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Research Article

Investigation of the bischofite gel reparative effect on the linear wound model

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Abstract

Introduction. The currently available information on the bischofite mineral pharmacodynamics makes it possible to assume that bischofite-based gels have wound-healing properties.

Materials and methods. Experiments were performed on 36 male rats. Using a blade, a linear wound of 50 mm long was modeled. Animals were divided into 4 groups: 1) Control; 2) Bishofit (500 mg of gel with bischofite on the wound area for 7 days); 3) Actovegine (500 mg of Actovegine gel on the wound area for 7 days); 4) Contractubex (500 mg of contractubex gel on the wound area for 7 days). The wound healing effect of the drugs was evaluated through studying the physicomechanical properties, assessing the morphological picture, determining the concentration of hydroxyproline (HP) and calculating the ratio of types I and III collagen.

Results. The strongest healing effect on the wound defect, significantly greater than that in the control ($p<0.01$), was obtained in the group that received bischofite gel (13.70±0.76 N). Actovegine also demonstrated a positive reparative effect (12.60±0.63 N, $p<0.05$). Significantly lower ($p<0.01$) healing effect than in the control group was obtained in the group of animals that had received contractubex (9.65±0.59 N). In the calorimetric analysis, it was found that the highest concentration of HP was in the tissues of wound defects in animals treated with contractubex; however, there was no statistically significant difference with the control group. Significantly lower in comparison with the control ($p<0.05$) concentration of HP, was found in tissues of simulated wounds in animals treated with bischofite gel (79.7% of the control). When assessing the ratio of types I and III collagen in the tissues of the wound defect, when stained with picrosirius red, it was found that by the number of mature collagen fibers, the studied groups can be arranged in the following descending order: Bishofite> Actovegine> Control> Contractubex. A similar trend is demonstrated by the morphological picture of tissues in the area of the wound defect.

Conclusion. The study showed that the best results were obtained with external use of bischofite gel. Actovegine has a less significant, but quite pronounced reparative, effect on this model. The least satisfactory results were obtained when applying contractubex.

Keywords

bischofite, linear wound, reparation, collagen, rats.

Introduction

Modern pharmacology has a wide arsenal of compounds with cytoprotective potential. In accordance with the requirements of aesthetic and cosmetic medicine, dermatotropic agents with wound-healing properties are being actively developed. One of these tools is a gel based on bischofite, a natural mineral, with significant deposits in the territory of the Lower Volga region. The pharmacological activity of this mineral, including as a component of gels, has been studied in detail for several decades (Sysuev 2006, Sysuev et al. 2011, 2013, Evseeva and Sysuev 2016, Spasov et al. 2009a, 2009b).

Materials and methods

The experiments were carried out on 36 male Wistar rats weighing 193-218 g, which had passed the 14-day quarantine and had been randomized in accordance with all bioethical norms and rules. Under anesthesia (chloral hydrate 300 mg/kg), after preliminary shaving (80×45 mm) and treatment with an antiseptic (70% solution of ethyl alcohol) in the dorsal region, a linear wound of 50±1 mm long was modeled by cutting the skin along the paravertebral line with a blade with a depth stop (2 mm), after which the edges of the wound were brought together by using three stitches of sterile suture (Mironov 2012).

Then the animals were divided into 4 equal groups:

I – *Control group* – imitation of rubbing the drug into the shaved area 10 minutes after modeling the wound and for the next 6 days (once per day)
II – *Bischofite* – rubbing 500 mg of gel with bischofite into the shaved wound area and adjacent tissues 10 minutes after modeling the wound and for the next 6 days (once per day)
III – *Actovegine* – rubbing 500 mg of Actovegine gel into the wound area and adjacent tissues 10 minutes after modeling the wound and for the next 6 days (once per day)
IV – *Contractubex* – rubbing 500 mg of Contractubex gel into the wound area and adjacent tissue 10 minutes after modeling the wound and for the next 6 days (once per day)

On the 8th day, the animals were taken out of the experiment by cranial dislocation under anesthesia, and then 4 skin grafts (total surface 25×45 mm) were removed from the dorsal surface for further research. The wound healing effect of the drugs was evaluated through studying the physicomechanical properties, assessing the morphological picture, determining the concentration of hydroxyproline (HP) and calculating the ratio of types I and III collagen.

