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THE FRIGOPROTECTIVE EFFECTS OF ETORICOXIB AND DICLOFENAC SODIUM IN A MODEL OF ACUTE GENERAL COOLING IN RATS: THE ROLE OF LEUKOTRIENES, INTERLEUKINS, AND NITRIC OXIDE SYNTHASE

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The aim: *The aim of the study was to evaluate the role of leukotrienes, interleukins, and nitric oxide synthase in frigoprotective effect of etoricoxib and diclofenac sodium in a model of acute general cooling in rats.*
Material and methods: *Acute general cooling was induced by exposing rats to −18°C for 2 hours without animal mobility restriction. 30 min before cold exposure animals were treated with etoricoxib (5 mg/kg) or diclofenac sodium (7 mg/kg). Body temperature was measured before and after acute general cooling modeling. In rat liver the following parameters were determined: 5-lipoxygenase (5-LOX), leukotriene B4 (LTB4), total leukotrienes (LTs), interleukins (IL-1β, IL-4, IL-6, IL-10), tumor necrosis factor-α (TNF-α), and nitric oxide synthase (NOS).*
Results*: activation of the lipoxygenase pathway of arachidonic acid metabolism was characterized by a significant increase in leukotriene levels (total and leukotriene B4) without substantial changes in proinflammatory cytokines (IL-1β, IL-6, TNF-α) but with a significant decrease in anti-inflammatory cytokines (IL-4, IL-10) in the liver. Etoricoxib and diclofenac sodium similarly reduced the severity of hypothermia, prevented the increase in leukotriene levels without affecting 5-LOX content. Etoricoxib and particularly diclofenac sodium significantly reduced IL-1β levels without substantial changes in the other cytokines. Both studied medicines restored NOS levels to those observed in the intact control group.*
Conclusions*: obtained results experimentally substantiate the possibility of diclofenac sodium and etoricoxib to decrease the severity of acute general cooling, while also demonstrating certain differences in the mechanisms underlying their frigoprotective effects*
Keywords*: frigoprotectors, acute general cooling, leukotrienes, interleukins, nitric oxide synthase, etoricoxib, diclofenac sodium*

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1. Introduction

Cold injury remains a significant medical and social problem requiring timely diagnosis and a systematic therapeutic approach [1–3]. In 2021, exposure to extreme temperatures resulted in 3.4 million cases of illness worldwide, including 36,000 deaths (0.4 fatalities per 100,000 population). In many regions, cold exposure accounts for a greater health burden than heat-related injury [4]. For example, there are approximately 1500 patients in the United States who have hypothermia noted on their death certificates [5, 6]. Moreover, between 2017 and 2022, the number of deaths associated with cold injuries more than doubled compared with 1999 levels [7]. In several European countries, the annual incidence of reported hypothermia cases ranges from 0.13 to 6.9 per 100,000 population [8]. Cold exposure also increases mortality risk during emergency situations [9, 10].

Cold injury is of particular relevance in Ukraine in the context of ongoing large-scale military conflict, as millions of individuals are deprived of adequate heating during winter due to damage to energy infrastructure and heating systems. According to the International Rescue Committee (IRC), up to 60% of households in Ukraine experience insufficient heating, and one in five individuals suffers from cold-related health problems [11].

The treatment of cold injury remains challenging [12, 13], underscoring the importance of identifying and investigating frigoprotective agents capable of providing comprehensive protection under low-temperature conditions. Inhibitors of the arachidonic acid cascade appear to be promising candidates, as inflammation plays a central role in the pathogenesis of cold injury [14]. Previous studies evaluating several nonsteroidal anti-inflammatory drugs (NSAIDs) have demonstrated that the most pronounced frigoprotective effects, in terms of attenuation of hypothermia severity, are observed with the non-selective cyclooxygenase (COX) inhibitor diclofenac sodium and the highly selective COX-2 inhibitor etoricoxib [15, 16]. Further investigations have revealed their modulatory effects on the cold stress response, as well as on renal, hepatic, and cardiac function [17], hemostasis [18], prostaglandin and energy metabolism [19], and cognitive function [20].

