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Experimental study of the antiulcer effect of cryopreserved placenta extract on a model of acetylsalicylic acid-induced ulcerogenesis

FEDIR VOL. HLADKYKH* 

Department of Experimental Cryomedicine, Institute for Problems of Cryobiology and Cryomedicine of the National Academy of Sciences of Ukraine, Kharkiv, Ukraine

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ABSTRACT

Introduction. The gastrototoxicity of nonsteroidal anti-inflammatory drugs is a leading side effect that significantly limits their clinical use, among other types of their toxicity (nephrotoxicity, hepatotoxicity, cardiotoxicity, etc.). Cryopreserved placenta extract has drawn our attention as a potential modifier of the ulcerogenic action of nonsteroidal anti-inflammatory drugs.

Aim. To characterize the cytoprotective properties of cryopreserved placenta extract by the condition of the mucous membrane of the proximal (esophagus and stomach) and distal (small and large intestine) parts of the gastrointestinal (GI) tract on the model of ASA-induced ulcerogenesis.

Material and methods. The study was performed using 28 male rats weighing 200-220 g. Subchronic ASA-induced ulcerogenesis of the digestive tract was reproduced by intragastrically administration to rats of ASA in a dose of 150 mg/kg. The effect of the studied drugs on the condition of the mucous membrane of the digestive tract was assessed macroscopically by the following criteria: edema, redness and hemorrhage on the surface of the mucous membrane. The ulcer index for each group of animals was calculated.

Results and discussion. Five doses of ASA 150 mg/kg cause damage to the esophagus, stomach, small and large intestines in all of the rats. The use of the proton pump inhibitor esomeprazole has pronounced gastrocytoprotective properties, but does not affect the ulcerogenic effect in the small intestine, and in the colon, it enhances it. This is indicated by ulcerative lesions of the colon in 57.1% of all rats administered ASA and esomeprazole, as well as in the folding of the gastric mucosa. In contrast, mild hyperemia of the gastric mucosa was seen in 28.6% of all rats and moderate hemorrhage in 57.1% of all rats due to the combined use of ASA and cryoextract placenta.

Conclusions. The use of cryopreserved placenta extract is statistically significantly ($p < 0.05$) inferior to the antiulcer activity of esomeprazole in the stomach. Thus, the ulcer index on the background of the use of ASA and cryopreserved placenta extract was 0.97, and on the background of the use of ASA and esomeprazole – 0.39. In the distal parts of the GI tract cryoextract placenta showed cytoprotective properties against the background of induced ASA ulcerogenesis, in contrast to esomeprazole.

INTRODUCTION

Gastrointestinal toxicity of nonsteroidal anti-inflammatory drugs (NSAIDs) is a leading side effect that limits their clinical use. Other side effects include nephrotoxicity, hepatotoxicity, cardiotoxicity, etc. [1,2]. The ulcerogenic action of NSAIDs in the gastrointestinal tract (GI tract)

is associated with the inhibition of physiological prostaglandins, which are known to be involved in regulating the smooth muscle tone of various organs, as well as in improving regional blood flow in the gastric mucosa (GM), intestine and renal cortex, and decreasing hydrochloric acid secretion and increasing the secretion of mucus in the stomach. In addition, they are involved in inhibiting platelet aggregation, etc. Currently, three isoforms of cyclooxygenase (COX)

* Corresponding author

e-mail: fedir.hladkykh@gmail.com

have been discovered and studied: structural (COX-1), induced (COX-2) and COX-3. It is believed that the anti-inflammatory effect of NSAIDs depends on the inhibition of COX-2, and their side effects are due to the suppression of COX-1 [3,4].

Given this, it is important to find ways of minimizing GI tract complications when taking NSAIDs. To date, combinations of NSAIDs with sucralfate, misoprostol, antacids, H₂-receptor antagonists, proton pump inhibitors (PPIs) and others have been studied. The combination of NSAIDs and PPIs is the most successful. However, this co-administration is not without its downsides. For example, PPIs protect only the upper GI tract, but do not protect against damage to the distal intestinal parts (NSAIDs-enteropathy) [5-7].

