EFFECTS OF NON-STEROIDAL ANTI-INFLAMMATORY AGENTS ON SYSTEMIC HEMOSTASIS DURING THE MOST ACUTE PERIOD OF COLD INJURY IN RATS

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Non-steroidal anti-inflammatory drugs (NSAIDs) have recently been considered promising agents for the prevention and treatment of cold injuries. The results of previous studies demonstrate a distinct frigoprotective effect of diclofenac sodium and etoricoxib.

The aim of the study: *to assess the impact of diclofenac sodium and etoricoxib, as the most effective frigoprotectors among NSAIDs, on coagulation indicators during the most acute phase of cold injuries using an acute model of general cooling in rats.*

Materials and Methods: *The experiment was carried out using 41 outbreed male rats weighing 310±10 g. Cold injury was induced by acute general cooling (exposure to –18 °C for 2 hours). Diclofenac sodium (7 mg/kg) and etoricoxib (5 mg/kg) were administered intragastrically 30 minutes before the onset of cold exposure. Rectal temperature was measured before and after cold exposure. Immediately after exposure, plasma was used to determine prothrombin time (PT), thrombin time (TT), activated partial thromboplastin time (APTT), fibrinogen levels, and in blood serum – the residual amount of prothrombin, thrombin, fibrinogen, as well as D-dimer using species-specific immunoenzymatic analysis kits.*

Results*: Etoricoxib and especially diclofenac sodium significantly reduced the degree of hypothermia (rectal temperature decreased by 1.3 % and 1.9 %, respectively, compared to a 5.4 % decrease in the control group, p<0.05). In the acute phase of cold injury in the untreated control group, there was a significant increase in D-dimer (by 2.7 times) and fibrinogen content (by 1.9 times) in blood serum, alongside a 21.7 % increase in thrombin time, indicating a heightened risk of thrombus formation and DIC syndrome development. The other coagulation indicators did not show significant changes. Both diclofenac sodium and etoricoxib significantly reduced elevated D-dimer and serum fibrinogen, normalizing thrombin time and indicating an antithrombotic effect. There was no significant difference in the effect of both NSAIDs on blood coagulation status.*

Conclusions: *The acute phase of cold injury demonstrates a dangerous shift in blood coagulation towards thrombus formation and DIC syndrome development. Prophylactic use of diclofenac sodium and, to a lesser extent, etoricoxib displays an anti-hypothermic effect, reducing the risk of thrombosis and DIC syndrome. This proves the expediency of using these NSAIDs for acute cold injury*

Keywords: *acute general cooling, diclofenac sodium, etoricoxib, blood coagulation, experiment*

1. Introduction

Acute cold injury (CI), including general hypothermia and frostbite, remains a relevant medical and social issue. Despite some progress in its prevention and treatment, many countries still report a significant number of acute cold-related incidents, often resulting in severe health disturbances and even death. For instance, in 2019 in the United States, among individuals aged 15 and older, between 0.2 to 8.6 fatal cases per 100,000 population due to hypothermia were registered, particularly in rural areas and among older individuals [1]. Over a 4-week period from December 2018 to January 2019, 1037 individuals sought medical assistance for CI in Ukraine, out of which 955 (92 %) were hospitalized [2]. High-risk groups for CI include military personnel, athletes, and homeless individuals. In 2019, the incidence

rate of CI reached 36.5 cases per 100,000 U.S. military personnel [3]. Among climbers, there are 366 cases of frostbite per 1000 individuals, while among skiers, the incidence of injuries reaches 20 % [4]. These figures, evidently, only partially characterize the frequency of CI, as a significant portion of cases, especially those that are not severe, may not be included in official statistics. In present-day Ukraine, CI is particularly relevant due to ongoing military operations during the cold season.