The mechanisms underlying the frigoprotective action of these agents are likely multifactorial. In particular, the potential influence of frigoprotectors on the lip-

oxygenase pathway of arachidonic acid metabolism, whose products, leukotrienes, are key mediators of the inflammatory cascade, warrants further clarification. In addition, the possible modulation of pro- and anti-inflammatory interleukins requires investigation. Given the importance of vascular responses in the pathogenesis of cold injury, assessment of nitric oxide synthase (NOS) activity is also of considerable interest.

Accordingly, the aim of the present study was to conduct an in-depth investigation of the roles of leukotrienes, cytokines, and nitric oxide synthase in the mechanisms underlying the frigoprotective effects of etoricoxib and diclofenac sodium in a rat model of acute general cooling.

2. Planning (methodology) of research

Based on the results discussed in the introduction, two non-steroidal anti-inflammatory drugs, etoricoxib and diclofenac sodium, were chosen for further in-depth investigation of mechanism underlaying their frigoprotective action on the model of acute general cooling in rats. The first stage was aimed to evaluate antihypothermic action of studied drugs by means of thermometric data. In the second stage of in-depth study, the investigation etoricoxib and diclofenac sodium on hepatic levels of 5-lipoxygenase, leukotrienes, interleukins, and nitric oxide synthase in rats with acute general cooling was carried out. The second stage involved evaluation of the quantitative balance between pro- and anti-inflammatory cytokines. This allowed to insight the role of the role of leukotrienes, interleukins, and nitric oxide synthase in the frigoprotective effect of etoricoxib and diclofenac sodium.

3. Materials and methods

3. 1. Animals

The study was conducted at the Educational and Scientific Institute of Applied Pharmacy, National University of Pharmacy (Kharkiv, Ukraine) during the period 2022–2023, in accordance with Directive 2010/63/EU of the European Parliament and of the Council on the protection of animals used for scientific purposes. The experimental protocol was reviewed and approved by the Bioethics Committee of the National University of Pharmacy (Protocol No. 5, March 25, 2021). The experiments were carried out on 30 adult male albino rats weighing 300–350 g. Male animals were used due to their greater sensitivity to acute cold exposure and the more pronounced response to frigoprotective agents compared with females [21]. The animals were housed under standard vivarium conditions with free access to food and water, controlled humidity, a 12:12 h light/dark cycle, and an ambient temperature of 22–23°C.

3. 2. Drugs and chemicals

Diclofenac sodium (Voltaren®, tablets; Novartis, Switzerland) and etoricoxib (Arcoxia®, tablets; Merck Sharp & Dohme Idea Inc., USA) were used. The doses were selected based on previous studies demonstrating maximal efficacy in a model of acute hypothermia [18, 19].

Animals were randomly assigned to the following experimental groups:

1. Control pathology group (n = 6): 30 min before cold exposure, rats received the vehicle (distilled water, 2 mL/kg) intragastrically (i.g.) via gastric gavage.

2. Etoricoxib group (n = 9): rats were administered etoricoxib (5 mg/kg, i.g.) as an aqueous suspension containing Tween-80, 30 min prior to cold exposure.

3. Diclofenac sodium group (n = 6): rats received diclofenac sodium (7 mg/kg, i.g.) as an aqueous suspension containing Tween-80 (2 mL/kg), 30 min before cooling.

4. Intact control group (n = 9): animals not subjected to cold exposure received distilled water (2 mL/kg, i.g.).

3. 3. Acute general cooling model and body temperature control

Acute general cooling was induced by exposing rats to −18°C for 2 hours in individual plastic chambers with a volume of 5,000 cm^3 that did not restrict animal mobility [18, 22]. Rectal temperature was measured immediately before and after cold exposure using a Gamma Thermo Base digital thermometer.

3. 4. Inflammatory markers

Five to ten minutes after completion of cold exposure and rectal temperature measurement, animals were euthanized under general anesthesia induced by thiopental sodium (40 mg/kg). The liver was excised, immediately frozen in liquid nitrogen, and stored at –70°C until further analysis.