Acetylsalicylic acid (ASA) has a special place among all NSAIDs, because in low doses (75-100 mg/kg) it reduces the risk of a range of cardiovascular events, and therefore it is recommended for primary prevention in patients with very high cardiovascular risk or in patients with concomitant diabetes [7].

One of the main limitations of ASA therapy is its discontinuation or initial "non-prescribing" in connection with the developed or potential side effects in the GI tract. In this case, the reasons for the discontinuation of ASA are not only serious complications such as gastrointestinal bleeding or perforations, but also dyspeptic phenomena accompanied by the development of aspirin-induced gastroenteropathy. The adverse sequelae of ASA withdrawal are well known: if the drug treatment in patients receiving it for the secondary prevention of cerebral ischemic events is interrupted for at least 10 days, the risk of this complications increases 3-fold, and in patients with coronary artery disease, the risk of myocardial infarction increases 2-fold. Moreover, discontinuation of ASA even for 8 weeks due to ulcer bleeding in patients receiving antiplatelet therapy for coronary artery disease and cerebrovascular disease has been associated with a significant increase in overall mortality [2,7].

The most appropriate way to reduce the risk of gastrointestinal complications is the administration of ASA in a minimally effective dose that does not exceed 75-81 mg/day; but this approach still does not guarantee the absolute gastrointestinal safety of therapy [2].

Cryopreserved extract of placenta (CEP) has drawn our attention as a potential modifier of the ulcerogenic action of NSAIDs. Our institution has developed a technology for obtaining CEP and a technology for its long-term storage in a cryobank. Years of research have shown that CEP acts upon target organs by stimulating their function, as well as increasing non-specific resistance to adverse environmental factors and stressors. What is more, CEP has been found to induce reparative cell properties [8-10]. Placental tissue contains a wide range of active substances, including hormones, proteins, polypeptides, nucleic acids, lipids, vitamins, etc., which either directly or indirectly can act upon the inflammatory processes (Tab. 1) [11,12].

A number of studies have demonstrated the presence of anti-inflammatory activity in CEP. This prompted researchers to study the effectiveness of CEP in inflammatory processes of various etiologies. The expediency of cryopreserved placenta in the complex therapy of acute

Table 1. Biologically active substances contained in the cryoextract of the placenta [8]

Name of biologically active substances	Characteristics	Content
Alpha-fetoprotein	Activator (or inhibitor) of growth of embryonic, transformed, activated immunocompetent cells	429±75 mIU/mL
Chorionic gonadotropin	Activator of the immune system, stimulates the production of steroid hormones (testosterone and estradiol)	26,8±8 mIU/mL
Estradiol	Reproductive function, cardioprotective action	755±48 pmol/mL
Progesterone	Reproductive function, cardioprotective effect	226±110 nmol/mL
Prolactin	Influence on the development of secondary sexual characteristics, erythropoietic action, regulation of fat metabolism	705±129 mIU/mL
Alpha-2-fertility microglobulin	Preparation for pregnancy, conception process, normal development of fetoplacental unit	1470±173 ng/mL
Lactoferrin	Stimulation of lactation	1270±223 ng/mL
Somatotropin	growth hormone, anabolic action	5.64 ng/mL
Luteinizing hormone	Pituitary hormone, secretion of estrogen, progesterone, testosterone	7.8±1.9 IU/l
Follicle-stimulating hormone	Pituitary hormone, promotes the maturation of follicles in the ovaries and spermatogenesis	7.1±2.3 mIU/l
Testosterone	Differentiation and functioning of the reproductive system, anabolic action	3.68±1.06 nmol/mL
Thyrotropic hormone	Stimulation of thyroid function, immunomodulatory effect	291±13 mIU/l
T3	Stimulation of metabolism, growth and differentiation of tissues, reproductive processes, hematopoiesis	2.1±0.6 pmol/L
T4	Stimulation of metabolism, tissue growth and differentiation, reproduction processes, hematopoiesis	5.6±0.99 pmol/l
Cortisol	Metabolism of proteins, carbohydrates, fats and nucleic acids	1392±515 nmol/l
G-CSF	Bone marrow cell proliferation	9.87 ng/mL
TNF-α	Inhibitor of cancer cell proliferation	84.5 pg/mL
IL-1β	Regulation of differentiation of poly potent stem cells, immunoendocrine system	201.7 pg/mL
IL-4	Regulation of differentiation of poly potent stem cells, immunoendocrine system	21.7 pg/mL
IL-6	Regulation of differentiation of poly potent stem cells, immunoendocrine system	114.9 pg/mL
Total protein	Plastic function	76.5±14 mg/g