The study of the physicomechanical properties of the wound defect was carried out using a tensile tester (Metrotest REM-0.2-1, Russia). The cut skin fragment was fixed in a special mounting device using strings and metal pins. After launching the device, the force (discreteness=0.1 N) necessary for tearing tissues along the wound line was monitored. This value characterizes the ultimate strength of the wound defect. In addition, data on the ultimate strain (tension at the time of rupture) of the skin graft were obtained by analyzing the force-tensile curve. This parameter allows estimating the elasticity of the wound defect.

Calorimetric analysis of the concentration of hydroxyproline in the tissues of the wound defect. In addition, to assess the degree of reparative reaction, the concentration of HP in tissues was determined. HP is one of the non-proteinogenic (non-coded) amino acids that make up the proteins of plants and animals, mainly collagens.

To determine the concentration of HP in the samples, there was used a colorimetric method for determining the reaction products of oxidized HP and Ehrlich reagent (Ignatieva et al. 2007). During the reaction, the pyrrolidine ring under oxidative dehydration with chloramine B in a buffer solution with a pH of about 6 becomes pyrrole, which, in turn, can be determined by a reaction with 4-(N,N-dimethylamino)-benzaldehyde. The resulting compound is intensely colored and is detected colorimetrically.

During the sample preparation, round skin areas without underlying tissues with a diameter of 5 mm and including all its layers were taken from the euthanized animals, using the Dermal Punch tool (USA). The samples were taken in such a way as to include the linearly scar, as large as possible, that was formed after the wound modeling and treatment. The samples were frozen in liquid nitrogen by immersion in it for 1-2 seconds and stored at –72° C in sealed tubes of the Eppendorf type.

On the day of the study, the samples were thawed for 3-5 hours at room temperature in the open air. The samples were then weighed and cut so that the weight of one of the fragments was about 20 mg.

Tissue hydrolyzate was prepared in the following way: tissue samples weighing 20 mg were placed in 5 ml glass ampoules and 400 µl of hydrochloric acid was added. The ampoules were sealed and placed for 25 minutes in an oil bath heated to a temperature of 166° C. After that, the ampoules were opened and in an incubator heated to 60° C were evaporated to dryness. The dry residue was diluted with 2 ml of distilled water.

To determine HP, 1 ml of chloramine B was added to 1 ml of the hydrolyzate, shaken and kept at room temperature for 20 minutes. Then 1 ml of perchloric acid was added, shaken again, and 1 ml of a 20% solution of Ehrlich reagent was added. The tubes were shaken again and placed in a water bath (60° C) for 20 minutes, then the reaction was terminated by immersing the tubes in an ice bath, and 5 ml of ethyl cellosolve was added. The optical density was determined at a wavelength of 557 nm. To prepare the standards, used crystalline hydroxyproline (Sigma-Aldrich, USA).

Morphological examination of the tissues of the skin graft taken from the wound area was performed in a standard way. The samples were fixed with 10% buff-

ered formalin. The sections were stained with hematoxylin-eosin and according to Van Gieson. Staining with hematoxylin-eosin allows for a general assessment of the histological pattern in the test drug. At the same time, coloring according to Van Gieson allows studying in detail the architectonics of the connective tissue, differentiating mature and immature collagen.

Assessment of the ratio of types I and III collagen in a polarizing microscope. For a quantitative assessment of the ratio of mature and immature collagen in the obtained histological preparations, the proportion of types I and III collagen fibers was studied. For this, the sections were stained with picrosirius red and were photographed using a polarizing microscope. For each slice, 10 fields of view were photographed at x400 magnification. The color relation of the differential staining was determined by automatically analyzing color histograms for each of the photomicrographs using the ImageJ program, followed by statistical processing. A lower ratio indicates a higher proportion of immature type III collagen (Lattouf et al. 2014, Bella 2016).

Results

After awakening and on further days of the study, the animals were active, the consumption of feed and food was within the normal range. There were no purulent complications, hemorrhages, excoriations or other undesirable effects.

