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Gastrocytoprotective Properties of Cryopreserved Placenta Extract in Combined Action of Low Temperatures and Inhibition of Cyclooxygenase

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SUMMARY

Introduction. Non-steroidal anti-inflammatory drugs (NSAIDs) are ranked first among the world's effective anti-inflammatory and analgesic drugs with anticipated side effects. That is why the prevention of development of adverse reactions associated with NSAIDs, in particular, of those related to their ulcerogenicity, remains a serious global problem.
Aims. To characterize the mechanisms of gastrocytoprotective activity of cryopreserved placenta in combined action of low temperatures and diclofenac sodium.
Material and methods. The study was performed on 42 male rats weighing 200 – 220 g. Acute diclofenac sodium-induced gastropathy was reproduced by a single injection of the latter in rats at the dose of 50 mg/kg. The content of malonic dialdehyde, catalase activity, prostaglandin synthase activity and the content of nitrogen monoxide metabolites in homogenates of gastric mucosa were determined by spectrophotometric method.
Results and discussion. The study showed that prophylactic administration of placental cryoextract in rats with diclofenac sodium-induced gastropathy is associated with increased activity of antioxidant system in gastric mucosa as demonstrated by an elevated catalase activity by 40.0% as compared with control rats. Modulation of antioxidant-prooxidant homeostasis is believed to be one of the principal mechanisms of gastrocytoprotective action in combined action of low temperatures and cryoextract of the placenta. This is shown by a statistically significant ($p < 0.05$) 2.2-fold increase of antioxidant-prooxidant index in the study group as compared with rats with diclofenac sodium-induced gastropathy. Administration of placental cryoextract was found to increase prostaglandin synthase activity in rats with diclofenac sodium-induced gastropathy by two times as compared with control rats, which would reduce iatrogenic prostaglandin deficiency in gastric mucosa. Also, the combined action of low temperatures and of placenta cryoextract was associated with a statistically significant ($p < 0.05$) increase in the level of metabolites of nitrogen monoxide (by 70.1%) as compared with rats with diclofenac sodium-induced gastropathy.
Conclusions. Modulation of prooxidant-antioxidant homeostasis in gastric mucosa and increase in contents of nitrogen monoxide metabolites and prostaglandin synthase activity are the leading

mechanisms of gastroprotective activity of cryopreserved placental extract in diclofenac sodium-induced gastropathy.

Keywords: **cryopreserved placenta extract, nonsteroidal anti-inflammatory drugs, ulcerogenicity, low temperature action**

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INTRODUCTION

Non-steroidal anti-inflammatory drugs (NSAIDs) are the world's leading effective anti-inflammatory and analgesic agents with anticipated side effects. That is why prevention of NSAID-associated adverse reactions, in particular of those related to their ulcerogenicity, remains a serious problem that is the subject to global debate. Also, because of these side effects, the therapeutic value of this class of drugs may be significantly limited (1, 2). The gastrocytoprotective efficacy of the combined use of NSAIDs with sucralfate, misoprostol, antacids, H_2-gastmin blockers, proton pump inhibitors and others has been demonstrated in previous research aimed at minimization of gastrointestinal complications. However, all these combinations are not devoid of their own side effects and do not always comply with the expected clinical effectiveness (1, 2).

Cryopreserved placenta extract has drawn our attention as a potential modifier of the ulcerogenic action of NSAIDs (CEP ("Cryocell-cryoextract of the placenta") State Enterprise "Interdepartmental Research Center of Cryobiology and Cryomedicine of the National Academy of Sciences of Ukraine, the National Academy of Medical Sciences of Ukraine and the Ministry of Health of Ukraine", Ukraine", Ukraine)).

Another equally important aspect of gastrocytoprotective therapy may be the effect of low temperatures (regional hypothermia) on gastric mucosa (GM), which may be implemented with the help of devices designed for use with endoscopes.

AIMS

The aim of the papaer was to characterize the metabolic mechanisms of gastrocytoprotective activity of cryopreserved placenta extract in combined action of low temperatures and diclofenac sodium.