λ-carrageenan-induced gastritis has been demonstrated, the clinical efficacy of CEP in intra-articular administration to patients with arthritis has also been revealed. Moreover, according to pathomorphological studies of the intestine, the use of placental drugs is effective in treating acute aseptic peritonitis [8,11,12].

The purpose of the present study is to characterize the cytoprotective properties of cryopreserved placenta extract according to the condition of the mucous membrane of the proximal (esophagus and stomach) and distal (small and large intestine) parts of the GI tract in the model of ASA-induced ulcerogenesis.

MATERIAL AND METHOD

Experimental model

Subchronic ASA-induced ulcerogenesis of the GI tract was reproduced by intragastric (i.g.) administration of ASA (Private Joint Stock Company «Pharmaceutical Company Darnitsa», Ukraine) to rats in a dose 150 mg/kg

(0,6 ulcerogenic dose (UD)₅₀) 5 times (3,1 UD₅₀) daily for three days [13,14].

Euthanasia of animals was performed 24hrs after administration of NSAIDs.

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

The study was performed on 28 male rats weighing 200–220 g, divided into 4 groups:

- Group I – intact rats (n = 7);
- Group II – rats with ASA-induced ulcerogenesis of the GI tract (control group, n = 7);
- Group III (n = 7) – rats with ASA-induced ulcerogenesis of the GI tract, which were administered 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) 5 injections 60 min. after each ASA administration);
- Group IV (n = 7) – rats with ASA-induced ulcerogenesis of the GI tract, which in a treatment and prevention regimen according to a scheme similar to the introduction of CEP, were i.g. injected esomeprazole, a proton pump inhibitor of the Vth generation (Joint Stock Company "Actavis", Iceland) at a dose of 50 mg/kg (5 injections for 3 days) [5].

The drug was administered with CEP in the «treatment and prevention» regimen – 5 injections 60 min. after each ASA administration. 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 [3,8,9]. 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.

Research methodology

Euthanasia of animals was performed by cervical dislocation under inhalation anesthesia. After removal of the esophagus from the organ complex, it was cut in the longitudinal direction and the MM structure was macroscopically evaluated, taking into account the recommendations of the international scientific nomenclature group Vevey (2011) on the severity of esophagitis and the expansion of the standard Los Angeles classification of gastroesophageal reflux disease hyperemia, edema of the MM esophagus) and N (no changes) [13,15,16].

After laparotomy along the white line of the abdomen (linea alba abdominis), a macroscopic evaluation of the size of the stomach (bloating) was performed, along with assessment for the presence of adhesions with adjacent organs as a sign of perforation. The removed stomachs were opened along the greater curvature and washed in 0.9% NaCl solution. The effect of the studied drugs on the condition of the MM of GI tract was assessed macroscopically by the following criteria: edema, redness and the presence of hemorrhages on the surface of the mucous membrane. For

each group, the percentage of experimental animals was calculated according to these characteristics, and the average value of the injury severity was assessed on the following scale [13]:

- 0 points – no signs of injury;
- 1 point – signs of injury are weakly expressed;
- 2 points – signs of injury are moderately expressed;
- 3 points – marked signs of injury.

In addition, GM condition was assessed on a scale as shown in Table 2 [13,14]. Calculation of the integrated GM condition index (an ulcerative index) was performed according to the formula:

$$\text{Ulcerative index} = (\text{Average score on the scale} \times \% \text{ rats with ulcers}) / 100.$$

The total number of ulcers in the small and large intestine was assessed, including the number of hemosiderin ulcers and the number of perforated ulcers.

