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MORPHOLOGICAL FEATURES OF A COLD SKIN WOUND UNDER THE INFLUENCE OF AN EXTRACT OF CRYOPRESERVED SKIN FRAGMENTS OF PIGLETS (EXPERIMENTAL STUDY)

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ABSTRACT

The aim of the study is to identify in an experiment the effect of an extract of cryopreserved fragments of piglets on the morphological state of a cold skin wound.

Materials and methods: Hairless six-month-old male rats were used in the study. They were divided into III groups: group I included 10 rats that had not been manipulated; group II was represented by 10 rats with cold wounds on the lateral surface of the thigh; group III was represented by 10 rats that were with a cold wound, followed by the injection of an extract of cryopreserved skin fragments of piglets into the abdominal cavity at a dose of 50 μg per 100 g of animal body weight (peptide concentration 100 μg/ml) once a day for 5 days from the time of wound modeling. Animals in groups I-III were withdrawn from the experiment on the 7th, 14th and 21st days. The material for the morphological study was the fragments of intact skin with underlying soft tissues from the thigh area in group I and the fragments of skin with underlying soft tissues from the thigh area directly from the zone of cryoexposure in groups II and III. Histological, histochemical and morphometric methods were used. Microspecimens were studied using an Olympus BX-41 microscope (Japan). Statistical processing was performed using the Statistica 6.0 and Microsoft Excel 2003 software package. Nonparametric methods were used to compare numerical values (Mann-Whitney U-test, Kruskal-Wallis test). The significance of differences between the average values of the indicators was taken at the level of $p<0.05$.

Results: The extract of cryopreserved skin fragments of piglets has an effective wound healing effect compared to the healing processes in a cold wound, which was not subjected to any therapeutic effects. It was manifested in the improved process of cleansing the wound from necrotic tissues that entered the zone of primary necrosis, as evidenced by 1,2 times decrease of the zone of primary necrosis on the 7th, 14th and 21st days; a decrease of the zone of secondary necrosis on the 7th, 14th and 21st days, respectively, – 1.2, 1.3, 1.2 times; growth and maturation of granulation tissue activation, as evidenced by an increase in the thickness of a granulation tissue layer on 7, 14, 21 days, respectively, – 1.9, 1.8, 1.2 times; activation of proliferative processes in the epithelial layer located in the marginal sections of the wound defect or covering the regenerate surface, as evidenced by more pronounced acanthotic growths in the underlying tissue and an increase in the thickness of the epithelial layer on the 7th, 14th and 21st days, respectively, – 2.1, 2.0, 2.2 times.

Conclusion: The extract of cryopreserved skin fragments of piglets has an effective wound healing effect and can be recommended for further research in order to study the possibility of its use in clinical practice.

KEY WORDS: cold wound, skin, morphological study, extract of cryopreserved skin fragments of piglets

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INTRODUCTION

The skin is the largest organ of the body that serves multiple functions [1]. It plays an important role in maintaining vital functions of the body through thermoregulation and water-electrolyte balance, acts as a barrier to external influences and represents a field of receptors with various types of sensitivity [2].

The study of the regenerative processes patterns in skin wounds is currently one of the important problems in modern theoretical and practical medicine. The wound process, as we know, is a complex multifaceted phenomenon in which there are three essential components: damage, inflammation and recovery. Many scientists distinguish three stages of the wound healing process: 1) traumatic inflammation, 2) formation of the connective (granulation) tissue, epithelial regeneration, 3) scar formation and remodeling [3].

The effectiveness of treatment is determined by the qualitative and quantitative characteristics of the morphological equivalent, including structural and functional assessment of the skin condition and underlying tissues, features and duration of the recovery period [4].

Features of skin wounds healing depend on many factors, including the nature of the damaging agent [5], one of which is the temperature factor. Cold wounds can occur as a result of atmospheric effects on the body or due to cooling the tissues for medicinal purposes, for example, in cryosurgery [6, 7].