Determination of the physicomechanical properties of the wound defect

When determining the force at the moment of rupture using a mechanical tensile tester (Metrotest REM-0.2-1, Russia), it was found that the average force required to rupture the skin graft along the wound defect in the control group was 11.76±0.71 N. The greatest strength of the wound defect, significantly greater than in the control ($p<0.01$), was obtained in the group treated with bischofite gel (13.70±0.76 N). Actovegine also demonstrated a positive reparative effect (12.60±0.63 N, $p<0.05$). Significantly lower strength ($p<0.01$) than in the control was obtained in the group using Contractubex (9.65±0.59 N) (Fig. 1).

Calorimetric analysis of the concentration of hydroxyproline in the tissues of the wound defect

In the calorimetric analysis, it was found that the highest concentration of HP was in the tissues of wound defects in animals treated with Contractubex; however, there was no statistically significant difference discovered from the control group. A significantly lower concentration of HP in comparison with the control ($p<0.05$) was in tissues of simulated wounds in animals treated with bischofite gel (79.7% of the control) (Tab. 1).

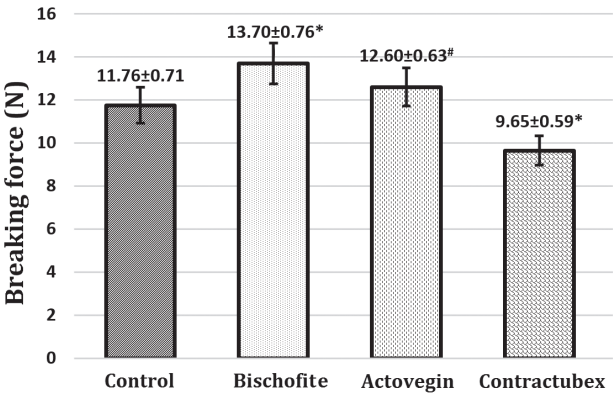

Figure 1. Force at the time of rupture (H) when assessing the strength of a wound defect using a mechanical tensile tester. *Note:* * – $p<0.01$ when compared with the control group; # – $p<0.05$ when compared with the control group

Taking into account the data obtained when determining the physicomechanical properties of tissues, the most likely reason for an increase in the concentration of HP in the tissues of animals treated with Contractubex is the presence of processes of secondary alteration and growth of granulation tissue.

On the other hand, a decrease in the concentration of HP in the wound defects of the group treated with bischofite gel indicates a decrease in secondary alteration and an accelerated repair.

Morphological examination of the skin graft tissue taken from the wound area

Control group. In the control group, a newly-formed connective tissue scar occupies a wide area; some areas of uneven maturation of the connective tissue can be visualized. The regenerated epidermis covering the wound is 3-4 times thicker than the epidermis of the adjacent intact skin (Fig. 2).

In the thickness of the epidermis, against the background of mitotic dividing cells of the basal layer, epithelial cells with pycnomorphic nuclei and karyolysis are visualized. The heterogeneity of the structure of the connective tissue scar should also be noted. In the central part, there is immature connective tissue, and on the outer sides, a large

Table 1. The Concentration of ydroxyproline (HP) in tissue samples of wound defects obtained on day 8 after the start of the experiment

Group	Control	Bischofite	Actovegine	Contractubex
Concentration of HP, mg/g	16.59±1.08	13.23±1.68	15.89±1.37	17.61±0.67

Note: * – presence of statistically significant differences when compared with the control group by the Mann-Whitney criterion with $p\leq0,05$

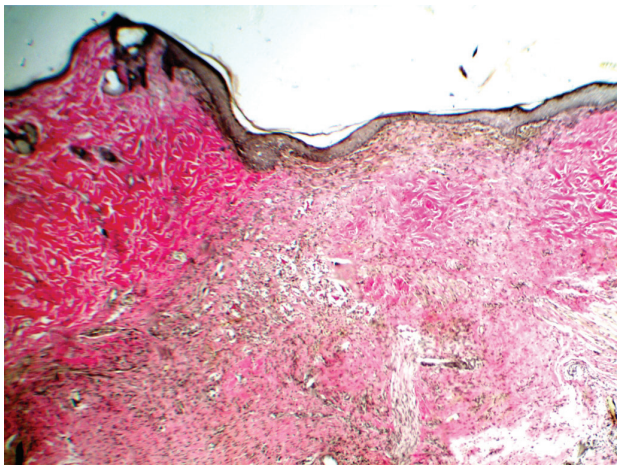

Figure 2. Photo micrograph of the skin slice in the wound area in the control group. Van Gieson stain. ×200

number of blood vessels can be visualized. Closer to the surface, local areas are observed where the collagen fibers are identical to those of the intact dermis by the degree of maturity (when assessing the intensity of color when staining using the Van Gieson method) (Fig. 3).