MATERIAL AND METHODS

Among the NSAIDs for the study, we selected diclofenac sodium (DN), which is represented by the largest number of trade names in the pharmaceutical market of Ukraine (from 69 to 80 names during 2014–2018) due to its strong clinical efficacy and affordability of generic drugs (3).

Experimental model

Acute diclofenac sodium-induced gastropathy was reproduced in rats by a single intravenous injection of Diclo (Limited Liability Company "Pharmaceutical Company "Zdorovia", Ukraine) at the dose of 50 mg/kg (Ulcerogenic dose $(UD)_{50}$ = 48 mg/kg) (4, 5). Euthanasia of animals was performed 24 h after administration of NSAIDs. Diclo in a tablet form was ground and emulsified in water for injections (Open Joint Stock Company "Halychpharm", Ukraine) with the addition of polysorbate Twin-80. The removed stomachs were opened and washed in 0.9% NaCl solution. To obtain a homogenate, GM was perfused with cold (+4^{0}C) isotonic 1.15% KCl solution and homogenized at 3000 rpm (Teflon glass) in a buffer solution at a ratio of 1:10 (weight/volume: portion 250 mg + 2,25 ml of 1.15% KCl solution); thus, a 10.0% homogenate was obtained.

Subject and design of the study (mode of administration of the studied drugs)

The study was performed on 42 male rats weighing 200 – 220 g, divided into 6 groups:

Group I – intact rats (n = 7) ;

Group II – rats with Diclo-induced gastropathy (n = 7) ;

Group III (n = 7) – rats with Diclo-induced gastropathy, which in the prophylactic mode intramuscularly (i.m.) were administered CEP (5 injections for 5 days before Diclo);

Group IV (n = 7) – rats with Diclo-induced gastropathy, in whom GM cryoirrigation was performed;

Group V (n = 7) – rats with Diclo-induced gastropathy, who were administered CEP i.m. in a prophylactic mode along with GM cryoirrigation;

Group VI (n = 7) – rats with Diclo-induced gastropathy, who were administered CEP i.m. in a prophylactic mode along with intragastric (i.g.) injection of esomeprazole (Joint Stock Company "Actavis", Iceland) at the dose of 50 mg/kg (5 injections) 5 days) (6).

The drug CEP according to the instructions is used in patients parenterally in a single dose of 1.8 ml. Accordingly, a single dose for rats is: (1.8 ml/70 kg) × 6.35 = 0.16 ml/kg body weight (5, 6). Before using the drug "Cryocell-cryoextract of the placenta", a single dose (0.16 ml/kg) ex tempore was diluted in

0.9% solution of NaCl (Private Joint Stock Company "Pharmaceutical Company Darnitsa", Ukraine) at a rate of 0.1 ml of 0.9% NaCl solution/100 g body weight.

Cryoirrigation was performed by local injection of liquid nitrogen vapour (temperature -120^{0}C) on GM for 10 seconds using a cryoapparatus "Cryo Pro Maxi" (CryoPro, Denmark).

RESEARCH METHODOLOGY

The concentration of malonic dialdehyde (MDA) was determined spectrophotometrically with the method of Asakawa T. et al. (7) by reaction with thiobarbituric acid (TBA), and was calculated from the optical density determined by light absorption at a wavelength λ = 535 nm, taking into account the molar extinction coefficient of the red-colored complex equal to 1.56 × 105 mol-1/cm^{-1} and expressed in μmol/kg tissue.

Catalase activity in GM was determined spectrophotometrically by the method (8) using light absorption at the wavelength of λ = 410 nm, comparing the test sample (0.1 ml of serum + 2 ml of 0.03 % H_2O_2) with the control (0.1 ml of serum + 2.0 ml of H_2O).

Antioxidant-prooxidant index (API) was calculated by the formula: API = Catalase activity × 100)/The content of malonic dialdehyde (MDA).

GH synthase activity in GM was determined spectrophotometrically by changing the concentration of the oxidized form of the adrenaline electron donor. The reaction was initiated by the addition of an alcoholic solution of 0.19 mm arachidonic acid and recorded after 1 min by light absorption at the wavelength of λ = 782 nm (9). PG synthase activity was expressed in μmol of arachidonic acid, oxidized 1 mg of protein per 1 min – μmol/min/mg of protein.

