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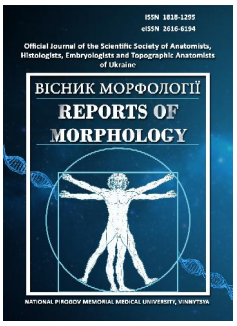

Morphological reasoning of the efficiency of application "Iruksan" in the experiment

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Treatment of infected and purulent wounds remains an actual problem nowadays. Scientific and practical interest is caused by the use of collagenase enzyme for wound debridement. Aim: to study morphological changes in contaminated and purulent wounds when using "Iruksan" ointment containing collagenase. To reproduce a wound infection, the wound was contaminated with a pathogenic strain of Staphylococcus aureus in combination with Pseudomonas aeruginosa. Microbiological, histological and statistical studies were conducted during the experiment. Control of contamination and identification of pathogens took place in all rats after contamination before use of ointment and at the day of exclusion from experiment. Due to microbiological findings, number of microorganisms in the wounds of animals of the control group remained practically unchanged ($p>0.05$) until the 10th day of observation, and in the experimental group ("Iruksan" ointment was used in the treatment of wounds), the number of microorganisms in the wounds has already decreased by the 7th day of the experiment ($p<0.05$). The obtained data correlate with the results of a morphological findings, especially with the severity of the inflammatory process. We noticed reduction of signs of the inflammatory process and the improvement of epithelization of the wound defect in the group of animals that were treated with "Iruksan" ointment containing collagenase. Due to the results of histological studies, complete coverage of the wound surface with newly formed epithelium was revealed on the 7th day of the experiment in the experimental group, in contrast to the control group where, on the 7th day, typical signs of the wound process for this time period were determined. In the control group of animals, epithelization of wounds was observed on the 10th day. The obtained data demonstrate expedience of use collagenase-based ointment for the treatment of wounds in the first phase of the wound process. It results in faster wound bed cleaning from necrotized tissues and enhance epithelialization.

Keywords: *purulent wound, infected wound, wound process, collagenase, morphological changes in the wound, surgical treatment of the wound.*

Introduction

The problem of diagnosis and treatment of purulent wounds, despite many years of experience and constant attention of scientists, does not lose its relevance. Currently, 30-40 % of patients in surgical departments are patients with purulent-inflammatory diseases. The frequency of purulent-inflammatory complications of postoperative wounds ranges from 2 to 4 % [3]. During operations on organs of the abdominal cavity and abdominal wall, it can reach even 21.1 % [4]. In the general structure of nosocomial infection, almost 45 % of cases are due to postoperative complications, and the mortality rate for purulent infection and its complications has practically not changed over the past 25-30 years and ranges from 35 to 40 % [6, 19]. It is worth taking into account the increase in the number of elderly patients with chronic diseases, namely diabetes, atherosclerosis of the vessels of the lower extremities and chronic venous insufficiency, which contributes to the increase in the number of patients with chronic wounds. A wide range of etiological factors causing the occurrence of chronic contaminated wounds requires a complex approach to treatment, which is based on the principles of pathogeneticity, timeliness and systematicity [5, 9, 17].

It is known that in the treatment of infected wounds, doctors adhere to fundamental principles that involve the use of a combination of surgical and medical treatment methods [13, 14, 15]. The modern approach to wound healing

offers a new therapeutic strategy based on stem cells [12]. At the same time, an important place in the treatment of soft tissue wounds belongs to multicomponent drugs for local use, the choice of which depends on the phase of the wound process. This allows you to ensure the maximum concentration of active substances in the focus of inflammation and stimulate reparative processes. The effectiveness of wound healing is determined not only by time, but also by the achieved aesthetic result. Because even after complete healing of wounds, undesirable consequences for patients may remain, such as, for example, excessive scarring, which in turn has a long-term negative impact and requires additional treatment [10, 18]. To accelerate the process of granulation and shorten the healing time of wounds, preparations of proteolytic enzymes are used, the basis of which is the ability to clean wounds from necrotic tissues and exudate [1]. The use of enzyme preparations as a part of external therapy has features due mainly to their instability, which makes it urgent to improve the existing dosage forms both in terms of stability and efficiency, as well as ease of use.