It is known that skin wounds caused by cryoexposure heal more slowly as compared to wounds of a different

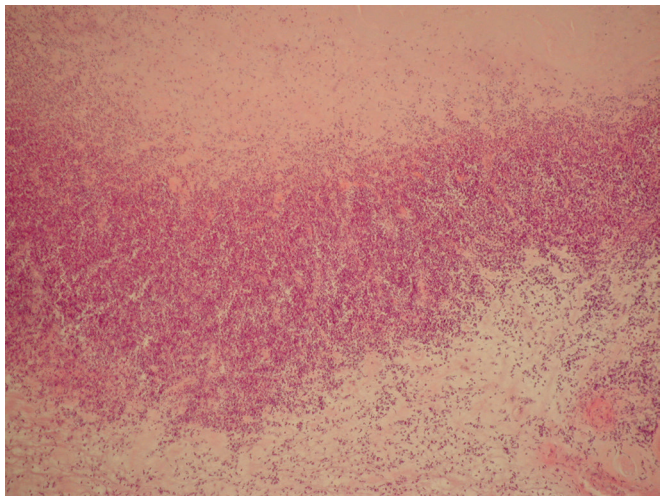

Fig. 1. The multilayer structure of the wound defect (a layer of primary necrosis, a demarcation leukocyte shaft, a layer of secondary necrosis) in the rat of group III on the 7th day of the experiment. Stained with hematoxylin and eosin, ×100.

genesis. This can be explained by the two-stage nature of tissue damage during cryodestruction. At the first stage this is a direct damaging effect of low temperatures. At the second stage the tissue damage is caused by vascular disorders [5].

Treatment of cold skin wounds is a long, laborious and costly process, which actualizes the problem of finding more effective ways to do with this pathology. A promising direction to improve treatment results may be the use of approaches from the arsenal of cell and tissue therapy, including drugs of xenogenic origin [8-10]. Thus, the study of the possibility to use the extract of cryopreserved fragments of the skin of piglets in treatment of cold skin wounds is of scientific and practical interest.

THE AIM

The aim of the study is to identify in an experiment the effect of an extract of cryopreserved fragments of piglets on the morphological state of a cold skin wound.

MATERIALS AND METHODS

Six-month-old male rats were used in the study. All manipulations with the animals were carried out in accordance with the Law of Ukraine «On Protection of Animals from Cruelty» (No. 3447-IV of 02.21.2006), subject to the requirements of the bioethics committee of the Institute for Problems of Cryobiology and Cryomedicine of the National Academy of Sciences of Ukraine, consistent with the provisions of the «European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes» (Strasbourg, 1986). During the study all animals were divided into III groups: group I (a control group) included 10 rats that did not undergo any manipulations; group II consisted of 10 rats, which had cold wounds simulated on the lateral surface of the thigh; group III was represented by 10 rats who had a cold wound simulated on the lateral surface of the thigh, followed by the injection of an extract of cryopreserved skin fragments into the abdominal cavity at a dose of 50 μg per 100 g of animal body weight (peptide concentration 100 μg / ml) once a day for 5 days from the moment of modeling the wound. Animals of groups I-III were withdrawn from the experiment on days 7, 14 and 21.

In groups II and III rats' skin underwent low-temperature impact by a cryo tool – cold accumulator, which made it possible to maintain the temperature of the applicator's working surface no higher than -50°C during the entire exposure period [11]. The cryoapplicator diameter was 10 mm. Time of cold exposure was 120 s.

The material for the morphological study was the fragments of intact skin with underlying soft tissues from the thigh area in group I and the fragments of skin with underlying soft tissues from the thigh area directly from the zone of cryoexposure in groups II and III. The obtained experimental material was fixed in a 10% formalin solution. Compaction of tissues fixed in formalin was achieved by passing through alcohols of increasing concentration, Nikiforov liquid (96% alcohol and diethyl ether in a 1:1 ratio), chloroform and paraffin filling. Serial sections of $4\text{-}5\times10^{-6}$ m thick were prepared from the blocks. Histological and histochemical staining methods such as hematoxylin and eosin, picrofuchsin according to van Gieson and Mallory were used.

The microspecimens were studied on an Olympus BX-41 microscope (Japan), followed by processing with the Olympus DP-soft version 3.1 software package. A morphometric study was carried out with it. During the study it was determined the thickness of the epithelial layer (in group I at an arbitrary place, in groups II and III of the areas bordering on the wound defect), the zone of primary necrosis, the demarcated leukocyte shaft, the zone of secondary necrosis, a layer of granulation tissue, a layer of connective tissue.

Statistical processing was performed using the Statistica 6.0 and Microsoft Excel 2003 software package. Nonparametric methods were used to compare the parameters (Mann-Whitney U-test, Kruskal-Wallis test). The significance of differences between the average values of the indicators was taken at a level of $p<0.05$.