The content of NO metabolites in GM was determined by spectrophotometric method, which was based on the oxidation of nicotinamide adenine dinucleotide phosphate (NADPH) during the reaction of NO formation with L-arginine. The decrease in NADPH was recorded by light absorption at the wavelength of λ = 340 nm. (10). NO synthase activity was expressed in mmol/g tissue.

Statistical analysis

Evaluation of the nature of the distribution of values in each group of the sample was performed using the W-test Shapiro-Wilk test. Homogeneity of dispersions was determined by Levene's test. To assess the significance of the identified differences in the studied indicators under different experimental conditions, statistical analysis was performed using parametric or nonparametric criteria.

In case of normal distribution of the independent values, the differences between the groups were determined in pairs by the Student's t-test. In case of abnormal distribution of at least one of the groups of independent quantities, the differences between them were determined in pairs by the nonparametric Mann-Whitney rank U-test. The obtained values were considered significant at a level above 95.0% ($p \leq 0.05$). Numerical data in case of normal distribution of values are given as "M ± m" (M ± SE), where M is an arithmetic mean, m (SE) is a standard error of the arithmetic mean or M (95% CI: 5% – 95%), where by 95% CI: – 95% is the confidence interval.

At abnormal distribution of the received sizes, the data are presented in the form of Me (LQ; UQ), where Me is the median, LQ is the upper limit of the lower quartile and UQ is the lower limit of the upper quartile (11).

Bioethical compliance

All experimental studies on laboratory animals were performed in accordance with the requirements of Good Laboratory Practice and in compliance with the basic provisions of the Council of Europe Convention on the Protection of Vertebrate Animals Used in Experiments and Other Scientific Purposes of 18 March 1986, European Parliament and Council Directive 2010/63/EU of 22 September 2010 on the protection of animals. The comprehensive research program was considered and approved by the Committee on Bioethics at the Institute of Cryobiology and Cryomedicine (excerpt from Protocol № 2 of March 11, 2020).

RESULTS AND DISCUSSION

The study showed that administration of an ulcerogenic dose of Diclo led to a statistically significant ($p < 0.05$) decrease in API values by 3.6 times as compared with intact animals (Table 1). These changes were due to oxidative stress in GM, as indicated by a statistically significant ($p < 0.05$) increase in MDA content by 102.5 ± 11.0% and inhibition of catalase activity by 71.1 ± 3.3% as compared with intact rats. Oxidative stress is one of the pathogenetic mechanisms of the development of

Table 1. *The effect of low temperatures, diclofenac sodium and CEP on the biochemical parameters of POL-AOC, M ± m (95% CI) or Me (LQ; UQ); n = 42*

The studied indicator, units of measurement	Experimental conditions					
	I group	II group	III group	IV group	V group	VI group
	Intact rats	Diclofenac sodium (Diclo)	Diclo + CEP	Diclo + cryo-irrigation	Diclo + CEP + cryo-irrigation	Diclo + esome-prazole
n	*7*	*7*	*7*	*7*	*7*	*7*
Malonic dialdehyde (MDA), μmol/kg of tissue	9.6 ± 0.37 (95% CI: 8.8 – 10.3)	19.1 ± 0.40 (95% CI: 18.4 – 19.9)*	12.7 ± 0,81 (95% CI: 10.1 – 12.8)*#	15.9 ± 0.59 (95% CI: 14.7 – 17.0)*#§	11.4 ± 0.69 (95% CI: 11.6 – 14.4)*# º	10.0 ± 0.44 (95% CI: 9.1 – 10.9)#
Catalase activity, mkat/kg of tissue	3.6 ± 0.18 (95% CI: 3.2 – 3.9)	2.0 ± 0.05 (95% CI: 1.9 – 2.1)*	2.8 ± 0.10 (95% CI: 2.6 – 3.0)*# º	2.7 ± 0.08 (95% CI: 2.5 – 2.9)*# º	3.6 ± 0.14 (95% CI: 3.3 – 3.9)#§μ	3.3 ± 0.07 (95% CI: 3.1 – 3.4)#
Antioxidant-prooxidant index (API)	38.0 ± 2.97 (95% CI: 32.2 – 43.8)	10.5 ± 0.42 (95% CI: 9.7 – 11.3)*	23.0 ± 2.11 (95% CI: 18.9 – 27.1)*#º	17.2 ± 0.84 (95% CI: 15.5 – 18.8)*#º	32.1 ± 2.08 (95% CI: 28.0 – 36.2)#	33.0 ± 1.03 (95% CI: 30.9 – 35.0)#