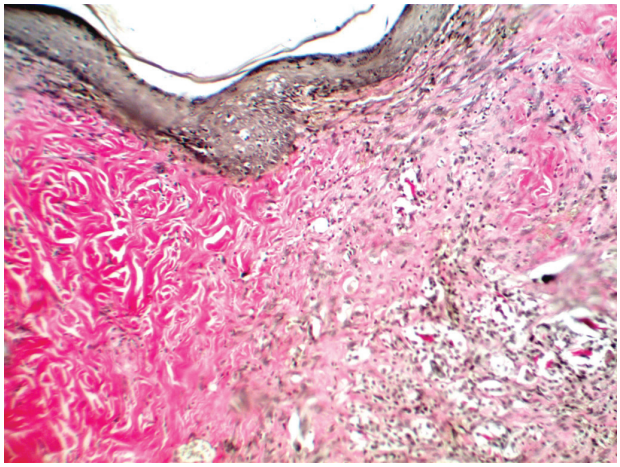

Figure 3. Photo micrograph of the skin slice in the wound area in the control group. Van Gieson stain. ×200

Bishofite. On histological slices from the animals group treated with bischofite gel, a thin connective tissue scar in the wound area is visualized, on the surface of which a complete regeneration of the epidermis is determined. The thickness of the newly formed multilayered epithelium is several times larger in wound area. In the scar zone, no derivatives are detected (Fig. 4).

Just below the epidermis, a wide band of connective tissue can be visualized, containing blood-filled vessels with local hemorrhages into the surrounding tissue.

Regarding the spatial organization of the newly formed connective tissue scar, the distal gradient should be noted in terms of the localization of the vertical newly formed vessels. Also the distortion of the skin layered structure should be mentioned. The newly formed connective tissue grows into the underlying hypodermis and muscle tissue (Fig. 5).

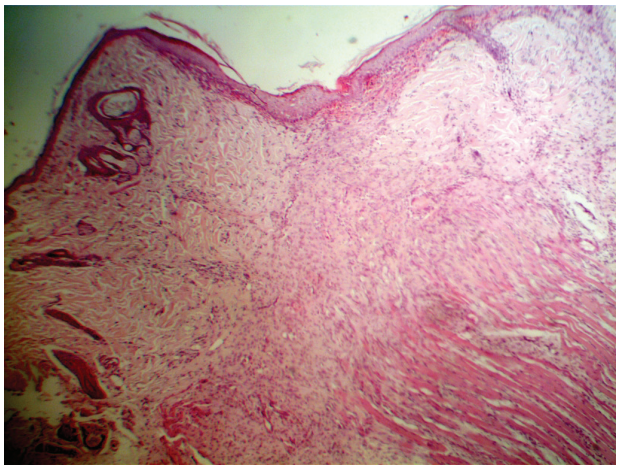

Figure 4. Photo micrograph of the skin slice in the wound area in the study group using bischofite gel. Stained with hematoxylin and eosin. ×200

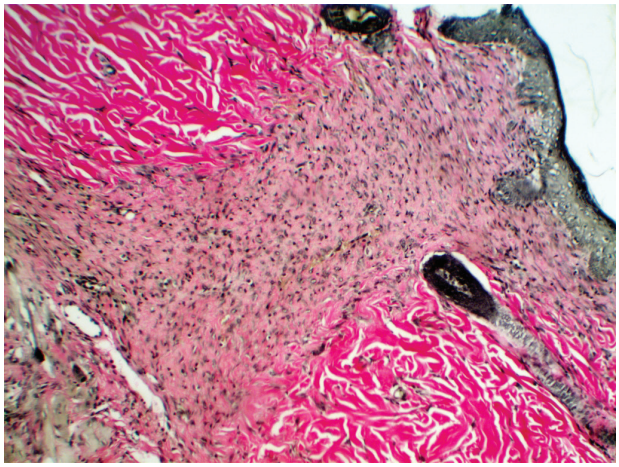

Figure 5. Photo micrograph of the skin slice in the wound area in the study group using bischofite. The growth of newly formed connective tissue into the underlying layers of skin. Van Gieson stain. ×200

From the lateral side of the newly formed scar, the border between the granulation tissue and the intact dermis is well defined. On the part of the dermis, disintegration of collagen fibers and growth into the newly formed granulation tissue can be observed. In the scar area, the cellular component predominates over the fibrous one. In the field of view, there are lymphocytes and fibroblastic cells. The tissue of the newly formed scar resembles loose fibrous connective tissue (Fig. 6).