RESULTS AND DISCUSSION

Surveying microscopy in groups II and III on days 7, 14, and 21 revealed a wide and deep wound defect in the area of cryoexposure, the depth of which reached the dermis, and in part of the visual fields, the hypodermis. This defect was characterized by the presence of a primary necrosis zone, a demarcation leukocyte shaft, a zone of secondary necrosis and a layer of granulation tissue (fig. 1). In a cold wound, as is known, the zone of primary necrosis is caused by the direct damaging effect of low temperatures, while the zone of secondary necrosis develops as a result of hemodynamic disturbances. The multilayer structure of a

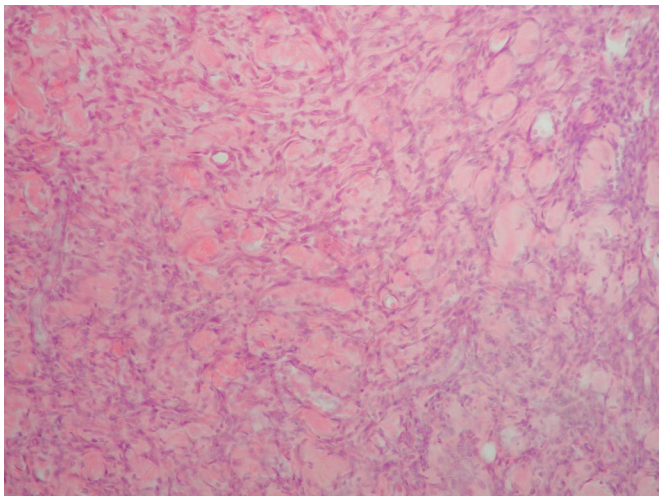

Fig. 2. Granulation tissue filling the wound defect in the rat of group III on the 14th day of the experiment. Stained with hematoxylin and eosin, ×200.

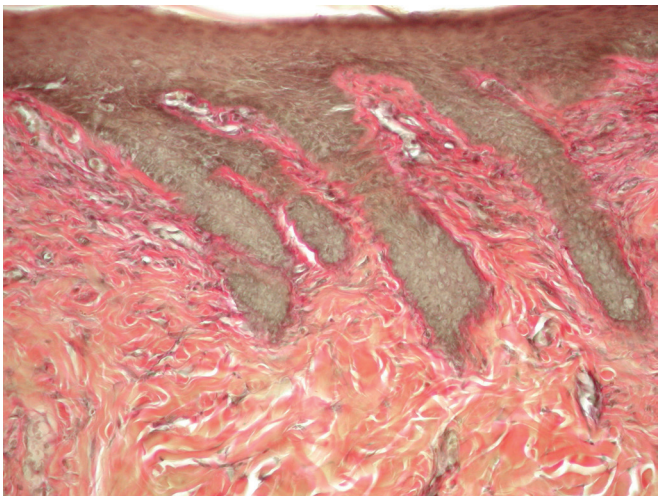

Fig. 3. Thickening of the epithelial layer and acanthotic growths in the underlying dermis tissue in the region of the marginal sections of the wound defect in the rat of group II on the 14th day of the experiment. Stained with picrofuchsin according to van Gieson, ×200.

cold wound noted by us was also described in our previous studies [12], as well as in the works of other scientists [13].

In groups II and III, the microscopic and morphometric features of the wound defect layers depended on the group and duration of the experiment.

When analyzing the thickness of the wound defect layers (table 1), it was found that the healing processes in group III proceeded faster than in group II. So, in group III compared with group II on the 7th, 14th and 21st days, the indicators of the thickness in the zone of primary and secondary necrosis were significantly ($p<0.05$) smaller, and the thickness of granulation tissue layer was significantly ($p<0.05$) larger. In group III, compared with group II, the thickness of the demarcation leukocyte shaft on days 7 and 14 did not differ much ($p>0.05$), and on day 21 it was significantly ($p<0.05$) larger. The healing of a wound defect in groups II and III with an increase in the duration of the experiment was indicated by a noticeable ($p<0.05$) decrease of the thickness of the primary necrosis zone, demarcation leukocyte shaft, zone of secondary necrosis and an increase of the thickness of the granulation tissue layer.