Table 2. Score condition GM

Points	Characteristics of the condition GM
0	No visible damage
1	The presence of one or more features from the list: <ul style="list-style-type: none"> • edema, • hemorrhage(s), • ulcer(s) up to 1 mm in diameter in an amount not exceeding three
2	More than three ulcers up to 1 mm in diameter or one ulcer up to 3 mm in diameter
3	The presence of at least one ulcer diameter up to 4 mm
4	Several ulcers up to 4 mm in diameter
5	Perforated ulcer

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 via 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.

The probability of differences between the percentages of qualitative parameters in the alternative form was determined by the value of the F-test of Fisher's angular transformation (F-test).

In case of normal distribution of the independent values, the differences between the groups were determined in pairs by administering the Student's t-test. In case of abnormal distribution of at least one of the group of independent quantities, the between differences were determined in pairs by applying the nonparametric Mann-Whitney rank U-test. The obtained values were compared with the critical ones with a probability level above 95.0% ($p \leq 0.05$), above 99.0% ($p \leq 0.01$), above 99.5% ($p \leq 0.005$) and above 99.9% ($p \leq 0.001$) and the probability of error was concluded. Numerical data in the case of normal distribution of values are given as "M±m" ($M \pm SE$), where M is the arithmetic mean, m (SE) is the standard error of the arithmetic mean or M (95% CI: 5% – 95%), where 95% CI: – 95% confidence interval.

In situations of abnormal distribution of the received sizes, the data are presented in the form of Me [LQ; UQ], where Me is the median, [LQ; UQ] is the upper limit of the lower quartile (LQ) and the lower limit of the upper quartile (UQ) [13].

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 No 2 of March 11, 2020).

RESULTS AND DISCUSSION

The study showed that three days of ASA administration at a dose of 150 mg/kg led to the development of damage of all parts of the GI tract. Here, the M-degree of damage to the esophagus according to the Los Angeles classification of gastroesophageal reflux disease [13,16] visually was found in 42.9% of all rats, while in rats injected with both CEP and rats administered esomeprazole, MM esophageal damage was not detected (Tab. 3). The detected signs of esophagitis may indicate the development of gastroesophageal reflux, which may be due to increased acidity of gastric juice and impaired evacuation of the stomach.

Macroscopic evaluation of the stomach showed that rats treated with ASA only showed a clear (3 points on the assessment scale) statistically significant (p < 0.05) bloating, hemorrhage, redness and GM edema with folding disorders in 100% of the animals (Fig. 1B).

We found that all animals that were injected ASA only (control group) revealed ulcerative defects GM. Accordingly, the average score on the scale [3] was 3.3±0.29 (Tab. 4). This was consistent with the literature that quadruple administration of ASA over three days results in model subchronic GI tract injury. [3,13].

A study of the MM of the intestine showed that in addition to subchronic damage to the stomach, ASA,

moreover, caused damage to the small and large intestine in 100% and 57.1% of animals, respectively. Thus, in the small intestine, ASA caused an average of 11.0±0.9 ulcers per 10 cm in length, and the colon – 5.9±2.2 ulcers per 10 cm in length (Tab. 4). In addition, within the small intestine, in contrast to the colon, perforated ulcers were also found, with an average length of 3.0±0.8 per 10 cm (Tab. 4).

The combined use of ASA and esomeprazole in the treatment and prevention regimen led to a statistically significant (p<0.05) attenuation of the ulcerogenic effect of the studied NSAIDs. We noted that rats injected with ASA and esomeprazole (Group IV) did not show bloating and hyperemia of MM or mild GM edema with folding disorders, and single hemorrhages were observed in 45.2% and 57.1% of all animals, respectively (Tab. 3, Fig. 1D).

Mild hyperemia of GM in 28.6% of all rats and moderate hemorrhage in 57.1% of all rats were observed on the background of combined use of ASA and CEP, but in contrast to rats of the combined use of ASA and esomeprazole, there was no swelling and fold GM (Tab. 3, Fig. 1D). This can be considered a sign of no inflammatory reaction in GM and is associated with the anti-inflammatory properties of CEP [8].