In this context, the search for effective frigoprotectors – medications that protect the body from the harmful effects of low temperatures – has significant importance. Considering the role of inflammation in the pathogenesis of CI [5], inhibitors of the arachidonic acid cascade emerge as promising frigoprotectors. Several of our research studies have been dedicated to this field [6–12]. The results from

these experiments compellingly demonstrate that among the 11 studied cyclooxygenase (COX) inhibitors, diclofenac sodium and etoricoxib prove to be the superior frigoprotectors during acute general cooling. The degree of the protective effect, evaluated by the criterion of preventing body temperature decrease, does not seem to depend on the selectivity of these medications towards COX isoforms. These mentioned medications improve cognitive functions, reduce the manifestations of cold stress, enhance the functional state of the heart and kidneys, and promote the activation of energy metabolism. At the same time, while their frigoprotective and anti-inflammatory actions are closely interlinked, they dissociate across a significant number of markers. In the case of CI, diclofenac sodium exerts a moderate influence on the levels of both COX isoforms in the liver of rats, tending towards normalization. It moderately increases the content of PGE2 and reduces PGF2α and TXB2 without affecting the elevated level of PGI2. In serum, it contributes to a decrease in the content of COX-1 to subnormal levels, with an impact on other biomarkers resembling that in the liver, except for a moderate decrease in PGI2. Regarding its impact on the cyclooxygenase pathway, diclofenac sodium is inferior to etoricoxib. In the liver, etoricoxib normalizes COX-1, COX-2, PGE2, PGI2 levels, significantly reducing PGF2α and TXB2 content to subnormal values. In serum, it decreases COX-1, COX-2, and PGE2 to subnormal levels, normalizes PGF2α and PGI2, and markedly reduces but does not normalize the content of TXB2 [12]. Additionally, on a CI model in mice, the anti-inflammatory effect of diclofenac is significantly weaker in a model of carrageenan-induced edema compared to normal conditions. However, this medication markedly reduces the degree of hypothermia [8]. Diclofenac sodium significantly reduces lactate acidosis, whereas etoricoxib alleviates lactate acidosis during CI [11].

These facts suggest that the frigoprotective action of NSAIDs may be associated not only with an impact on the synthesis of inflammatory mediators but also with other mechanisms that require further investigation.

Thromboses have been observed during CI [5, 13]. Coagulation dysfunction, along with hypothermia and acidosis, constitute the 'triad of death' in CI [14]. Most NSAIDs possess anti-thrombotic properties, suggesting that the mechanisms behind their frigoprotective effects might be partially associated with their positive influence on blood rheology. Such a link in the mechanism of frigoprotective effect has been established for glucosamine hydrochloride, which inhibits both platelet aggregation and the blood coagulation cascade [13, 15]. Regarding NSAIDs, recommendations exist for using ibuprofen to alleviate rheological disturbances in CI [16]. However, this drug is inferior to diclofenac sodium and etoricoxib in terms of integral frigoprotective activity [6]. Given the vital importance of rheological disruptions in CI, a direct comparison of NSAIDs that offer maximum protection against hypothermia in their impact on blood coagulation would be advisable.

The aim of the study: to investigate the effect of diclofenac sodium and etoricoxib as the most effective frigoprotectors among NSAIDs on blood coagulation in-

dicators in the most acute period of CI on a model of acute general cooling in rats.

2. Research Design (Methodology)

Based on the results of previous experiments discussed in detail in the introduction, it is worthwhile to investigate the impact of NSAIDs on the state of the coagulation system during acute general cooling. For this purpose, two drugs were selected: diclofenac sodium – a non-selective COX inhibitor, and etoricoxib – a highly selective COX-2 inhibitor. These drugs are leaders in frigoprotective activity screening by increasing the lifespan of mice in the CI model. In-depth studies ensure minimal temperature reduction in rats during cold exposure, especially with diclofenac. Moreover, they improve cognitive functions (especially diclofenac), reduce inflammatory response and lactate acidosis (especially etoricoxib), and exert cardio- (especially diclofenac) and nephroprotective, stress-protective effects. The research was conducted on rats using the model of aerial hypothermia, representing acute general cooling [17].

Stages of the study:

1. Analysis of publications regarding the state of hemostasis and its correction in cases of hypothermia.

2. Modelling hypothermia, monitoring body temperature dynamics.

3. Blood samples were collected from animals after the completion of cold exposure.

4. Determining the parameters of the blood coagulation system (prothrombin time, thrombin time, APTT, prothrombin, thrombin, fibrinogen, D-dimer).