We revealed the presence of a pronounced demarcation leukocyte shaft in group III compared with group II, especially on the 21st day of the experiment, during a histological and morphometric examination. The presence of a pronounced demarcation zone can be associated with the accelerated formation of a granulation tissue and epithelization of the skin wound [3].

Wound healing is a complex process taking place at various levels (molecular, subcellular, cellular, tissue and organ), the ultimate goal of which is to repair damage with maximum restoration of the anatomical structure with minimal functional loss. In the case of a deep wound defect development, its cavity, as a rule, is replaced by granulation tissue, which then matures and turns into connective tissue [14]. Granulation tissue is characterized by the presence of cellular, vascular and fiber components and, during its maturation, the specific volume of the fiber component increases, while the specific volumes of the cellular and vascular components decrease [2].

In groups II and III in the granulation tissue filling the wound cavity, the cellular, vascular and fiber components were determined (fig. 2), the ratio of which depended on the duration of the experiment. We noticed that in all groups with an increase in the observation time in the granulation tissue, the fiber component began to prevail over the cellular and vascular, which indicated its maturation. The above feature was more pronounced in group III compared with group II.

In the course of survey microscopy, we noted a higher content of the vascular component in granulation tissue in all experimental periods in group III as compared to group II, which, in our opinion, led to an improvement in granulation tissue trophism and, consequently, to accelerated healing of the wound defect.

In the studied groups the character of polymorphic cell infiltration was different in granulation tissue. So, on day 7, cell infiltration was represented by a significant number of leukocytes and an insignificant number of macrophages, lymphocytes and the cells of fibroblastic differon. With an increase in the observation period, the number of leukocytes was decreasing, while the number of lymphocytes, macrophages and, especially, fibroblast cells was increasing. Over the course of all periods, a greater number of macrophages, lymphocytes and fibroblast cells were detected in the granulation tissue in group III compared with group II.

Macrophages, as is known, carry out not only phagocytosis of tissue detritus, but also secrete various biologically active substances that regulate intercellular and cell-tissue interactions. Macrophages are also able to isolate angiogenesis inducers that stimulate proliferation of endotheliocytes. In addition, macrophages stimulate fibronectin, which enhances chemotaxis of fibroblasts [1, 15].

Table 1. The thickness of the layers (×10-6m) of the wound defect in groups II and III

Group number	Layer name	The term of the animals withdrawn from the experiment, days		
		7	14	21
II	zone of primary necrosis	1975.88±93.729	1530.96±62.474 $p_1<0.05$	579.94±22.044 $p_1<0.05$
	leukocyte demarcation shaft	329.46±33.185	249.83±15.238 $p_1<0.05$	94.61±5.736 $p_1<0.05$
	zone of secondary necrosis	482.71±18.972	393.25±9.189 $p_1<0.05$	203.28±4.244 $p_1<0.05$
	granulation tissue layer	148.33±20.419	203.83±12.581 $p_1<0.05$	541.77±23.999 $p_1<0.05$
III	zone of primary necrosis	1617.17±42.181 $p_2<0.05$	1330.97±27.187 $p_1<0.05$ $p_2<0.05$	487.42±4.798 $p_1<0.05$ $p_2<0.05$
	leukocyte demarcation shaft	331.40±15.093 $p_2>0.05$	277.77±6.783 $p_1<0.05$ $p_2>0.05$	162.83±4.486 $p_1<0.05$ $p_2<0.05$
	zone of secondary necrosis	397.80±12.580 $p_2<0.05$	292.63±2.939 $p_1<0.05$ $p_2<0.05$	171.75±3.065 $p_1<0.05$ $p_2<0.05$
	granulation tissue layer	275.47±7.384 $p_2<0.05$	368.97±3.481 $p_1<0.05$ $p_2<0.05$	667.97±3.458 $p_1<0.05$ $p_2<0.05$

Note: p1 – the significance of the differences compared with the previous term of the animals withdrawn from the experiment, p2 – the significance of the differences compared with group II.

Table 2. The thickness of the epithelial layer (×10-6m) in groups I-III

Group number	The term of the animals withdrawn from the experiment, days		
	7	14	21
I	21.33±0.711		
II	26.75±1.154 $p_1<0.05$	32.92±1.268 $p_1<0.05$ $p_2<0.05$	39.60±1.079 $p_1<0.05$ $p_2<0.05$
III	56.10±2.023 $p_1<0.05$ $p_3<0.05$	65.40±1.704 $p_1<0.05$ $p_2<0.05$ $p_3<0.05$	85.43±2.185 $p_1<0.05$ $p_2<0.05$ $p_3<0.05$

Note: p1 – the significance of the differences compared with group I, p2 – the significance of differences compared to the previous period of animal withdrawal from the experiment, p3 – the significance of the differences compared with group II.