In addition, we saw GM ulcerative defects in 57.1% of all rats administered ASA and CEP and in 42.9% of all rats

Table 3. GM condition due to ASA-induced gastropathy (M ± m or Me [LQ; UQ], n=28)

Experimental conditions		Indicator				
		Flatulence	Hemorrhages	Hyperemia	Edema	Folding disorders
Intact rats (n=7)	Abs. (%)	0/7 (0)	0/7 (0)	0/7 (0)	0/7 (0)	0/7 (0)
	Points	0	0	0	0	0
ASA (n=7)	Abs. (%)	7/7* (100)	7/7* (100)	7/7* (100)	7/7* (100)	7/7* (100)
	Points	3 [2; 3]	3 [2; 3]*	3 [3; 3]*	3 [2,5; 3]*	3 [3; 3]*
ASA + CEP (n=7)	Abs. (%)	0/7# (0)	4/7* (57,1)	2/7# (28,6)	0/7# (0)	0/7# (0)
	Points	0#	2 [0; 3]*	0 [0; 0,5]	0#	0#
ASA + Esomeprazole (n=7)	Abs. (%)	0/7# (0)	4/7* (57,1)	0/7# (0)	3/7*# (42,9)	2/7# (28,6)
	Points	0#	1 [0; 2]*#	[;]	0 [0; 2] #	0 [0; 1] #

Notes
 1 * - p<0.05 relative to intact animals;
 2 # - p<0.05 relative to rats that received only ASA;
 3 Abs. - absolute numbers

Table 4. The state of the MM of the proximal and distal parts of the GI tract in ASA-induced damage (M ± m, n=28)

Parts of the gastrointestinal tract:		Esophagus				Stomach			Small intestine			Colon		
Indicator	Number of animals, n	Number of animals with M-degree of damage, abs. (%)	Number of animals with ulcers, abs. (%)	Average score in the group	Ulcerative index	Number of animals with ulcers, abs (%)	The number of ulcers per 10 cm in length			Number of animals with ulcers, abs. (%)	The number of ulcers per 10 cm in length			
							total number	with hemosiderin	perforated		total number	with blood clots	perforated	
Intact rats	7	0 (0)	0 (0)	0	0	0/7 (0)	0	0	0	0/7 (0)	0	0	0	
ASA	7	3/7* (42.9)	7/7* (100)	3.3 ±0.29*	3.3	7/7* (100)	11.0 ±0.9*	6.0 ±1.0*	3.0 ±0.8*	4/7* (57.1)	5.9 ±2.2*	2.6 ±1.0*	0	
ASA + CEP	7	0/7# (0)	4/7# (57.1)	1.7 ±0.29*# 0	0.97	0/7# 0 (0)	0# 0	0# 0	0*# 0	0/7# 0 (0)	0 #0	0# 0	0	
ASA + Esomeprazole	7	0/7# (0)	3/7# (42.9)	0.9 ±0.26*#	0.39	7/7# (100)	7,3 ±1.0*#	3,4 ±1.3*	1,6 ±0.4*	7/7 (100)	10,4 ±1.3*	1,7 ±0.5*	0	

Notes
 1 * - p<0.05 relative to intact animals;
 2 # - p<0.05 relative to rats that received only ASA;
 3 0 - p<0.05 relative to rats treated with ASA and esomeprazole