Inflammatory markers were quantified in liver homogenates using enzyme-linked immunosorbent assay (ELISA). The following parameters were determined: 5-lipoxygenase (5-LOX), leukotriene B4 (LTB4), total leukotrienes (LTs), interleukins (IL-1β, IL-4, IL-6, IL-10), tumor necrosis factor-α (TNF-α), and nitric oxide synthase (NOS). Species-specific ELISA kits were used, including Rat 5-lipoxygenase (5-LO) ELISA Kit, Rat Leucotriene B4 (LTB4) ELISA Kit (Competitive ELISA), Rat Total Leucotriene (LT) ELISA Kit (Competitive ELISA), Rat Interleukin 1 Beta (IL1b) ELISA Kit, Rat Interleukin 4 (IL4) ELISA Kit, High Sensitivity Rat Interleukin 6 (IL6) ELISA Kit, Rat Interleukin 10 (IL10) ELISA Kit, Rat TNF alpha PicoKine ELISA Kit, Rat Nitric Oxide Synthase (NOS) PicoKine ELISA Kit (MyBioSource, USA).

3. 5. Statistical analysis

Statistical analysis was performed using ANOVA Test software. Data distribution was assessed using the Shapiro-Wilk test as well as skewness and kurtosis coefficients. As the assumption of normality was not met, differences between groups were analyzed using the Kruskal–Wallis test followed by post hoc pairwise comparisons using the Mann–Whitney test. Differences were considered statistically significant at $p < 0.05$. Quantitative data were presented as an arithmetic mean with standard error of the mean ($M \pm m$), medians with 25% and 75% percentiles (Me [Q25; Q75]).

4. Research results

4. 1. Antihypothermic action of diclofenac sodium and etoricoxib after acute general cooling

The thermometric data demonstrate the frigoprotective effects of etoricoxib and diclofenac sodium (Table 1). Baseline rectal temperature in all experimental groups corresponded to physiological norms and did not differ significantly between groups.

In the control pathology group, rectal temperature decreased significantly by an average of 2.1°C after 2 hours of cold exposure compared with baseline values ($p < 0.01$).

In contrast, pretreatment with etoricoxib or diclofenac sodium significantly attenuated the severity of hypothermia. In these groups, body temperature decreased by only 0.47°C ($p < 0.05$) and 0.45°C ($p < 0.05$), respectively, compared with the control pathology group.

Table 1

Effects of etoricoxib and diclofenac sodium on rectal temperature in rats in a model of acute general cooling ($M \pm m$; Me [Q25; Q75])

Group	Rectal temperature, °C		
	before	after cold exposure (2 hours at –18°C)	difference
Intact control (n = 9)	36.8 ± 0.32 36.7 [36.6; 37.1]		
cold exposure (acute general cooling)			
Control pathology (n = 6)	37.1 ± 0.46 37.1 [36.9; 37.4]	34.9 ± 1.53$^{\&\&}$ 35.05 [34.85; 35.8]	−2.1 ± 1.68 −1.5 [−2.2; −1.1]
Etoricoxib (n = 9)	37.0 ± 0.61 36.9 [36.7; 37.6]	36.5 ± 0.79 36.8 [36.6; 37.0]	−0.47 ± 0.58$^{\#}$ −0.4 [−0.75; −0.2]
Diclofenac sodium group (n = 6)	36.8 ± 0.61 36.9 [36.3; 37.25]	36.4 ± 0.89 36.4 [35.85; 37.1]	−0.45 ± 0.42$^{\#}$ −0.3 [−0.7; 0.1]

Note: statistically significant differences: $^{\#}$ – $p < 0.05$ vs. control pathology group; $^{\&}$ – $p < 0.05$; $^{\&\&}$ – $p < 0.01$; $^{\&\&\&}$ $p < 0.001$ vs. baseline values of the same group; n – number of animals per group.

4. 2. Influence of diclofenac sodium and etoricoxib on hepatic levels of 5-lipoxygenase, leukotrienes, interleukins, and nitric oxide synthase in rats with acute general cooling

Acute general cooling in the control pathology group was accompanied by the development of a systemic inflammatory response associated with activation of the lipoxygenase pathway of the arachidonic acid cascade (Table 2). Total hepatic leukotriene levels increased 1.7-fold ($p < 0.01$), while leukotriene B4 (LTB4) increased 1.5-fold ($p < 0.01$) compared with the intact control group. At the same time, hepatic 5-lipoxygenase (5-LOX) levels remained comparable across all experimental groups, with no statistically significant differences detected.

