ORIGINAL ARTICLE

The effect of meloxicam and cryopreserved placenta extract on initial inflammatory response – an experimental study

Vliv meloxikamu a extraktu z kryokonzervované placenty na počáteční zánětlivou reakci – experimentální studie

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Summary

Alteration is the first phase of the inflammatory process triggering the entire cascade of inflammation and causing destructive changes in the affected tissues. Therefore, suppression of inflammation at this point is essential for the success of anti-inflammatory therapy. Previous experimental studies have shown that the combined use of nonsteroidal anti-inflammatory drugs and drugs with pleiotropic mechanisms of action may potentiate their anti-inflammatory properties. The purpose of my work was to characterize the combined effect of cryopreserved placenta extract and meloxicam on the alterative phase of the inflammatory process in a model of aseptic skin and subcutaneous tissue inflammation in rats. Subcutaneous administration of acetic acid and of dextran was found to result in formation of necrotic ulcers in 100% of rats by day 7 of the experiment. The most pronounced antialterative effect (23.9%) on day 7 of the experiment was observed with the combined use of meloxicam and cryopreserved placenta extract – the area of necrotic ulcers was 1.3-fold smaller ($p < 0.05$) as compared with control rats (without treatment). On day 27 of the experiment, the antialterative effect of combined treatment and prophylactic use of meloxicam and cryopreserved placenta extract was 1.7 times higher than the effect in meloxicam monotherapy group, and 1.2 times higher than that in the placenta cryoextract monotherapy group.

Key words: cryopreserved placenta extract • meloxicam • inflammation • alteration

Souhrn

Alterace je první fází zánětlivého procesu, která spouští celou kaskádu zánětu a způsobuje destruktivní změny v postižených tkáních. Proto je potlačení zánětu v této fázi nezbytné pro úspěch protizánětlivé léčby. Předchozí experimentální studie ukázaly, že použití nesteroidních protizánětlivých léčiv v kombinaci s léčivy s pleiotropními účinky může zesílit jejich protizánětlivé vlastnosti. Cílem mé práce bylo charakterizovat kombinovaný účinek extraktu z kryokonzervované placenty a meloxikamu na alterativní fázi zánětlivého procesu v modelu aseptického zánětu kůže a podkožní tkáně u potkanů. Bylo zjištěno, že subkutánní podání kyseliny octové a dextranu vedlo k tvorbě nekrotických vředů u 100 % potkanů do 7. dne experimentu. Nejvýraznější antialterativní účinek (23,9 %) 7. den experimentu byl pozorován při kombinovaném použití meloxikamu a kryokonzervovaného extraktu z placenty – plocha nekrotických vředů byla 1,3krát menší ($p < 0,05$) ve srovnání s kontrolními potkany (bez léčby). V 27. dnu experimentu byl antialterativní účinek kombinované léčby a profylaktického použití meloxikamu a kryokonzervovaného placentárního extraktu 1,7krát vyšší než účinek ve skupině s monoterapií meloxikamem a 1,2krát vyšší než ve skupině s monoterapií placentárním kryoextraktem.

Klíčová slova: kryokonzervovaný extrakt z placenty • meloxikam • zánět • alterace

Introduction

Inflammation is known to be a protective living organism reaction, but considering the deleterious effects of its chronicity, artificial suppression of

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inflammation may be required. The largest body of evidence in terms of anti-inflammatory efficacy belongs to the group of nonsteroidal anti-inflammatory drugs (NSAIDs). All members of this group posess a nonspecific anti-inflammatory activity, the leading mechanism of which is the supression of prostaglandin synthesis by inhibition of cyclooxygenase and of the arachidonic acid cascade. However, along with cyclooxygenase inhibition, the anti-inflammatory effects of NSAIDs are also associated with supression of lipid peroxidation, inhibition of lysosomal enzymes, inhibition of synthesis of macroergic compounds of oxidative phosphorylation (i.e., reduction of energy supply in inflammatory process and inhibition of cell chemotaxis to the inflammatory focus), suppression of neutrophil aggregation and hence decrease of release of inflammatory mediators (e.g., bradykinin, lymphokines, leukotrienes, complement components), suppression of the proliferative inflammation phase by inhibiting the lymphocyte transformation production, rheumatoid factor production, and this list is still not complete.