5. Processing and analysis of the obtained results.

6. Identification of promising areas for further research.

3. Materials and methods

The work was carried out in the Educational and Scientific Institute of Applied Pharmacy of the National University of Pharmacy in accordance with Directive 2010/63/EU of the European Parliament and of the Council of the EU "On the protection of animals used for scientific purposes" [18]. The protocols of the experiments were approved by the bioethics committee of the National University of Pharmacy (Protocol No. 5, 25.03.2021).

41 adult male outbred white rats, aged 1 year with a weight of 310±10 g, were used in the study. The selection of male rats was based on the fact that during cold-induced injuries, the shift of coagulation parameters toward thrombosis is more pronounced in males compared to females [13]. The animals were housed in standard polypropylene cages at a temperature of 20–26 °C and relative humidity of 50 % in properly ventilated rooms with a 12-hour day/night cycle and free access to food and water.

The following drugs and substances were used in the study: diclofenac sodium (Voltaren® tablets, Novartis, Switzerland), etoricoxib (Arcoxia®, tablets, Merck Sharp&Dohme Idea Inc, USA). Rats were randomly divided into 4 groups: the intact control group (*n*=9), the untreated control group (*n*=8), the groups of animals that received intragastrically (i.g.) diclofenac sodium at a dose of 7 mg/kg (*n*=10) etoricoxib at a dose of 5 mg/kg (*n*=14).

These doses have been found as the most effective in increasing the life expectancy of animals in acute hypothermia [6, 7]. The drugs were ground and suspended with the addition of Tween-80, administered through an intragastric probe in a volume of 0.5 ml per 100 g of rat body weight 30 minutes before cold trauma exposure. Animals of the intact control and the untreated control groups received i.g. drinking water in an equivalent volume.

The model chosen for CI is air hypothermia, which represents acute general cooling [17]. The animals were placed in separate transparent plastic containers (5000 cm^3) without the restriction of motor activity and air access. Containers with rats underwent exposure at –18 °C for 2 hours in the freezer "Nord Inter-300." The rectal temperature was measured 5 minutes before and 5 minutes after cold exposure using a medical digital thermometer, specifically the Gamma Thermo Base model.

After the final temperature measurement, the animals were anesthetized with sodium thiopental (40 mg/kg intraperitoneally). After 5–10 minutes, they were decapitated, and blood samples were collected to obtain plasma and serum, where the parameters of the coagulation system were determined. In the plasma, prothrombin time (PT), thrombin time (TT), activated partial thromboplastin time (APTT), as well as fibrinogen were measured using BioSystem S.A. kits (Spain). In the serum, the residual amount of rat prothrombin, thrombin, fibrinogen, and D-dimer were determined by immunoenzymatic analysis using species-specific kits: Rat Prothrombin ELISA Kit, Rat TM (Thrombin) ELISA Kit, Rat FG (Fibrinogen) ELISA Kit, Rat D-Dimer ELISA Kit (MyBioSource, USA) on the LAB Analyt M201 Microplate Reader. It should be noted that the study of coagulation factors in serum has attracted attention for more than 60 years [19].

During coagulation, fibrinogen is converted into fibrin, and typically, serum is considered free of fibrinogen. However, we supplemented routine fibrinogen determination in plasma with a special methodological approach – measuring it in serum to characterize possible residual amounts and comparing it with the content of D-dimer.

Statistical processing of the results was done using the program "Statistica 10.0". The hypothesis of normality was rejected according to the Shapiro-Wilk test as well as asymmetry and excess coefficients values. The central tendencies of independent samples were compared using the Kruskal-Wallis test and the Mann-Whitney U test. The differences in dynamics within the individual groups were assessed by the paired samples Wilcoxon test. The differences were considered statistically significant at $p<0.05$. Quantitative data were presented as arithmetic means with standard errors of the mean ($M\pm m$), medians with 25 % and 75 % percentiles (Me [Q25; Q75]).