* – $p < 0.05$ relative to intact animals;
\# – $p < 0.05$ relative to rats treated only with diclofenac sodium;
§ – $p < 0.05$ relative to rats treated with diclofenac sodium and CEP;
μ – $p < 0.05$ relative to rats treated with diclofenac sodium and performed cryoirrigation;
º – $p < 0.05$ relative to rats treated with diclofenac sodium and esomeprazole.

Table 2. *The effect of low temperatures, diclofenac sodium and CEP on GHG synthase activity and the content of NO metabolites in homogenates of GM rats, M ± m (95% CI) or Me (LQ; UQ); n = 42*

The studied indicator, units of measurement	Experimental conditions					
	I group	II group	III group	IV group	V group	VI group
	Intact rats	Diclofenac sodium (Diclo)	Diclo + CEP	Diclo + cryo-irrigation	Diclo + CEP + cryo-irrigation	Diclo + esome-prazole
n	*7*	*7*	*7*	*7*	*7*	*7*
Activity PG synthase, μmol/min/mg protein	18.6 ± 0.72 (95% CI: 17.2 – 20.0)	8.4 ± 0.57 (95% CI: 7.3 – 9.5)*	17.0 ± 1.2 (95% CI: 15.0 – 19.0)#º	10.1 ± 0.63 (95% CI: 8.9 – 11.4)*§º	20.1 ± 0.67 (95% CI: 18.8 – 21.5)#§μº	12.6 ± 0.78 (95% CI: 11.0 – 14.1)*#§μ
Metabolites NO, mmol/g tissue	920 (905; 925)	550 (520; 555)*	780 (760; 790)*#	820 (810; 835)*#§º	940 (905; 950)#§ μº	710 (700; 715)*#§

* – $p < 0.05$ relative to intact animals;
\# – $p < 0.05$ relative to rats treated only with diclofenac sodium;
§ – $p < 0.05$ relative to rats treated with diclofenac sodium and CEP;
μ – $p < 0.05$ relative to rats treated with diclofenac sodium and performed cryoirrigation;
º – $p < 0.05$ relative to rats treated with diclofenac sodium and esomeprazole.

NSAID-induced gastropathy, which determines the appropriateness of the use of antioxidants (mexidol, hypoxen, etc.) or drugs with antioxidant properties (1). The presence of proven antioxidant activity in CEP (2) allows us to consider it as a means of pathogenetic therapy of NSAID-induced gastropathy: five days of prophylactic administration of CEP lead to a significant decrease in the intensity of Diclo-induced oxidative stress in GM, which was demonstrated by a statistically significant ($p < 0.05$) increase in API by 2.2 times as compared with rats with "pure" Diclo-induced gastropathy, mainly due to the

1.4-fold increase in catalase activity in GM. Similar changes in antioxidant-prooxidant homeostasis were observed with Diclo and esomeprazole (Group VI), where the MDA content in GM decreased by 1.9 times and catalase activity increased by 1.7 times as compared with rats with Diclo-induced gastropathy. However, these changes were pathogenetically related to the acid-suppressive activity of PPIs, because PPIs do not have a pronounced antioxidant activity.

In contrast to CEP, GM cryoirrigation in rats with Diclo-induced gastropathy resulted in a less pronounced increase in API (1.6 and 2.2 times, respectively), which may be associated with activation of the prooxidant system due to destruction of gastric epithelocytes by the action of low temperatures. At the same time, prophylactic administration of CEP and cryoirrigation to rats with Diclo-induced gastropathy led to the most pronounced changes in the antioxidant system - catalase activity with GM increased to the level of intact rats and amounted to 3.6 ± 0.14 mcat/kg tissue, which statistically significantly ($p = 0.01$) performed better than the animals who were injected CEP without cryorigation. The API value in rats who had combined administration of CEP and subsequent cryoirrigation (32.1 ± 2.08) and the API value in rats injected with esomeprazole (33.0 ± 1.03) were not significantly different. It should be noted that in contrast to esomeprazole, the leading mechanism of gastrocytoprotective action of combined administration of CEP and GM cryoirrigation is the modulation of the prooxidant-antioxidant system, while the action of esomeprazole is aimed at reducing the aggressiveness of gastric juice.