Actovegine. On the preparations, there is a fully formed wedge-shaped connective tissue scar. A complete closure of the wound defect with a stratified squamous epithelium can be observed (Fig. 7).

In the epidermis, a large number of mitoses can be visualized in the basal and spinous layers (Fig. 8).

In the enlarged apical part in the papillary layer, there are a large number of blood vessels with margination.

Contractubex. In the study of microscopic specimens of the skin, obtained from the animals treated with Contractubex, a wide area of the scar defect can be visuali-

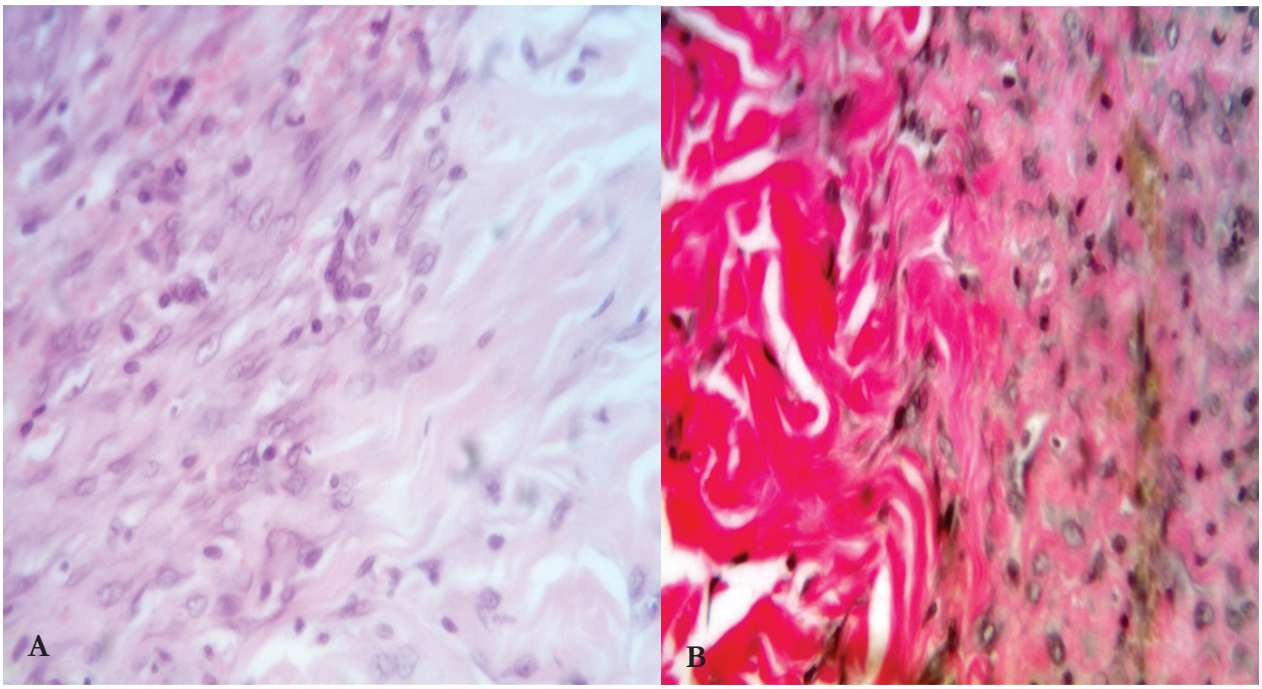

Figure 6. Photo micrograph of the skin slice in the wound area in the study group using bischofite. The transition zone of the connective tissue scar and intact dermis surrounding the scar. A – stained with hematoxylin and eosin. x400. B – Van Gieson stain. ×200.