Collagenases are one of the most effective proteolytic enzymes, as they have the ability to break down collagen, the main component of wounds and scars. Collagen is the strongest protein of tissue detritus [14, 16]. It is also one of the components of the formation of the extracellular matrix of granulation tissue in the proliferative phase of wound healing [8, 11]. According to the results of numerous studies, there is no universal method for performing surgical treatment. Modern approaches combine surgical, mechanical, autolytic and biological methods. At the same time, wounds containing collagen fibers are poorly amenable to cleaning with the help of other proteolytic enzymes - trypsin, chymopsin, streptokinase. It is worth noting that in the United States of America, for example, the above-mentioned enzymes are generally prohibited for use as means for local application. At the same time, collagenase has a selective effect on one protein of wound detritus - collagen, which makes it safe to use as a topical preparation for enzymatic lysis of necrotic tissues [2, 8, 13, 14].

Currently, there are combined medicinal products widely available, the composition of which includes collagenase, which significantly accelerates the purchase of the inflammatory process, accelerates the formation and maturation of granulation tissues with a simultaneous active process of epithelization. However, clear indications for their use and evidence of the effectiveness of use in the treatment of infected and purulent wounds require further research, including morphological.

The purpose of the work: to study the morphological changes in the tissues of contaminated and purulent wounds of rats in the experiment during their treatment with "Iruksan" gel, which contains collagenase.

Materials and methods

Research was carried out on the basis of the department of general surgery, the department of microbiology and the experimental clinic (vivarium) of Vinnytsia National Pirogov Memorial Medical University, Vinnytsia. The Bioethics Committee of the Vinnytsia National Pirogov Memorial Medical University, Vinnytsia, as a result of the examination conducted in accordance with the provisions of the committee, established that the research materials do not contradict the basic bioethical norms of the Council of Europe Convention on the Protection of Vertebrate Animals Used in Experiments and for Other Scientific Purposes dated March 18, 1986, EEC Directive № 609 dated November 24, 1986 and Order of the Ministry of Health of Ukraine № 281 dated November 1, 2000 (Protocol № 6 dated October 3, 2022).

The experimental study was conducted on 32 white laboratory rats (males) with a body weight of 200 to 250 g. The rats were kept in accordance with generally accepted norms. All animals were kept in the same conditions. Before conducting the research, the animals selected for the experiment were quarantined for two weeks on a standard diet, water was not limited. On the day of the experiment, the animals were not fed. After premedication, anesthesia was performed by intramuscular injection of ketamine. In the interscapular area, a standardized wound measuring 1.5 x 1.5 cm was modeled with excision of the skin, subcutaneous tissue, and superficial fascia.

To reproduce a wound infection, the wound was contaminated with a pathogenic strain of Staphylococcus aureus in combination with Pseudomonas aeruginosa at a concentration of 1 billion microbial bodies in 1 ml of liquid. Then the wound was closed with an aseptic gauze bandage. The rats were dynamically observed for 2 days. 2 days after the creation of the experimental wound and its contamination, a macroscopic assessment of local changes and control of the bacterial spectrum of the wound for contamination by the investigated flora was carried out.

Subsequently, the rats were divided into 2 groups of 16 animals each. The distribution of animals was as follows: 0 - control group (without treatment); And - Iruksan gel was used for treatment. The study drug and an aseptic gauze bandage were applied to the wound surface of the animals of the 1st group, in the control group only the replacement of the aseptic bandage was performed. Daily dressings were performed for the next 14 days in all animals. After removing the bandage, local changes in the wound and in the surrounding tissues were evaluated, step-by-step photofixation of the examined wounds was performed.

Four rats from each group were removed from the experiment on the third, seventh, tenth and fourteenth day. Euthanasia was performed by an overdose of intramuscular ketamine anesthesia. Also, on these days of the experiment, bacteriological culture was carried out from the investigated wound. Bacteriological cultures were carried out with the help of Amies transport medium with subsequent identification of the culture at the Department of Microbiology of the National Pirogov Memorial Medical

University, Vinnytsia.

To assess morphological changes, tissues were collected for histological examination by excision of a fragment of the skin with underlying tissues from the location of the wound defect (2.0 x 2.0 cm with a depth of up to 4 mm), departing from its edges by a distance of at least 0.5 cm, with subsequent fixation in 10 % in a solution of neutral buffered formalin for at least 48 hours. The preparations were prepared with further processing in the automatic tissue processor Diapath Donatello TM Series 2 (made in Italy), pouring in paraffin using the pouring dosing console Amos scientific TEC 2800-M (made in Italy). Histological sections with a thickness of 4 μm prepared on an automatic rotary microtome Diapath Galileo AUTO Series 2 (made in Italy) were stained with hematoxylin and eosin.