Evaluation of GH synthase activity in GM showed (Table 2) that the introduction of Diclo led to a statistically significant ($p < 0.05$) inhibition of its activity by 53.6 ± 4.8% relative to intact rats and was 8.4 ± 057 μmol/min/mg protein. These changes were consistent with the literature (1) that the inhibition of GHG synthase activity of NSAIDs is one of the mechanisms of their ulcerogenic action. It is known that in addition to the synthesis of GHG, cyclooxygenase of the first type (COX-1), provides blood supply to GM and the duodenum but also stimulates the formation of bicarbonates that perform gastroprotective and trophic functions. With the use of NSAIDs and blockade of COX-1, all these functions are disrupted, and GHG synthesis is inhibited by reducing the activity of GHG synthases, depletion of their reserves in tissues, resulting in the development of iatrogenic prostaglandin deficiency (1).

The introduction of esomeprazole (Group VI), as well as cryoirrigation (Group IV) in rats with Diclo-induced gastropathy, led to a comparable increase in the activity of GH synthases by 1.2 and 1.5 ($p = 0.001$) times, respectively, relative to rats in the control group (8.4 ± 057 μmol/min/mg protein) and was 10.1 ± 0.63 μmol/min/mg protein and 12.6 ± 0.78 μmol/min/mg protein, respectively.

The most pronounced changes in GH synthase activity were observed in rats with Diclo-induced gastropathy, which were administered CEP - the study rate increased by 2.0 times in the group of rats administered Diclo and CEP and 2.4 times in animals treated with Diclo, CEP and performed cryo-irrigation. The established intergroup differences indicate the ability of CEP to neutralize the inhibitory effect of Diclo on the activity of GHG synthases in GM, which may be one of the mechanisms of its gastroprotective activity under NSAID-induced ulcerogenesis.

A study of the content of NO metabolites in GM (Table 2) showed that the introduction of Diclo led to a statistically significant ($p < 0.05$) decrease in their content by 41.5% relative to intact rats, which is consistent with the literature on the ability of NSAIDs to inhibit nitrogen synthesis of monoxide by inhibiting the activity of NO synthases, which leads to disruption of microcirculation and actual damage to GM (1, 9).

Processes such as platelet aggregation and neutrophil migration through the vascular wall, relaxation of vascular intima cells, neoangiogenesis, neoneurogenesis, and others are associated with NO. It is the hypothesis of the gastroprotective role of NO that became the basis for the creation of a new subgroup of NSAIDs – cyclooxygenase inhibiting NO-donating drugs (CINODs). NO plays an important role in ensuring the motor function of the gastrointestinal tract, as well as the regulation of bile flow into the intestine. In particular, NO reduces the motility of the gastrointestinal tract, relaxes the sphincter of Oddi and the lower esophageal sphincter. Therefore, attempts to create a drug that would locally increase the concentration of NO in GM and compensate for the lack of prostaglandins are in great demand today (12).

The introduction of CEP (Group III) and cryoirrigation of GM (Group IV) led to a statistically

significant ($p < 0.05$) increase in the content of NO metabolites by 41.8% and 49.0%, respectively, relative to rats with Diclo-induced gastropathy. However, the most pronounced increase in the content of the studied mediator was observed against the background of the combined action of low temperatures and the introduction of CEP - the level of NO metabolites statistically significantly ($p < 0.05$) increased by 70.1% and amounted to 940 (905; 950) mmol/g of tissue.

CONCLUSIONS

1. Preventive management of co-preserved placenta extract in rats with diclofenac sodium-induced gastropathy increases the activity of the antioxidant system in gastric mucosa, as reflected by a 40% increase in catalase activity as compared with the control group rats.