4. Results

As seen in Table 1, the initial body temperature in all groups of rats had simi-lar values, with average values ranging from 36.78 to 37.18 °C. After a 2-hour exposure at –18 °C, the temperature in the untreated control group of rats decreased on average by 2.01 °C (5.4 %), $p<0.001$ compared to the initial temperature. On the background of etoricoxib, the degree of hypothermia was significantly lower (0.69 °C, or 1.9 %, $p<0.05$). The smallest temperature reduction was observed in the diclofenac sodium group, just 0.48 °C (1.3 %, $p<0.05$).

There were changes in certain coagulation parameters (Table 2). In the untreated control group, the thrombin time (TT) significantly increased (on average by 21.7 %, $p<0.001$ compared to intact control), while under the influence of NSAIDs, it increased only as a trend by 2.9 % (diclofenac) and 8 % (etoricoxib).

These values were significantly inferior to the pathology control group. The other plasma parameters (prothrombin time, activated partial thromboplastin time, fibrinogen) remained virtually unchanged. It is worth noting only a statistically non-significant increase in fibrinogen content by 11.4–14.5 % in all animals subjected to cold exposure.

However, there is a significant (2.7 times) increase in the D-dimer content in the serum of the untreated rats ($p<0.001$). On the background of both NSAIDs, this increase was significantly lower (1.8 times). In the diclofenac group, the D-dimer content was significantly higher than in the intact animals but lower compared to the control pathology ($p<0.05$). On the background of etoricoxib, due to the higher dispersion of significant differences, this indicator did not show noticeable changes. The level of serum fibrinogen was significantly lower than plasma fibrinogen (ng/ml and g/l, respectively) and underwent similar changes.

It was highest in the untreated control group (almost 2 times higher than in the intact control, $p<0.01$), while in the diclofenac and etoricoxib groups, the increase was on average 1.5 times ($p<0.05$ and $p<0.01$, respectively). The residual amount of prothrombin increased in all groups, especially in the untreated control group, where this biomarker exceeded the intact rats' indicator by 2.4 times ($p>0.05$), while in other groups, the increase was less pronounced. The level of thrombin remained almost unchanged.

Table 1

The impact of diclofenac sodium and etoricoxib on the rectal temperature (°C) of rats in a model of acute general air hypothermia ($M\pm m$, Me[Q25; Q75])

Group, number of animals	Body temperature, °C		
	Initial temperature	After cold influence	Difference
Intact control, ($n=9$)	36.78±0.14 36.7 [36.6; 37.0]	–	–
Acute general cooling			
Untreated control ($n=8$)	37.18±0.12 37.1 [37.0; 37.4]	35.16±0.47$^{\&\&\&}$ 35.4 [35.0; 36.0]	–2.01±0.46 –1.7 [–1.93; –1.3]
Diclofenac sodium ($n=10$)	36.82±0.16 36.85 [36.5; 37.1]	36.34±0.28$^{\&}$ 36.55 [35.9; 37.0]	–0.48±0.23$^{\#\#}$ –0.3 [–0.8; –0.1]
Etoricoxib ($n=14$)	37.06±0.17 37.1 [36.7; 37.6]	36.45±0.31$^{\&}$ 36.75 [36.0; 37.1]	–0.69±0.23$^{\#}$ –0.4 [–1.0; –0.2]

Note: statistically significant differences: compared to the untreated control group values: $^{\#}$– $p<0.05$, $^{\#\#}$ – $p<0.01$ (Mann-Whitney criterion); compared to the initial indicator of the respective group values: $^{\&}$ – $p<0.05$, $^{\&\&\&}$ – $p<0.001$ (Wilcoxon criterion)

Table 2

Effect of sodium diclofenac and etoricoxib on coagulation parameters in rats with cold injury (*M*±*m*, Me[Q25; Q75])