Microscopy of histological preparations was carried out using an OLIMPUS BX 41 light microscope (Ministry of Health of Ukraine, State Registration Certificate № 8120/ 2008, code 9011800000) using 40, 100, 200, and 400 times magnification. Image visualization and morphometry were performed using the morphometric program Quickphoto micro 2.5 (license agreement № 925113924). During the microscopic examination, the morphological state and composition of the skin tissue at the edges and bottom of the wound defect, the presence of pathological and reparative changes, and their nature were evaluated.

Statistical data analysis was performed using the STATISTICA 64 v12 program. Stat Soft. Inc. and Microsoft Office Excel 2016.

Results

After infection of the simulated wounds, all rats were checked for contamination and identification of the pathogen before the start of the study drug and on the day the rats were removed from the experiment. The results of microbiological research are shown in Table 1.

As can be seen from Table 1, the number of microorganisms in group 0 remained practically unchanged ($p>0.05$) until the 10th day of the experiment. However, in the 1st group, their number decreased ($p<0.05$) until the 7th day, and from the 10th day of the experiment, flora was not sown. The obtained data correlate with the results of a morphological study, namely with the concentration of the pathogen and the severity of the inflammatory process, which gradually decreases up to the 10th day and is completely absent on the 14th day of the experiment in the control group, where complete epithelization of the edges of the wound defect is noted. At the same time, in the 1st (experimental) group, the negative result of the bacteriological examination, associated with complete epithelization of the wound, was present already on the 10th day.

During the morphological study, the following morphometric indicators of tissues in the area of the bottom of the wound defect in animals of both groups were studied: the density (number per 1 mm^2) of blood vessels and the density (number per 1 mm^2) of elements of the inflammatory cell infiltrate (segmented nuclear leukocytes, plasma cells, macrophage elements).

Table 1. Dynamics of changes in the concentration of microorganisms in simulated rat wounds.

Groups of animals	Concentration of pathogens, microbial bodies in 1 ml				
	0 day	3 day	7 day	10 day	14 day
0 group	St. aur. 10^4 Ps. aer. 10^4	St. aur. 10^5	St. aur. 10^5	St. aur. 10^4	-
I group	St. aur. 10^5 Ps. aer. 10^5	St. aur. 10^4	St. aur. 10^2	-	-

Table 2. Density (quantity per 1 mm^2) of blood vessels and leukocyte elements of inflammatory cell infiltration in the tissues of the bottom of the wound defect.

Groups of animals	Morphometric indicators			
	3 day	7 day	10 day	14 day
0 group	vessels - 376 leukocyte - 625	vessels - 538 leukocyte - 332	vessels - 263 leukocyte - 625	epithelialization
I group	vessels - 523 leukocyte - 247	epithelialization	epithelialization	epithelialization

Quantitative results of the obtained data indicate a faster process of epithelization of the wound in animals of the I group, which were treated with gel with collagenase (Table 2).

The results of histological studies showed that on the third day in the control group, inflammatory cell infiltration, signs of unexpressed swelling of tissues, fullness of blood vessels at the edges and bottom of wounds were morphologically determined. The surface layer of necrotized tissues was separated from the underlying tissues by a

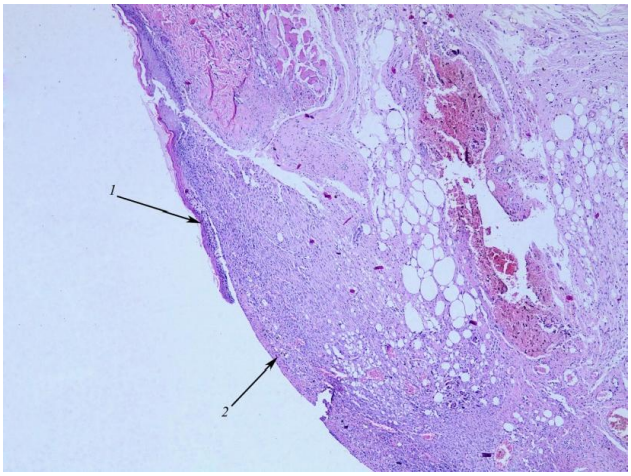

Fig. 1. Morphological changes of tissues in the area of the wound defect. The third day. Control group. The newly formed epidermis at the edge of the wound defect (1), the zone of deposition of fibrinoid substance (2) in the tissues of the wound bed. Hematoxylin-eosin. Ocular 10. Objective 4.