NSAIDs are known to suppress mainly the exudation phase. The most potent drugs also act on the proliferation phase (by reducing collagen synthesis and subsequent tissue sclerosis), but this action is weaker than that on the exudative phase. NSAIDs have the least pronounced effect on the alteration phase, as they are not able to actively stabilize cell membranes and lysosomes, but to some extent they still inhibit lysosomal enzymes. The latter is the mechanism of their action upon the alteration phase.

Since alteration is the first phase of the inflammatory process triggering the entire cascade of inflammation and causing destructive changes in the affected

Table 1. Biologically active substances contained in the cryoextract of the placenta[4)]

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 polypotent stem cells, immunoendocrine system	201.7 pg/mL
IL-4	regulation of differentiation of polypotent stem cells, immunoendocrine system	21.7 pg/mL
IL-6	regulation of differentiation of polypotent stem cells, immunoendocrine system	114.9 pg/mL
Total protein	plastic function	76.5 ± 14 mg/g

tissues, the suppression of inflammation at this stage is important for the success of anti-inflammatory therapy[1].

Previous experimental studies have shown that the combined use of NSAIDs and of drugs with multivector mechanisms of action may potentiate their anti-inflammatory properties. Not only artificially synthesized molecules with multifunctional activity (thiotriazoline, phenycaberan), but also substances of natural origin, e.g., flavonoids (including quercetin), or biological drugs (including cryopreserved placenta extract [CEP]) can reduce the side effects of NSAIDs (gastrotoxicity, hepatotoxicity, nephrotoxicity, etc.)[2, 5].

Our institution has developed a technology for obtaining CEP as well as a technology for its long-term storage in a cryobank. Years of research have shown that CEP affects target organs by stimulating their function, and increases non-specific resistance to adverse environmental factors and stressors, stimulates reparative cell properties, possesses an anti-inflammatory activity, but to date there is no information about the differential effect of CEP on separate phases of an inflammation[4, 6].

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 (Table 1).

For the present study we have selected meloxicam which is an enolic acid derivative. In addition to inhibiting the activity and expression of cyclooxygenase (predominantly type II), meloxicam inhibits the activity of collagenase and reduces the expression of matrix metallopeptidases-1, which may explain its antiproliferative potential[7].

The aim of the present sudy was to characterize the combined effect of cryopreserved placenta extract and meloxicam on the alterative phase of the inflammatory process in a model of aseptic skin and subcutaneous tissue inflammation in rats.

Experimental part

Materials and methods

Experimental model of aceto-dextran skin ulcers in rats

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

- **I** – rats (n = 7) with aceto-dextran skin ulcers without treatment (control group)
- **II** – rats (n = 7) with aceto-dextran skin ulcers which were injected intramuscularly (i.m.) 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"; 0.16 mL/kg 5 times (Fig. 1)
- **III** – rats (n = 7) with aceto-dextran skin ulcers, which were administered intragastrically (i.g.) meloxicam ("Meloxicam – Astrapharm", Limited Liability Company "Astrapharm", Ukraine), 9.1 mg/kg 7 times (Fig. 1)
- **IV** – rats (n = 7) with aceto-dextran skin ulcers administered meloxicam (i.g.) and CEP (i.m.)

The effect of CEP on the antialterative effect of meloxicam was studied in a model of aseptic inflammation of the skin and subcutaneous tissue in rats, which manifests the formation of necrotic ulcers and allows to monitor the effectiveness of treatment