Biomarkers	Intact control (*n*=6)	Acute general cooling		
		Untreated control (*n*=6)	Diclofenac sodium (*n*=6)	Etoricoxib (*n*=6)
Blood plasma				
PT, s	18.10±0.82 18.0 [16.9; 19.6]	17.62±0.59 17.7 [17.1; 18.0]	19.83±1.05 19.4 [18.6; 21.1]	17.38±0.78 17.4 [16.9; 18.4]
TT, s	44.98±1.78 44.9 [43.4; 45.5]	54.73±1.09*** 54.2 [53.6; 55.4]	46.28±3.17# 45.3 [40.7; 49.7]	48.58±1.07## 49.6 [48.0; 50.1]
APTT, s	21.05±0.96 21.2 [19.2; 22.1]	21.88±1.52 23.0 [19.0; 24.3]	23.42±1.10 23.3 [21.5; 25.5]	21.58±1.61 21.1 [19.3; 22.1]
Fibrinogen, g/l	4.82±0.15 4.9 [4.8; 5.0]	5.50±0.50 5.6 [5.2; 6.3]	5.52±0.39 5.3 [4.9; 6.3]	5.37±0.54 5.1 [5.1; 6.3]
Blood serum				
Biomarkers	Intact control (*n*=9)	Untreated control (*n*=6)	Diclofenac sodium (*n*=6)	Etoricoxib (*n*=9)
Prothrombin, ng/ml	119.0±29.2 125.2 [69.8; 141.3]	281.2±74.1 228.9 [176.4; 290.2]	246.2±79.6 206.7 [146.2; 293.3]	228.8±52.4 217.8 [121.7; 239.9]
Thrombin, ng/ml	38.69±1.44 39.6 [36.2; 41.3]	41.95±2.67 41.3 [38.1; 45.2]	42.70±3.47 41.3 [39.0; 43.0]	38.51±1.55 39.6 [36.2; 41.3]
Fibrinogen, ng/ml	159.9±17.5 159.9 [152.5; 185.2]	306.8±34.4** 325.5 [244.6; 361.6]	237.1±29.2* 239.2 [185.3; 267.4]	248.2±15.3**## 244.8 [223.3; 266.8]
D-dimer, ng/ml	905.3±98.9 786.5 [713.8; 1110.0]	2454.0±250.3*** 2412.0 [2142.5; 2652.3]	1660.0±293.6*# 1462.0 [1229.0; 2193.0]	1635.6±386.2 1161.0 [801.8; 2142.0]

Note: * – *p*<0.05, ** – *p*<0.01, *** – *p*<0.001 compared to the intact control group values; # – *p*<0.05, ## – *p*<0.01 compared to the untreated control group values; n – number of animals in the experiment

5. Discussion

The body temperature of the rats exposed to cold under the influence of both investigated NSAIDs significantly decreased to a lesser extent compared to the untreated animals, especially under the influence of diclofenac sodium. The minimal degree of hypothermia observed with this non-selective COX inhibitor is consistently noted in all other hypothermia studies [9–12], confirming its most pronounced frigoprotective effect. Etoricoxib slightly trails behind diclofenac in this aspect. The shifts in blood coagulation during hypothermia require special discussion. Under the influence of stress-realizing factors, the adhesion-aggregation properties of platelets increase, and the release of platelet-derived chemical mediators, which potentiate cellular aggregation and blood protein clotting, is stimulated. Hypothermia leads to disturbances in the blood clotting and fibrinolysis system, promoting thrombosis [20]. Tissue freezing and reperfusion contribute to tissue damage, crystallization of the extracellular space, leading to increased oncotic pressure, leakage of fluid from cells causing dehydration, cellular membrane instability, vasoconstriction, increased blood viscosity, and subsequent microvascular damage, all culminating in an inflammatory response [5]. This cascade of events concludes with thrombosis, tissue ischemia, swelling, and endothelial damage.