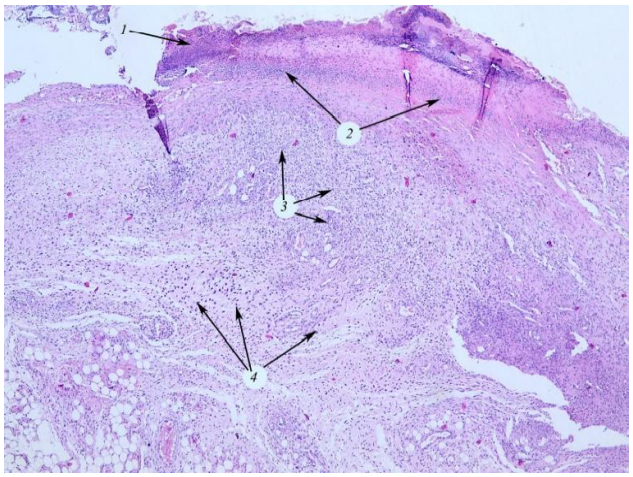

Fig. 2. Morphological changes of tissues in the area of the wound defect. The third day. Control group. Necrotized tissues (1), demarcated by a demarcation leukocyte shaft (2), uneven inflammatory cell infiltration (3), active fibroblasts (4) in the tissues of the wound bed. Hematoxylin-eosin. Ocular 10. Objective 4.

well-defined demarcation leukocyte shaft. On the greater part of the segment of the wound defect, the necrotic tissues were mostly already rejected, only a thin zone of surface deposition of fibrinoid substance remained. In the inflammatory cell infiltrate, neutrophilic and single eosinophilic leukocytes, macrophage elements, and lymphocytes were present. A small number of active fibroblasts were determined in the periwound zone. Regenerative proliferation of multi-layered flat epithelium from the edges of wounds in the form of a thin layer of crawling epitheliocytes was revealed (Figs. 1, 2).

On the third day, the formation of granulation tissue was histologically detected in the I (experimental) group, the presence of areas with a newly formed, thicker than in

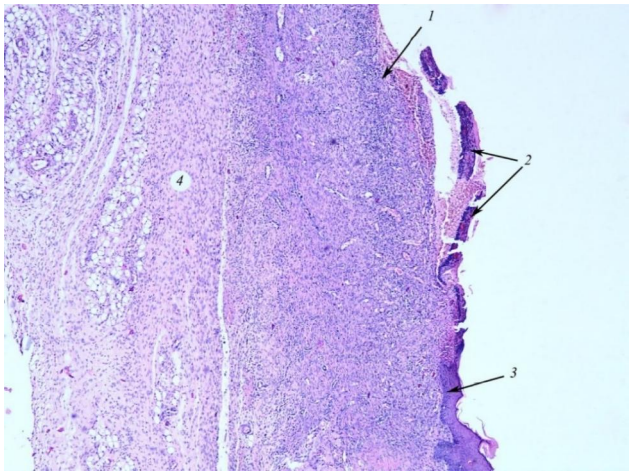

Fig. 3. Morphological changes of tissues in the area of the wound defect. I group. The third day. The tissues of the bottom of the wound (1) are covered with a wound "crust" (2), newly formed epithelium (3) from the edge of the wound. Hematoxylin-eosin. Ocular 10. Objective 4.

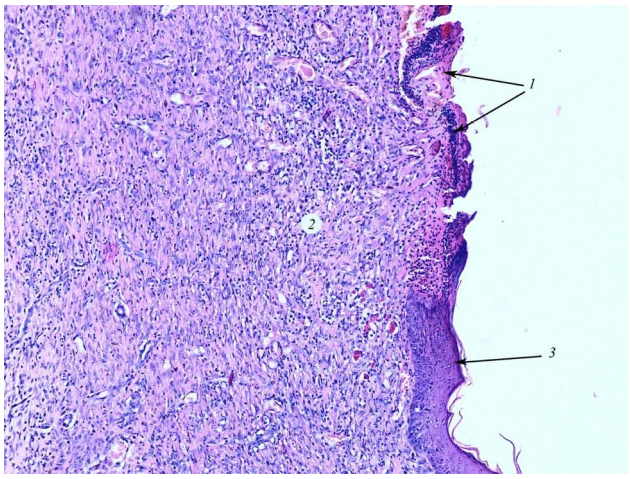

Fig. 4. Morphological changes of tissues in the area of the wound defect. I group. The third day. Wound "crust" (1), covering tissues rich in blood vessels (2), newly formed epithelium (3) from the edge of the wound. Hematoxylin-eosin. Ocular 10. Objective 4.