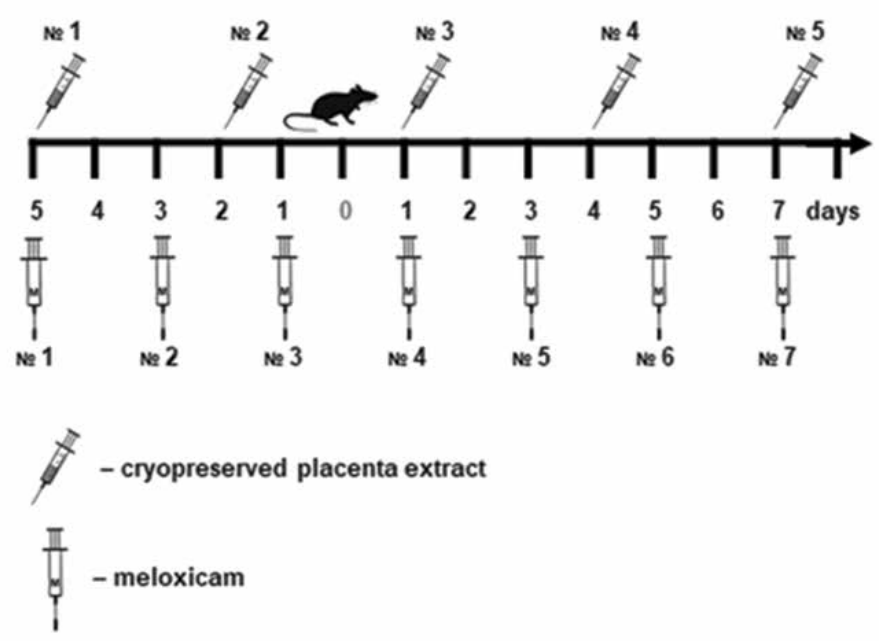

Fig. 1. Scheme of study of antialterative activity of CEP and meloxicam in a model of acetic acid-dextran skin ulcers in rats

for alternative inflammation. The alternative phase of inflammation was caused by subcutaneous injection in a pre-depilated area of the back 1.0 mL of 9.0% acetic acid solution with simultaneous intraperitoneal injection of 6.0% solution (solution) of dextran at a dose of 300 mg/kg per animal, causing coagulation necrosis of tissues, resulting in functional and trophic disorders and the formation of trophic ulcerative defects[7, 10]. The day of administration of acetic acid and dextran was considered day zero ("0") of the experiment.

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

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[7]. 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.

CEP was administered intravenously in the treatment-and-prophylactic mode – the administration was started 5 days before the simulation of the inflammatory-degenerative process (2 injections with an interval of 3 days) and continued (3 injections with an interval of 3 days, the first – 6 hours after introduction of acetic acid and dextran) to the formation of the maximum area of the ulcer on day 7 of the experiment (Fig. 1).

Meloxicam was administered at a dose of 9.1 mg/kg (ED_{50} for antiexudative activity) intravenously with an interval of 2 days, starting 5 days before the simulation of the inflammatory-degenerative process and for 7 days after administration of acetic acid and dextran (total 7 in actions)[1, 8].

Research methodology

Antialterative activity was assessed planimetrically by the total area of ulcers on the 7th, 12th, 17th, 22nd and 27th days of the experiment[1, 8]. To determine the area of acetic-dextran ulcers in rats, a transparent film was applied to the wound surface and the contour of the wound was applied, after which the film was digitized by scanning and its area in mm^2 using publicly available BioVision 4.0 software in Microsoft Windows. According to the results of measurements of the area of wound defects, antialterative activity was calculated[8, 10]:

The antialterative effect (AAE,%) was determined by the formula:

$$AAE = ((S_C - S_S) / S_C) \times 100\ \%,$$

where *AAE* – antialterative effect (%), S_C – defect area in rats of the control group (mm^2), S_S – the area of the defect in the rats of the experimental group (mm^2).

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 the normal distribution of the independent values, the differences between the groups were determined in pairs by the Student's t-test. In the case of an 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 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 concluded that the probability of error. Numerical data in the case of normal distribution of values are given as "M ± m" (M ± SE), where M is the arithmetic mean, m (SE) is the standard error of the mean or M (95% CI: 5–95%), where 95% CI is the 95% 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; UQ) is the upper limit of the lower quartile (LQ) and the lower limit of the upper quartile (UQ).

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 (Protocol № 2 of 11 March 2020).