The results of our study confirm these findings and indicate that in the acute phase of modelled hypothermia in the untreated control group of rats, thrombosis occurs, leading to acute disseminated intravascular coagulation (DIC). This is directly indicated by the significant (almost three-fold) increase in the blood level of D-dimer – an important thrombosis marker [21], associated with the observed increase in prothrombin time (PT) seen in the untreated control group. PT characterizes the transformation of fibrinogen into fibrin—the final stage of the general blood clotting pathway. The increase of this indicator in animals with hypothermia might be linked to the presence of fibrin degradation products in the blood, indicated by the high D-dimer content. The rise in PT may suggest a swift transition to the hypocoagulation stage of DIC. The absence of significant changes in activated partial thromboplastin time (APTT) and PT, which are usually observed in DIC, could be related to the dynamic nature of changes in the blood coagulation system's state. The slightly (trend-wise) elevated plasma fibrinogen level by 14 % might be associated with the relatively short duration of exposure to low temperatures, during which a pronounced increase in this component of the blood coagulation system does not have enough time to occur. Rapid development of DIC in hypothermia has been observed in experimental [13, 22] and clinical studies [14, 23]. The results of our study, which differ in that the blood coagulation status was evaluated during the acute phase of hypothermia, support these findings.

Fibrinogen is typically measured in plasma. Assessing fibrinogen in serum might initially seem unnecessary since it is converted to fibrin during clot formation. However, fibrinogen is present in serum, although its levels are minimal, typically measured in ng/dl. Alongside the determination of D-dimer, this marker gains particular importance as it could be interpreted as a product of fibrin degradation. In this case, its increase further demonstrates thrombosis activation. Studies have shown that an increase in serum fibrinogen is associated with the development of arterial thrombosis [24]. Therefore, the nearly twofold increase observed in our experiment in the serum fibrinogen levels of the untreated rats may have unfavourable prognostic significance, indicating a risk of thrombotic complications in the most acute phase of a CI model.

Both sodium diclofenac and etoricoxib significantly mitigated the adverse shift in the blood coagulation state during the most acute phase of hypothermia. Under the influence of each NSAID, the prothrombin time (PT) normalized, and there were no changes observed in thrombin time (TT), activated partial thromboplastin time (APTT), or plasma fibrinogen. Nearly identical reductions in D-dimer content by 32–33 % compared to the untreated control group and fibrinogen by 19–23 % indicate a reduction in the risk of thrombosis and disseminated intravascular coagulation (DIC) during acute hypothermia, starting from its most acute phase.

The nearly equivalent antithrombotic efficacy between the non-selective COX inhibitor sodium diclofenac and the highly selective COX-2 inhibitor etoricoxib fails to explain the slightly higher frigoprotective (antihypothermic) efficacy of diclofenac in terms of greater inhibition of thrombus formation. It appears that a combination of mechanisms, each with varying intensity in each drug, plays a role. However, considering the overall criteria demonstrated (reduction in hypothermia, alleviation of cold-induced stress responses and inflammation, changes in energy metabolism, condition of internal organs – especially the heart – and cognitive impairments, as well as the antithrombotic effect), both sodium diclofenac and etoricoxib could be recommended for the prevention and treatment of hypothermia.

Study limitation. The blood coagulation status under the influence of NSAIDs with different selectivity for COX has been investigated only in the most acute stage of hypothermia.

Further research prospects. Investigating the comparative impact of diclofenac sodium and etoricoxib on blood coagulation indicators during the dynamics of hypothermia.

6. Conclusions

1. Etoricoxib (5 mg/kg) and especially diclofenac sodium (7 mg/kg) significantly reduce the degree of hypothermia in the acute period of cold injury induced by a 2-hour exposure of rats at –18 °C.

2. Immediately after cold exposure, there is a significant increase in D-dimer and fibrinogen levels in the serum of the untreated rats against the background of prolonged thrombin time, indicating an increased risk of thrombus formation and the development of DIC syndrome.

3. Both diclofenac sodium and etoricoxib markedly decrease the levels of D-dimer and serum fibrinogen, normalize the thrombin time, indicating an antithrombotic effect in the acute phase of cold injury.

Conflict of interest

The authors declare that they have no conflict of interest in relation to this research, whether financial, personal, authorship or otherwise, that could affect the research and its results presented in this article.

Data availability
Data will be made available on reasonable request.

Use of artificial intelligence
The authors confirm that they did not use artificial intelligence technologies when creating the currrent work.

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