, although these aspects certainly need direct verification under the conditions of CT.

Thus, there is evidence that the significant frigoprotective activity of diclofenac sodium can be caused by the beneficial effect on hemorheological parameters and vascular tone, which can be realized through the prostacyclin-thromboxane system modulation as well as other mechanisms.

It is also necessary to note the changes in the thermoregulatory centre, which can be targeted by prostanoids. It has long been known that under the influence of cold in animals (such as cats or rabbits) the changes in the synthesis of prostaglandins in the CNS do not influence thermoregulation significantly in the absence of fever[38, 39]. On the other hand, the

significance of prostaglandin $E_2$ for thermogenic responses under the influence of cold in the absence of fever is discussed concerning laboratory animals as well as humans[40]. In the context of the widespread use of NSAIDs, their possible negative effects under hypothermia are emphasized in the study[40]. Further research is needed to resolve the discrepancy between these data and the frigoprotective activity of certain NSAIDs.

Our results have demonstrated the increase in 8-isoprostane in the liver and blood serum, and this change of the biomarker, associated with the arachidonic acid cascade, indicates the significant oxidative stress in the tissues of rats of the untreated control group. It should be noted that in another immersion model of hypothermia, namely during prolonged swimming in cold water (+5 °C) the level of isoprostane in erythrocytes is also increased, and this effect is sex-dependent, being more pronounced in females than in males[41]. Nevertheless, the fact that the incomplete normalizing effect of diclofenac sodium on the content of 8-isoprostane does not counteract the prevention of hypothermia indicates that antioxidant activity is not determinant for high frigoprotective activity, especially considering that the sampling was done immediately after modelling of AGC. It is possible that such an effect is more relevant during further periods after cold injury and is expected to be important for the treatment of its consequences. Also, the modulatory influence of the drug on the adaptation processes is essential in this context. Thus, in volunteers exposed to low temperatures during whole-body cryostimulation (–130 °C for 3 min), the increase in the concentration of 8-isoprostane in the blood plasma was more pronounced after the first session, then the direction of the response changed, and the level of this biomarker decreased in response to cold exposure[42]. This has been regarded as the indication of adaptation mechanism formation. And importantly, the ability of etoricoxib to increase the non-enzymatic formation of isoprostanes, as shown *in vitro* with human LDL[43], was not seen in our model. Diclofenac did not demonstrate this effect even at high concentrations, which are hardly achievable *in vivo*[43].

Thus, there is a dissociation between the frigoprotective effect of NSAIDs and their influence on the inflammatory cyclooxygenase pathway of the arachidonic acid cascade: diclofenac sodium is more effective than etoricoxib in maintaining the body temperature in rats exposed to CT but has less effect on the values of the mentioned pathway in the liver and blood serum. A similar dissociation, as already noted, also exists between the frigoprotective and anti-inflammatory action of diclofenac sodium, which is significantly reduced in AGC[16]. Complex mechanisms could be supposed as a reason for the maximum frigoprotective efficacy of diclofenac sodium among 8 other NSAIDs with different selectivity for COX isoforms. Among these mechanisms, it is expedient to consider the antiplatelet effect inherent mainly in non-selective COX inhibitors (which can contribute to the correction of microcirculatory disorders in CT, although by frigoprotective activity, diclofenac sodium surpasses acetylsalicylic acid – a commonly known antiplatelet drug[12, 13], COX-dependent and independent mechanisms of vascular tone regulation, the influence on the lipoxygenase pathway of the arachidonic acid cascade, on the central mechanisms of thermoregulation, as well as on the state of energy metabolism in skeletal muscles and internal organs. The involvement of specific pharmacological properties of certain NSAIDs, in particular diclofenac sodium, into their frigoprotective activity needs clarification and will be the subject of our further research.

## Conclusions

In the model of acute general cooling in rats (exposure at –18 °C for 2 hours), non-selective COX inhibitor diclofenac sodium (7 mg/kg, administered intragastrically 30 minutes before cold injury modeling) effectively prevents hypothermia, surpassing highly selective COX-2 inhibitor etoricoxib by frigoprotective activity. After cold exposure in the liver of rats of the untreated control group, there is a moderate elevation in COX-1 level and a highly significant one – in COX-2 level, the concentration of PGE2 is reduced, while the content of prostaglandins of other classes, such as PGF2α and especially PGI2 and TXB2 is increased. In the blood serum, COX-1 level is, in contrast, decreased, and changes in COX-2 and prostaglandins concentrations are similar to those in the liver. Under the conditions of cold injury in rats, diclofenac sodium has a moderate normalizing effect on the level of both COX isoforms in the liver, moderately increases the content of PGE2 and reduces those of PGF2α and TXB2, does not change the elevated level of PGI2. In the blood serum, diclofenac sodium reduces COX-1 level to subnormal values, and the effect on other biomarkers is similar to that in the liver except for a moderate decrease in PGI2. Considering the effect on the cyclooxygenase pathway, diclofenac sodium is inferior to etoricoxib, which normalizes COX-1, COX-2, PGE2, and PGI2 in the liver, significantly reduces the content of PGF2α and TXB2 in the liver to subnormal values. At the same time, in the blood serum, such changes are seen as the decrease in COX-1, COX-2, and PGE2 to subnormal values, normalization of PGF2α and PGI2, s significant reduction (but not a complete normalization) of TXB2 content. Regardless of belonging to highly selective COX-2 inhibitors, etoricoxib in the cold injury model reduces the level of both COX-1 and COX-2. A comparison of the intensity of the studied NSAIDs' influence on the pro-inflammatory cyclooxygenase pathway and body temperature in animals that underwent acute general cooling (air hypothermia) indicates a dissociation between their anti-inflammatory and frigoprotective action. This substantiates the expediency of further elucidation of the NSAID's antihypothermic effect mechanisms. The content of 8-isoprostane in the liver of rats of the untreated control

group is significantly increased after cold exposure. Diclofenac sodium moderately reduces this biomarker of oxidative stress, and etoricoxib completely normalizes it. The identified antioxidant effect of both NSAIDs is not determinative for frigoprotective activity since diclofenac, despite the weaker antioxidant effect, still exerts the maximum antihypotermic activity.

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