Etoricoxib exerted a pronounced anti-inflammatory effect, as evidenced by a significant reduction in total leukotrienes ($p < 0.01$) and LTB4 ($p < 0.05$) compared with the control pathology group. In the diclofenac sodium group, a significant decrease in LTB4 levels ($p < 0.05$) was observed, along with a downward trend in total leukotrienes; however, total leukotriene levels did not differ significantly from those of the intact control group.

The inflammatory response to acute general cooling was not characterized by marked changes in proinflammatory cytokines. In the control pathology group, hepatic levels of IL-1β, IL-6, and TNF-α did not differ significantly from those in the intact control group, although TNF-α showed a 1.5-fold increase at the level of a statistical trend. In contrast, anti-inflammatory cytokines were significantly reduced: IL-4 decreased 1.6-fold ($p < 0.01$) and IL-10 decreased 1.6-fold ($p < 0.001$) compared with intact controls.

The frigoprotective effects of the studied drugs were associated with a significant reduction in IL-1β levels. Etoricoxib decreased IL-1β concentrations 1.5-fold ($p < 0.05$) compared with the control pathology group and 1.4-fold ($p < 0.05$) relative to intact controls. Diclofenac sodium reduced IL-1β levels 1.8-fold ($p < 0.01$) compared with control pathology and 1.6-fold ($p < 0.001$) compared with intact controls.

Neither etoricoxib nor diclofenac sodium produced a significant effect on IL-6 levels. TNF-α concentrations under treatment with both drugs were comparable to those observed in the intact group. However, the levels of anti-inflammatory cytokines (IL-4 and IL-10) remained significantly lower than in intact controls and did not differ significantly from those in the control pathology group (see Table 2).

The quantitative balance between pro- and anti-inflammatory cytokines was further evaluated. Acute general cooling resulted in a pronounced cytokine imbalance. The ratio of total proinflammatory cytokines to total anti-inflammatory cytokines increased 1.82-fold compared with the intact control group (Table 3). These changes were primarily attributable to a statistically significant decrease in anti-inflammatory cytokines (IL-4 and IL-10), accompanied by a trend toward increased levels of proinflammatory cytokines (IL-1β and TNF-α). No significant changes in IL-6 levels were observed (Table 3).

Both etoricoxib and diclofenac sodium significantly reduced IL-1β levels without exerting statistically significant effects on the other examined cytokines. The frigoprotective effects of these agents appear to be associated with a shift toward predominance of anti-inflammatory cytokine activity. Accordingly, the pro-/anti-inflammatory cytokine ratio decreased 1.63-fold in the etoricoxib group and 1.76-fold in the diclofenac sodium group compared with the control pathology group, approaching the values observed in intact animals.

It is well established that acute general cooling in rats is accompanied by suppression of nitric oxide production as one of the adaptive mechanisms aimed at vasoconstriction to reduce heat loss [23]. The findings of the present study also reflect this adaptive response (Table 2). Specifically, hepatic NOS levels in the control pathology group were significantly decreased by 1.3-fold compared with the intact control group ($p < 0.01$).

Table 2

Effects of etoricoxib and diclofenac sodium on hepatic levels of 5-lipoxygenase, leukotrienes, interleukins, and nitric oxide synthase in rats with acute general cooling ($M \pm m$; Me [Q25; Q75])