Results and discussion

The study showed that subcutaneous administration of acetic acid with simultaneous administration of dextran leads to the formation of necrotic ulcers in 100% of rats on day 7 of the experiment with an average total area of 317.1 mm^2 followed bygradual healing of skin defects. Thus, on day 27 of the experiment, the area of necrotic ulcers in rats of the control group decreased to 60.0 mm^2, but no individual had been seen with complete healing within the specified period (Table 2).

Among the groups of rats with aceto-dextran skin ulcers treated (groups II–IV) on day 7 of the experiment, the lowest AAE was observed in the meloxicam group (13.5%), which was significantly lower than that in rats

of the CEP monotherapy group (18.6%). The identified antialterative properties of CEP were consistent with the literature on its ability to stimulate reparative processes and anti-inflammatory properties[4].

The most pronounced antialterative effect (23.9%) on day 7 of the experiment was observed with the combined use of meloxicam and cryopreserved placenta extract – the area of necrotic ulcers was was 1.3-fold smaller ($p < 0.05$) as compared with control rats (without treatment). On day 27 of the experiment, the antialterative effect of combined treatment and prophylactic use of meloxicam and cryopreserved placenta extract was 1.7 times higher than the effect in the meloxicam monotherapy group, and 1.2 times higher than that in the placenta cryoextract monotherapy group. The above data indicate the ability of CEP in the therapeutic and prophylactic mode of administration to cause anti-alterative effect on the model of aseptic inflammation of the skin and subcutaneous tissue in rats and consider its AAE as one of the mechanisms of its anti-inflammatory activity.

The revealed AAE of meloxicam is consistent with the data[11] where a comprehensive study of the anti-inflammatory effect of this drug was performed on a model of experimental wounds in rats. At the same time, it should be noted that time frames of action of different NSAIDs are not the same. Another study has shown that diclofenac sodium did not affect the healing processes of aceto-dextran ulcers in rats by day 8 of the experiment[1].

Subsequent observations of serial changes showed that the temporal dynamics of healing of necrotic-ulcerative skin defects in rats of all groups was linear and the defect area decreased by an average of 8.7% (values of animals in the control group) every subsequent 5 days, respectively, i.e., on the 12th, 17th, 22nd and 27th days of the experiment (Fig. 2).

This suggests that the treatment-and-prophylactic regimen of both meloxicam and CEP have an effect on the value of the initial defect area in the peak time of formation of aceto-dextran skin ulcers in rats (7–8 days) and then their natural regression. This indicates the feasibility of a preventive regimen of CEP to achieve AAE in the early stages of inflammatory-degenerative processes.

A study of the area of ulcerative defects of the skin in rats on day 27 of the experiment showed that in monotherapy with meloxicam complete healing of acetic-dextran ulcers was observed in 42.9% of animals, and the average area of remaining defects was 2.0 times lower than that in the animals of the control group (30.0 mm^2 vs 60.0 mm^2, respectively).

Table 2. The effect of CEP and meloxicam on the dynamics of the area of necrotic skin ulcers in rats, mm^2 (M ± m (95% CI) or Me [LQ; UQ], n = 28)

Experimental conditions	*n*	The term of the study							Number of animals with healed wounds on day 27, abs. (%)
		7 day		12 day	17 day	22 day	27 day		
		mm^2	AAE				mm^2	AAE	
Control group	7	317.1± 14.1 (95% CI: 289.4–344.8)	–	152.9 ± 3.6 (95% CI: 145.8–159.9)	118.6 ± 3.4 (95% CI: 111.9–125.2)	92.9 ± 5.2 (95% CI: 82.6–103.1)	60.0 [55.0; 70.0]	–	0/7 (0)
CEP	7	257.1 ± 4.2 (95% CI: 248.9–265.4)*	18.9%	75.7 ± 6.9 (95% CI: 62.3–89.1)*§	58.6 ± 5.1 (95% CI: 48.6–68.5)*§	37.1 ± 2.9 (95% CI: 31.5–42.7)*§	0.0 [0.0; 25.0]*§	81.4%	4/7 (57.1)
Meloxicam	7	274.3 ± 6.5 (95% CI: 261.6–287.0)*#	13.5%	115.7 ± 5.7 (95% CI: 104.5–126.9)*#§	81.4 ± 3.4 (95% CI: 74.8–88.1)*#§	55.7 ± 3.7 (95% CI: 48.5–62.9)*#§	30.0 [0.0; 50.0]*§	58.1%	3/7 (42.9)
Meloxicam + CEP	7	241.4 ± 5.5 (95% CI: 230.6–252.3)*#°	23.9%	61.4 ± 2.6 (95% CI: 56.3–66.5)*°§	40.0 ± 3.1 (95% CI: 34.0–46.0)*#°§	21.4 ± 2.6 (95% CI: 16.3–26.5)*#°§	0.0 [0.0; 0.0]*°§	100.0%	7/7 (100)