the previous group, multilayered flat epithelium oriented from the edges of the wound. Non-epithelialized areas of the bottom of the wound were covered with a scab. Within the tissues of the wound bed, leukocyte infiltration remained, with a significant proportion of lymphohistiocytic elements (Figs. 3, 4).

On the seventh day, in the preparations of the zero (control) group, mature granulation tissue was identified, which formed the bottom of the wound defect. This tissue, in addition to numerous blood vessels, contained thin collagen fibers, inflammatory and proliferative cells. From the edges of the wounds, a thin, but considerable in length, epithelial layer was located on the surface of the granulations (Fig. 5).

In contrast to the given data for the zero group, in the 1st (experimental) group, on the seventh day, complete

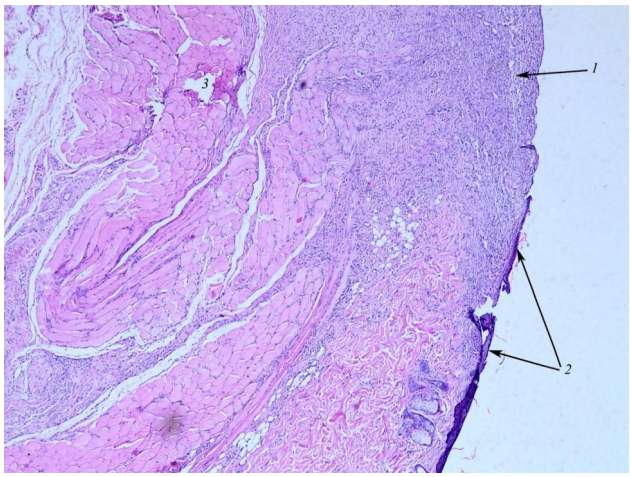

Fig. 5. Morphological changes of tissues in the area of the wound defect. Control group. The seventh day. Mature granulation (1), extensive epithelization (2) of the wound from its edges. Hematoxylin-eosin. Ocular 10. Objective 4.

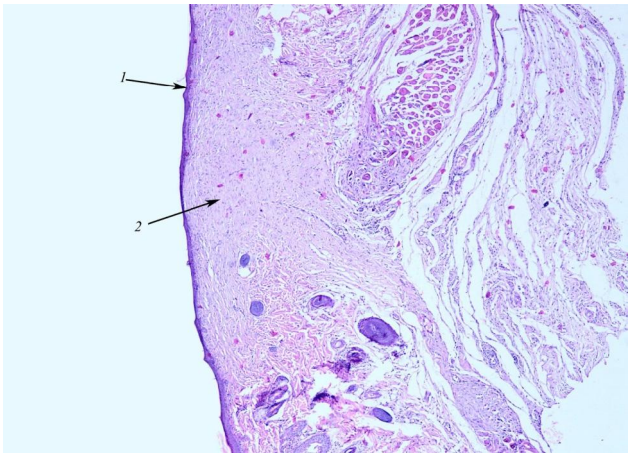

Fig. 6. Morphological changes of tissues in the zone of the bottom of the wound defect. I group. The seventh day. The newly formed epithelium (1) completely covers the scar tissue (2). Hematoxylin-eosin. Ocular 10. Objective 4.

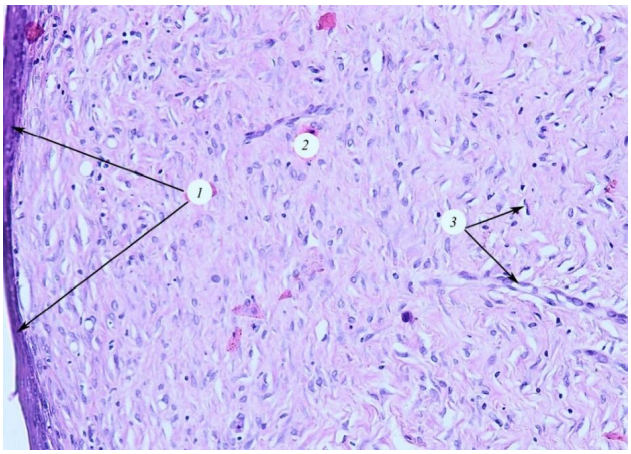

Fig. 7. Morphological changes of tissues in the area of the wound defect. I group. The seventh day. The newly formed epithelium (1) completely covers the scar tissue (2) with thin-walled blood vessels (3). Hematoxylin-eosin. Ocular 10. Objective 4.