Immunocompetent cells play an important role in ensuring the development of reparative processes. Lymphocytes are capable of producing lymphokines that inhibit or stimulate proliferation and functional activity of fibroblasts [3, 15].

At present stem cells, organ-specific progenitor cells (prefibroblasts), differentiating fibroblasts, reparative fibroblasts, myofibroblasts, fibroclasts, fibrocytes are referred to the cells of fibroblastic differon. Stem cells and organ-specific progenitor cells are cambial, reserve cells. Mature fibroblasts are the differentiated cells, the most active in the functional respect, ensure the maintenance of homeostasis, perform synthetic, resorptive and regulatory functions when updating or remodeling tissues. They synthesize fiber proteins (elastin, collagen), components of the intercellular matrix (fibronectin, glycosaminoglycans). Studies have shown the presence of two types of fibroblasts. The most numerous type of them includes short-lived fibroblasts, characterized by intense proliferation. They are the most important in wound healing. The second type is long-lived fibroblasts, which are characterized by a lower level of synthetic processes [16, 17].

A thickened epithelium with the signs of hyperplasia in the marginal sections of the wound defect was noted in groups II and III (fig. 3). The results of our morphometric study has revealed that the average thickness of the epithelial layer significantly ($p<0.05$) increased in groups II and III with an increase in the experimental time compared with group I (table 2). The epithelial layer thickness index was significantly ($p<0.05$) larger at all experimental periods in group III compared with group II.

Epithelial regeneration is characterized by three interconnected processes: cell migration, proliferation and differentiation. The regenerating epithelium is characterized by the presence of glycogen granules in the cytoplasm, an increased content of ribonucleoproteins and redox enzymes. With the differentiation and formation of a multilayer epidermal layer, these qualities are lost [3].

During survey microscopy we also noted that the epithelial layer in the marginal sections of the wound defect or the epithelial layer covering the regenerate surface formed acanthotic growths in the underlying tissue (fig. 3). The

number of acanthotic growths, both shallow and deep, was as large as possible in group III compared with group II. Many scientists who studied the morphological features of the skin wounds healing in the experiment also noted acanthotic growths in their studies. It is known that the formation of skin derivatives is associated with submerged growth of the epithelium in prenatal ontogenesis; therefore, the appearance of protrusions in the regenerating epidermis is regarded by many as realization of morphogenetic potentials of the wound epidermis under new conditions [3].

Thus, the structural and functional states of the cold skin wound in rats, exposed to the extract of cryopreserved fragments of piglet skin, were objectively assessed in the course of a comprehensive morphological study of the experimental material. High capability of the extract of cryopreserved fragments of the piglets' skin to stimulate the healing processes in cold skin wounds has been shown. Our results are consistent with the studies showing that the intraconjunctival use of a pig skin extract can significantly improve and accelerate the regenerative processes in the corneal tissue after alkali burns [18].

CONCLUSIONS

The extract of cryopreserved fragments of piglets' skin has an effective wound healing effect, which was manifested in improvement in the processes of cleansing the wound from the necrotic tissues that entered the zone of primary necrosis, as evidenced by a 1.2-fold decrease of the area of primary necrosis on 7, 14, 21 days; a decrease of the zone of secondary necrosis on 7, 14, 21 days, respectively, 1.2, 1.3, 1.2 times; activation of growth and maturation of the granulation tissue, as evidenced by an increase in the thickness of the granulation tissue layer on 7, 14, 21 days, respectively, 1.9, 1.8, 1.2 times; activation of proliferative processes in the epithelial layer, located in the marginal sections of the wound defect or covering the regenerate surface, as evidenced by more pronounced acanthotic growths in the underlying tissue and an increase of the thickness of the epithelial layer on days 7, 14, 21, respectively, 2.1, 2.0, 2.2 times.

The studied extract of cryopreserved fragments of the piglets' skin can be recommended for further research in order to study its possible application in clinical practice.

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Conflicts of interest:
Authors declare no conflict of interest.

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