2. Modulation of antioxidant-prooxidant homeostasis is one of the mechanisms of gastrocytoprotective action in combined action of low temperatures and cryopreserved placenta extract. This is demonstrated by a statistically significant ($p < 0.05$) increase in antioxidant-prooxidant index which is 2.2 times higher as compared with the rats with diclofenac sodium-induced gastropathy.

3. The administartion of cryopreserved placenta extract resulted in a 2-fold increase of prostaglandin synthases activity in rats with diclofenac sodium-induced gastropathy as compared with control rats, thus preventing the iatrogenic prostaglandin deficiency in gastric mucosa.

4. The combined action of low temperatures and the introduction of cryopreserved extract of the placenta was associated with a statistically significant 70% increase ($p < 0.05$) in the levels of nitrous monoxide metabolitesas compared in rats with diclofenac sodium-induced gastropathy.

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Gastroprotektivne karakteristike krioprezerviranog ekstrakta placente kod kombinovanog delovanja niskih temperatura i inhibicije ciklooksigenaze

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SAŽETAK

Uvod. Nesteroidni antiinflamatorni lekovi (NSAIL) prvi su na listi efektivnih antiinflamatornih i analgetskih lekova sa očekivanim neželjenim efektima. Zbog toga, prevencija neželjenih efekata ovih lekova, naročito ulcerogenosti, ostaje ozbiljan globalni problem.

Cilj . Cilj rada bilo je opisivanje mehanizama gastroprotektivne aktivnosti krioprezervirane placente kod kombinovanog delovanja niskih temperatura i diklofenak-natrijuma.

Metode. Studija je sprovedena nad 42 pacova muškog pola, težine 200 g – 220 g. Akutna gastropatija indukovana diklofenak-natrijumom izazvana je kod pacova jednom injekcijom diklofenak-natrijuma u dozi od 50 mg/kg. Sadržaj malonil-dialdehida, aktivnosti katalaze, aktivnosti prostaglandin sintaze, kao i sadržaj metabolita nitrogen-monoksida u homogenatu sluzokože želuca određeni su spektrofometrijskom metodom.

Rezultati. Ova studija pokazala je to da je profilaktičko davanje krioekstrakta placente pacovima sa gastropatijom izazvanom diklofenak-natrijumom povezano sa povećanom aktivnošću antioksidativnog sistema sluzokože želuca, što je potvrđeno povišenom aktivnošću katalaze za 40% u poređenju sa ovom aktivnošću kod pacova kontrolne grupe. Modulacija antioksidativno-prooksidativne homeostaze smatra se jednim od glavnih mehanizama gastroprotektivnog delovanja kombinovane primene niskih tempaeratura i krioekstrakta placente. Ovo je prikazano statistički značajnim povećanjem antioksidativno-prooksidativnog indeksa 2,2 ($p < 0,05$) puta u ispitivanoj grupi u poređenju sa ovim indeksom kod pacova kod kojih je gastropatija bila izazvana diklofenak-natrijumom. Utvrđeno je to da davanje krioekstrakta placente povećava aktivnost prostaglandin sintaze 2,0 puta kod pacova kod kojih je gastropatija bila izazvana diklofenak-natrijumom u poređenju sa ovom aktivnošću kod pacova kontrolne grupe, što bi smanjilo deficijenciju prostaglandina izazvanu jatrogenim putem na sluzokoži želuca. Takođe, kombinovano delovanje niskih temperatura i krioekstrakta placente povezano je sa statistički značajnim povećanjem ($p < 0,05$) nivoa metabolita nitrogen-monoksida (za 70,1%) u poređenju sa ovim nivoima kod pacova kod kojih je gastropatija izazvana diklofenak-natrijumom.

Zaključak. Modulacija prooksidativno-antioksidativne homeostaze sluzokože želuca i povećanje sadržaja metabolita nitrogen-monoksida i aktivnosti prostaglandin sintaze vodeći su mehanizmi gastroprotektivne aktivnosti ekstrakta krioprezervirane placente kod gastropatije izazvane diklofenak-natrijumom.

Ključne reči: **ahalazija, simptomi, disfagija, manometrija, donji ezofagijalni sfinkter, kvalitet života, dvadesetčetvoročasovni monitoring**