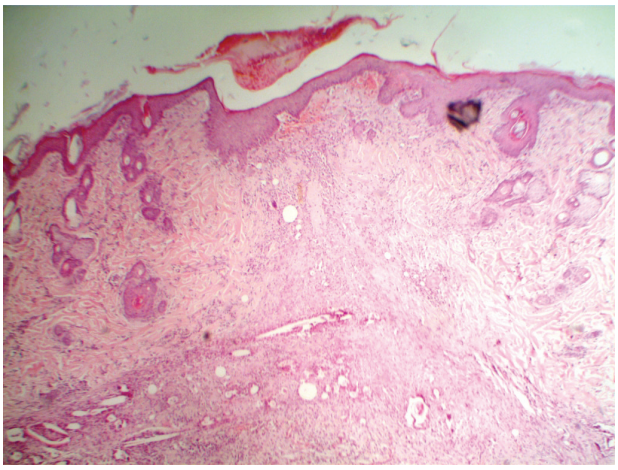

Figure 7. Photo micrograph of the skin slice in the wound area in the study group using Aktovegin. Stained with hematoxylin and eosin. ×200

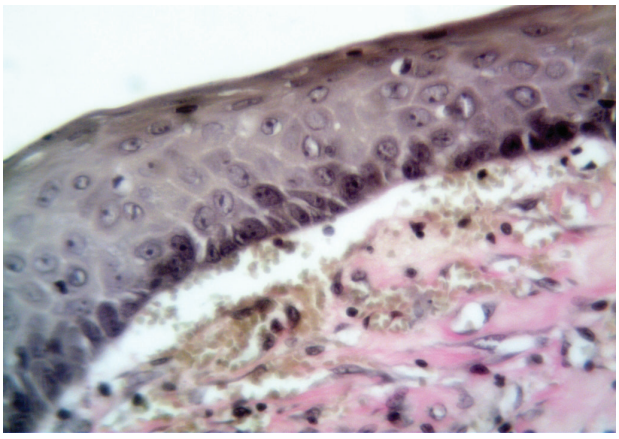

Figure 8. Photo micrograph of the skin slice in the wound area in the study group using Actovegin. On the surface of the newly formed connective tissue scar, the epidermis is well formed, with a clear stratification. Van Gieson stain. ×400

zed, a well as the complete filling of the wound defect with granulation tissue. The newly formed connective tissue without a clear boundary passes into the connective tissue of the intact skin surrounding the wound defect (Fig. 9).

Over the entire surface of the scar, detachment of the newly formed thickened epithelium can be observed. Heterogeneity in the spatial organization of the connective tissue scar should also be taken note of. There are also local areas with a high degree of maturity of the newly formed connective tissue (Fig. 10).

At the same time, collagen fibers, randomly and loosely arranged, are interspersed with areas of single fat cells with different diameters, not prone to fusion.

Assessment of the ratio of types I and III collagen in a polarizing microscope

When assessing the ratio of types I and III collagen in the tissues of the wound defect when staining with picrosirius red, it was found that, by the number of mature collagen fibers, the study groups can be arranged in the following descending order: Bishofite>Actovegine>Control>Contractubex (Tab. 2, Fig. 11).

Statistically significant differences were found in the Bischofite and Contractubex groups. In the group that received Contractubex, there is an increased relative content of type III collagen, which indicates a delay in the maturation of collagen and that there is the tendency to form scar tissue.

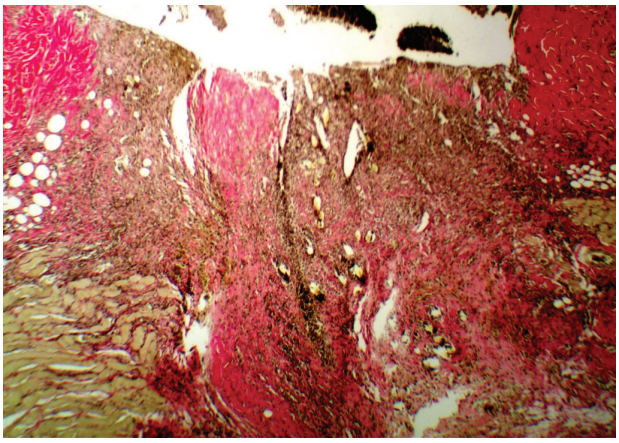

Figure 9. Photo micrograph of the skin slice n in the wound area in the study group using Contractubex. Zone of wound defect and surrounding intact skin. Van Gieson stain. ×200