Biomarker	Intact control (n = 9)	Acute general cooling		
		Control pathology (n = 6)	Etoricoxib (n = 9)	Diclofenac sodium (n = 6)
5-LOX, ng/g	163 ± 12.20 164 [157; 168.5]	169 ± 14.15 169 [163; 180.5]	167 ± 12.98 174 [158.5; 176]	168 ± 6.9 166 [164; 171]
Total leukotrienes, pg/g	1969 ± 842 2109 [1135; 2380]	3342 ± 643** 3354 [2786; 3776]	1780 ± 986 ## 1515 [1011; 2566]	2328 ± 577 2046 [1985; 2784]
Leukotriene B4 (LTB4), pg/g	727 ± 149.9 800 [569; 817]	1078 ± 151.8 ** 1058 [984; 1132]	769 ± 373.9 # 819.5 [598; 942]	826 ± 150.7 # 847.5 [753; 928]
IL-1β, ng/g	6.29 ± 0.87 6.03 [5.86; 7.04]	6.85 ± 2.82 5.91 [5.525; 7.515]	4.52 ± 1.13 * # 4.19 [3.80; 4.08]	3.86 ± 0.87 *** ## 3.44 [3.34; 4.70]
IL-6, ng/g	0.20 ± 0.065 0.22 [0.13; 0.24]	0.14 ± 0.08 0.11 [0.10; 0.15]	0.15± 0.04 0.13 [0.11; 0.19]	0.14 ± 0.05 0.15 [0.10; 0.16]
TNF-α, ng/g	1.70 ± 0.54 1.59 [1.24; 2.03]	2.58 ± 1.14 2.48 [1.695; 3.33]	1.64 ± 0.55 1.58 [1.26; 1.89]	1.74 ± 0.14 1.73 [1.64; 1.82]
IL-4, ng/g	8.57 ± 2.31 7.81 [6.52; 10.22]	5.50 ± 0.75 ** 5.30 [5.01; 5.60]	5.89 ± 0.87 ** 5.85 [5.54; 6.46]	5.76 ± 1.89 * 5.34 [4.45; 6.88]
IL-10, ng/g	1.81 ± 0.24 1.87 [1.72; 1.99]	1.13 ± 0.19*** 1.11 [1.01; 1.24]	1.25 ± 0.35** 1.28 [1.09; 1.45]	1.21 ± 0.21** 1.15 [1.08; 1.36]
NOS, ng/g	162 ± 32.87 163 [150; 181]	124 ± 21.65** 122 [106; 143]	151 ± 23.22 # 155 [130; 169]	156 ± 9.56 156 [148.5; 163.5]

Note: statistically significant differences: – $p < 0.05$; ** – $p < 0.01$; *** – $p < 0.001$ vs. intact control; # – $p < 0.05$; ## – $p < 0.01$ vs. control pathology group; n – number of animals per group.*

Table 3

Changes in the quantitative balance between pro- and anti-inflammatory cytokines in the liver of rats with acute general cooling and the effects of diclofenac sodium and etoricoxib

Group	Total content (ng/g)		Pro-/anti-inflammatory cytokine ratio
	pro-inflammatory cytokines (IL-1β, IL-6, TNF-α)	anti-inflammatory cytokines (IL-4, IL-10)	
Intact control	8.19	10.38	0.79
Acute general cooling			
Without treatment (control pathology)	9.57	6.63	1.44
Etoricoxib	6.31	7.14	0.88
Diclofenac sodium	5.74	6.97	0.82

Administration of etoricoxib resulted in a statistically significant increase in NOS levels compared with the control pathology group (1.2-fold, $p < 0.05$). Diclofenac sodium demonstrated only a tendency toward an increase in this parameter. Importantly, in both treatment groups, NOS levels did not differ significantly from those of the intact control group.

Thus, under conditions of acute hypothermia, the frigoprotective effect of both drugs, particularly etoricoxib, appears to be complemented by a potential endotheliprotective action.

5. Discussion of research results

The results of our study indicate that the studied drugs, etoricoxib and diclofenac sodium, are almost equally effective in preventing the decrease in body temperature in rats under conditions of acute general cooling. Our findings are consistent with previous reports demonstrating the frigoprotective properties of these agents [18, 19].

Acute general cooling was characterized by a significant increase in total leukotrienes and leukotriene B4 levels, while 5-lipoxygenase (5-LOX) content remained unchanged. In the present experiment, we assessed the concentration rather than the enzymatic activity of 5-LOX. Therefore, the observed elevation in leukotriene levels is most likely attributable to increased 5-LOX activity [24]. The frigoprotective effects of etoricoxib and diclofenac appear to be mediated, at least in part, by a reduction in hepatic total leukotrienes and leukotriene B4 levels. Notably, etoricoxib reduced total leukotriene levels to a greater extent than diclofenac. However, the 5-LOX content under treatment with both NSAIDs remained virtually unchanged across all experimental groups, which is consistent with previous findings for diclofenac [24].