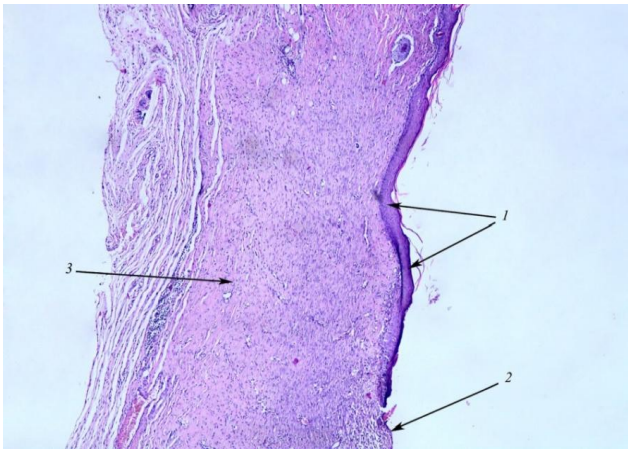

Fig. 8. Morphological changes of tissues in the zone of the bottom of the wound defect. Control group. The tenth day. Newly formed epidermis (1) on the surface of the scar (3), granulation tissue without an epithelial cover (2) in the central parts of the wound. Hematoxylin-eosin. Ocular 10. Objective 4.

coverage of the wound surface with newly formed epithelium was noted. The layer of the epithelium was thin (in some places - only three layers of flattened epitheliocytes), scar tissue with few cells of the lymphohistiocytic series and thin-walled vessels was located under it (Figs. 6, 7).

In the control group of animals, on the tenth day, the wounds were also completely epithelialized. Completion of stratification of the epidermis and maturation of scar tissue was morphologically determined, although in two animals there were centrally small areas without an epithelial cover (Fig. 8).

Discussion

According to the ideas existing today, the wound process after hemostasis takes place in the form of the following phases: inflammation, proliferation and reorganization of the scar with epithelization [22, 23]. In the inflammatory phase, vascular reactions and successive cellular mechanisms of inflammation occur with the participation of neutrophil leukocytes, macrophages, and lymphocytes, and then the wound is cleaned of dead tissues.

In the second phase, the formation of an extracellular matrix of granulation tissue is observed, in which keratinocytes, macrophages, fibroblasts and endothelial cells play a significant role, and collagen is one of the components of this granulation tissue. In the last phase of the wound process, reorganization of scar tissue occurs.

The results of morphological studies obtained by us in the course of experimental work demonstrate some differences in the healing process of contaminated wounds in rats of the control and experimental groups. At the same time, it is important to emphasize that the contamination of wounds in experimental animals was carried out with a pathogenic strain of Staphylococcus aureus in combination with Pseudomonas aeruginosa - pathogens that are typical etiological agents of wound infection in surgical patients.

According to the results of morphometric and histological studies, it was found that the use of the combined drug "Iruksan" has a positive effect on the indicators of the number of leukocyte elements in the focus of inflammation of the wound, which indicates a lower expressiveness of the inflammatory infiltrate when treated with this drug.

Thus, with the help of a histological examination, it was established that on the third day in the control group of animals, inflammatory cell infiltration, signs of unexpressed tissue swelling, blood vessels in the edges and bottom of the wound were found, which is similar to the data highlighted in the work of R. G. Sibbald and co-authors [20]. In the inflammatory cell infiltrate, neutrophilic and single eosinophilic leukocytes, macrophage elements, and lymphocytes were determined, which are characteristic signs of an inflammatory reaction. Regenerative proliferation of multilayered flat epithelium from the edges

of wounds in the form of a thin layer of epitheliocytes was observed. At the same time, on the third day in the experimental group, the formation of granulation tissue, the presence of areas with a thicker, multilayered flat epithelium than in the control group was found. Leukocyte infiltration with a significant proportion of lymphohistiocytic elements remained within the bottom of the wound. The data obtained by us coincide with the data obtained by some scientists [7].