*p < 0.05 relative to the animals of the control group

#p < 0.05 relative to rats treated with CEP

°p < 0.05 relative to rats treated with meloxicam

§p < 0.05 relative to the indicators on the 7th day of the experiment (T – Wilcoxon test)

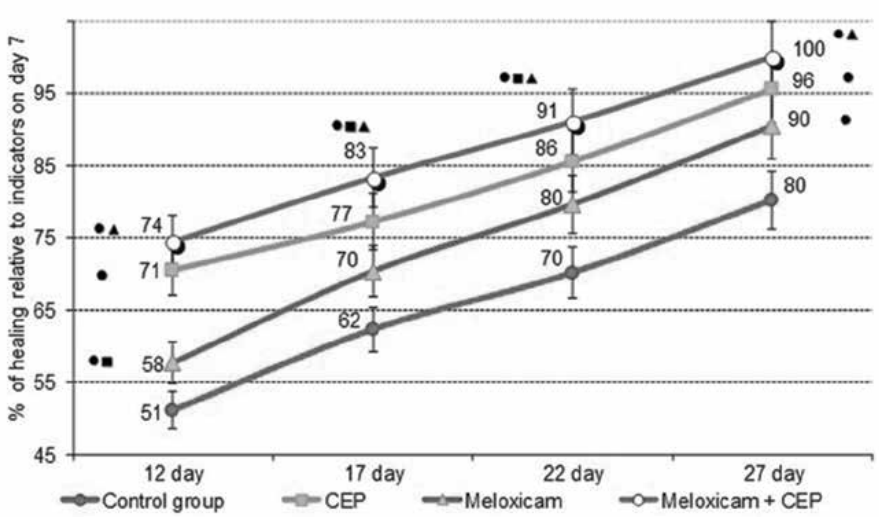

Figure 2. The effect of CEP and meloxicam on the dynamics of healing of necrotic skin ulcers in rats,%

● *p ≤ 0.05 relative to the indicators of animals of the control group*

■ *p ≤ 0.05 relative to rats treated with CEP*

▲ *p ≤ 0.05 relative to rats treated with meloxicam*

AAE of CEP on day 27 of the experiment was 81.4%, which was 1.4 times higher than in rats of the monotherapy group with meloxicam, and complete healing of ulcerative skin defects was observed in 57.1% of animals.

The most pronounced AAE on day 27 of the experiment was observed in the group receiving combined treatment and prevention of meloxicam and CEP (Table 2) – complete healing of ulcerative skin defects was observed in 100.0% of rats, which is 1.7 times higher than in the rats of meloxicam monotherapy and 1.2 times higher than in the CEP monotherapy group. The obtained data were proportionally consistent with the initial values of the area of aceto-dextran skin ulcers in rats on day 7 of the experiment.

Conclusions

1. Subcutaneous administration of acetic acid with simultaneous administration of dextran leads to the formation of necrotic ulcers in 100% of rats on day 7 of the experiment.
2. The most pronounced AAE (23.9%) on day 7 of the experiment was observed on the background of the combined use of meloxicam and cryopreserved placenta extract – the area of necrotic ulcers was statistically significant ($p < 0.05$) in 1.3 was less than in control rats (without treatment).
3. On day 27 of the experiment, the AAE on the background of the combined treatment and prophylactic use of meloxicam and CEP was 1.7 times higher than in rats of the meloxicam monotherapy group and 1.2 times higher than in the CEP monotherapy group.

Conflict of interest: none.

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