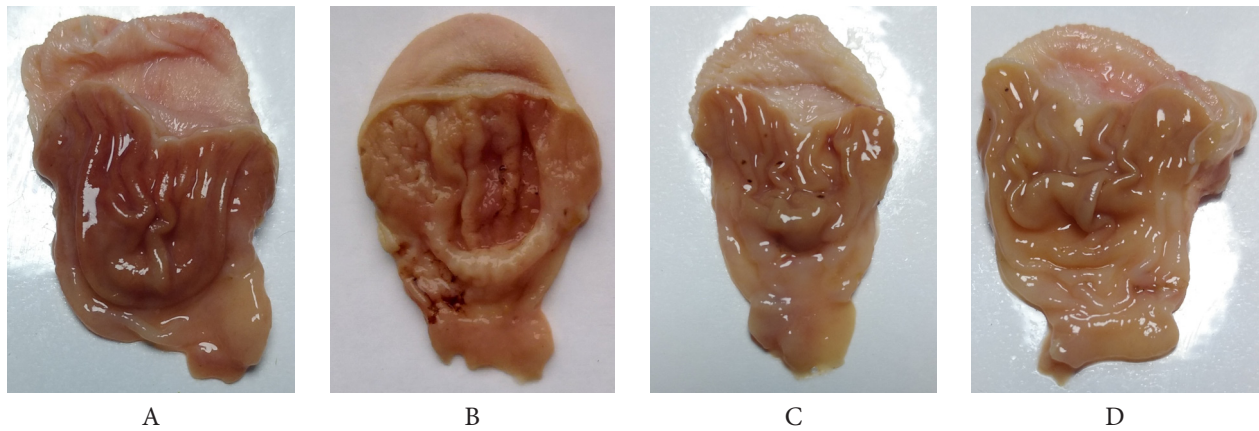


Figure 1. GM Condition: A – intact rat (Group I); B – rats with ASA-induced ulcerogenesis (Group II); C – rats with ASA-induced ulcerogenesis administered CEP (Group III); D – rats with ASA-induced ulcerogenesis administered esomeprazole (Group IV)

injected with ASA and esomeprazole. Herein, the ulcer index was 0.97 and 0.39, respectively, which were 3.4 and 8.5 times lower, respectively, than that in the control rats (3.3) (Fig. 1C). The obtained data are consistent with the literature reports describing the pronounced gastroprotective effect of esomeprazole, in particular, in GM lesions caused by NSAIDs, due to its acid-suppressive effect that may be mediated by interaction with vanilloid receptors type 1 [3,8].

The results of macroscopic assessment of the condition of the MM distal parts of the GI tract deserve special attention. We discovered that the combined use of ASA and esomeprazole had little effect on the ulcerogenic effect of the studied NSAIDs in the small intestine, and in the colon it was even associated with a more pronounced damage of MM. Thus, ASA caused colon damage in 57.1% of all control rats, and in the combined use of ASA and esomeprazole – in 100% of all rats, and the average number of ulcers was 1.8 times higher than that of control rats and was, respectively, 10.4 ± 1.3 and 5.9 ± 2.2 ulcers per 10 cm in length (Tab. 4). The obtained data are consistent with the literature data on the ability of PPI to enhance the damaging effects of NSAIDs in the distal parts of the GI tract, in particular, by increasing the aggressiveness of the microbiota [5].

No damage to the small and large intestine was detected on the bodies of combined use of ASA and CEP in the treatment-and-prophylactic regimen, which indicates the presence of the cytoprotective properties of the latter (Tab. 4).

CONCLUSIONS

1. Fivefold administration of ASA at a dose of 150 mg/kg causes damage to the esophagus, stomach, small and large intestines in 100% of the rats.
2. The use of the proton pump inhibitor esomeprazole has pronounced gastrocytoprotective properties, but does not affect the ulcerogenic effect in the small intestine, and even enhances it in the colon. This is demonstrated by ulcerative lesions of the colon in 57.1% of all rats that were administered ASA and esomeprazole.
3. The use of cryopreserved placenta extract is statistically significantly ($p < 0.05$) inferior to the antiulcer activity of esomeprazole in the stomach. Thus, the ulcer index

on the background of the use of ASA and cryopreserved placenta extract was 0.97, and on the background of the use of ASA and esomeprazole – 0.39.

4. In the distal parts of the GI tract, cryoextract of the placenta showed cytoprotective properties against induced ASA ulcerogenesis, in contrast to esomeprazole.

ABBREVIATIONS

ASA – acetylsalicylic acid;
 CEP – Cryopreserved extract of placenta;
 COX – cyclooxygenase;
 GI tract – gastrointestinal tract;
 GM – gastric mucosa;
 i.g. – intragastrically;
 MM – mucous membrane;
 NSAIDs – nonsteroidal anti-inflammatory drugs;
 PPIs – proton pump inhibitors;
 UD – ulcerogenic dose.

ORCID iDs

Fedir Vol. Hladkykh  <https://orcid.org/0000-0001-7924-4048>

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