It should be noted that in the dynamics of the experimental group, a faster epithelization of the wound defect was observed: on the 7th day, the wound surface was already completely covered by the newly formed epithelium. These results are significantly different from the indicators of the control group, where mature granulation tissue with proliferative elements was determined on the 7th day, which is a typical manifestation of the course of the wound process in this time period. Later, in the control group of animals, the wounds were also epithelialized on the 10th day of observation.

Thus, a beneficial effect on the early development of granulation tissue and, at the same time, early onset of the phase of wound epithelization in the I (experimental) group compared to the control group was revealed. In our opinion, it is obviously related to the presence of collagenase enzyme in the composition of "Iruksan". After all, it is known that successive changes in the structure of the extracellular matrix require a clear balance between the processes of synthesis and breakdown of collagen [21, 23].

The results of the conducted research form the basis for further study of the role of collagenase in the treatment of contaminated and purulent-necrotic wounds of soft tissues.

Conclusions

1. The combined gel "Iruksan" based on collagenase showed its effectiveness when used at different stages of the wound process compared to the control group, as evidenced by better regenerative activity of tissues in the affected area, which was manifested in the rapid removal of inflammatory cell infiltration and cleansing of the wound from necrotic detritus already on the third day of the experiment and the subsequent accelerated formation and maturation of granulation tissue, simultaneous active process of epithelization of the bottom of the wound defect on the seventh day.

2. The effect was found when using the preparation with collagenase in the treatment of wounds that were contaminated by typical causative agents of wound infection in medical institutions of a surgical profile.

3. The obtained results indicate the expediency of using this drug in the treatment of wounds in the first phase of the wound process, which allows to reduce the time of cleaning the wound surface from purulent-necrotic tissues and epithelization.

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МОРФОЛОГІЧНЕ ОБҐРУНТУВАННЯ ЕФЕКТИВНОСТІ ВИКОРИСТАННЯ ЛІКУВАЛЬНОЇ СУМІШІ "ІРУКСАН" В ЕКСПЕРИМЕНТІ

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Лікування інфікованих та гнійних ран залишається актуальною проблемою на сьогоднішній день. Науковий і практичний інтерес викликає застосування ферменту колагенази у препаратах для санації рани. Мета: вивчити морфологічні зміни тканин контамінованих та гнійних ран щурів в експерименті під час їх лікування гелем "Іруксан", що містить колагеназу. Для відтворення ранової інфекції її контамінували патогенним штамом Staphylococcus aureus у комбінації із Pseudomonas aeruginosa, котрі є типовими збудниками ранової інфекції у хірургічних пацієнтів. У процесі експерименту застосовані мікробіологічні, гістологічні, статистичні методи дослідження. Після інфікування модельованих ран у всіх щурів проводили контроль контамінації та ідентифікації збудника перед початком дії досліджуваного препарату та в день виводу щурів з експерименту. За допомогою мікробіологічних досліджень виявлено, що кількість мікроорганізмів у ранах тварин контрольної групи залишалась практично незмінною (p>0,05) до 10 дня спостереження, а у дослідній групі (для лікування ран застосовували гель "Іруксан") кількість мікроорганізмів у ранах зменшувалась (p<0,05) уже до 7-ї доби експерименту. Отримані дані корелюють з результатами морфологічного дослідження, а саме з виразністю запального процесу. Виявлено, що зменшення ознак запального процесу і розвиток епітелізації ранового дефекту відбувався швидше у дослідній групі тварин. За результатами гістологічних досліджень встановлено повне покриття ранової поверхні новоутвореним епітелієм уже на 7-му добу експерименту у дослідній групі, що суттєво відрізняється від показників контрольної групи, де на 7-му добу визначались типові ознаки перебігу ранового процесу для цього часового проміжку. В контрольній групі тварин епітелізація ран спостерігалася на 10-ту добу. Отже, отримані дані свідчать про позитивний ефект використання препарату з колагеназою ("Іруксан") при лікуванні ран, а саме про доцільність його застосування у першій фазі ранового процесу, що дозволяє скоротити терміни очищення поверхні рани від гнійно-некротичних тканин та пришвидшити її епітелізацію.

Ключові слова: *гнійна рана, інфікована рана, рановий процес, колагеназа, морфологічні зміни в рані, хірургічна обробка рани.*