Cold exposure did not induce significant changes in proinflammatory cytokines (IL-1β, IL-6, TNF-α) in the experimental groups, suggesting complex regulation of the cytokine response during hypothermia and the absence of a classical early proinflammatory reaction [25, 26]. The decrease in energy-dependent anti-inflammatory mediators (IL-4, IL-10) may reflect the characteristic metabolic suppression observed under conditions of acute general cooling [27]. The mechanism underlying the frigoprotective effects of etoricoxib and particularly diclofenac sodium involves a reduction in IL-1β levels and a relative predominance of anti-inflammatory cytokines. Our findings are consistent with previous reports on the effects of diclofenac sodium on interleukin levels in acute cold injury [24].

Thus, the frigoprotective mechanism of etoricoxib appears to be primarily associated with its antileukotriene properties, whereas diclofenac sodium exerts a more pronounced suppressive effect on the proinflammatory cytokine IL-1β. In addition, normalization of hepatic NOS levels under acute hypothermic conditions contrib-

utes to the frigoprotective mechanism of both NSAIDs, indicating potential endothelioprotective properties of these agents in acute general cooling.

Overall, the obtained results experimentally substantiate the potential of arachidonic acid cascade inhibitors, diclofenac sodium and etoricoxib, to attenuate the severity of acute general cooling, while also demonstrating certain differences in the mechanisms underlying their frigoprotective effects.

Practical relevance. The results of the study provide experimental evidence supporting the potential use of diclofenac sodium and etoricoxib as frigoprotectors.

Research limitations. A limited number of interleukins have been identified (only 5). Also, NO metabolism has not been extensively studied.

The impact of wartime conditions. The war in Ukraine has made the problem of cold injuries more urgent. This led to the implementation of this study, which continues a series of works devoted to acute cold trauma. Martial law also limited the scope of the study, which is given above.

Prospects for further research. Expanding the number of studied markers of the cytokine link in the pathogenesis of acute cold injury. Elucidation of acute general cooling effect on the mechanisms of interaction of interleukins, leukotrienes and prostaglandins. Determining the specifics of the effect of NSAIDs on the regulation of vascular tone in acute cold injury. Further in-depth studies concerning the molecular mechanisms of the frigoprotective activity of NSAIDs with different mechanisms of action. Moreover, it is important to assess the potential influence of individual COX inhibitors on inflammatory mechanisms and vascular tone regulation depending on sex and sex steroid levels, since the male body is more sensitive to acute cold injury than the female body, and the cryoprotective effect of certain cryoprotectants (in particular glucosamine [21, 28, 29]) is more pronounced in males.

6. Conclusions

1. In the ultra-acute phase of acute general cooling (general air hypothermia at −18°C for 2 hours) in rats, activation of the lipoxygenase pathway of arachidonic acid metabolism was observed. This was characterized by a significant increase in leukotriene levels (total leukotrienes and leukotriene B4) without substantial changes in proinflammatory cytokines (IL-1β, IL-6, TNF-α), but with a significant decrease in anti-inflammatory cytokines (IL-4, IL-10) in the liver.

2. Etoricoxib (5 mg/kg) and diclofenac sodium (7 mg/kg), administered intragastrically 30 minutes before cold exposure, similarly reduced the severity of hypothermia. Both agents prevented the increase in leukotriene levels (with diclofenac sodium showing a less pronounced effect) without affecting 5-lipoxygenase (5-LOX) content. At the same time, etoricoxib and particularly diclofenac sodium significantly reduced IL-1β levels without substantial changes in the other studied cytokines.

3. A decrease in hepatic NOS levels, reflecting reduced production of the endothelial relaxing factor, contributes to the pathogenesis of acute general cooling. Both frigoprotective NSAIDs restored NOS levels to those observed in the intact control group.

Conflicts of interest

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

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Data availability

The manuscript has no associated data.

Use of artificial intelligence

The authors used artificial intelligence technologies within reasonable limits in the “Introduction” section to search for statistical data from open-access sources over the past five years regarding the prevalence of cold-related injuries.

Authors contributions

Sergii Shtrygol’: Conceptualization, Methodology, Validation, Investigation, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition; **Olesia Kudina:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Visualization; **Dmytro Lytkin:** Methodology, Validation, Formal analysis, Investigation; **Andrii Taran:** Investigation; **Tetiana Yudkevych:** Investigation.

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