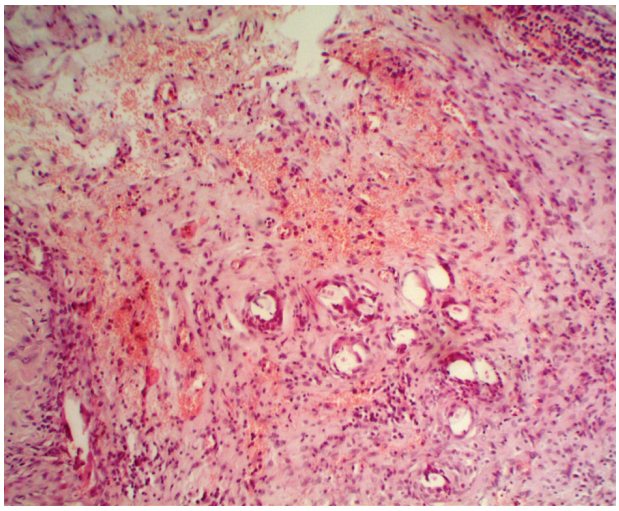

Figure 10. Photo micrograph of the skin slice in the wound area in the study group using Contractubex. In the newly formed granulation tissue, there are a great number of blood vessels with hemorrhages. Stained with hematoxylin and eosin. ×200

Table 2. The Ratio of I and III Types Collagen in Tissue Samples of Simulated Wounds Obtained on Day 8 After the Start of the Experiment

Group	Control	Bischofite	Actovegine	Contractubex
The ratio of type I collagen / type III collagen	0.56±0.012	0.73±0.023*	0.67±0.017	0.38±0.02*

Note: * – p≤0.05 when compared with the control group

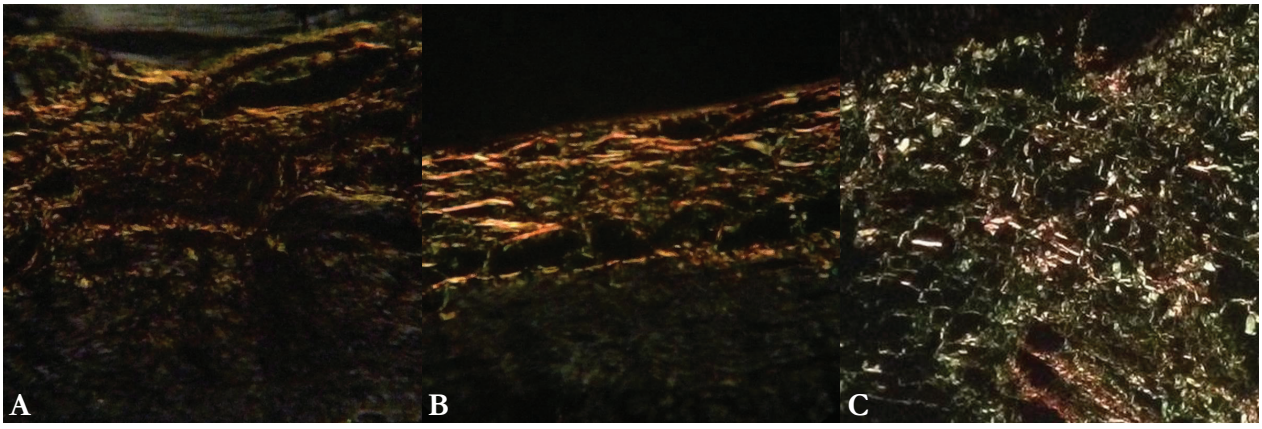

Figure 11. Microscopic picture of the skin in the area of the simulated wound in the control group (A), in the group treated with Bishofite (B), and in the group treated with Contractubex (C). Polarization microscopy. Staining Sirius Red. ×400

Conclusion

The study showed that the best results were obtained when using the bischofite gel externally. The wound defect in this group was characterized by the greatest strength, a good histological picture and a lower tendency to form a scar, which can be proved by a low concentration of hydroxyproline and type III collagen. Actovegine has a less significant, but pronounced reparative effect on this model. Actovegine gel showed a positive effect on the macro- and microscopic picture of the wound defect, as well as wound strength, and when using laboratory research methods, its preventive effect on the excessive formation of scar tissue was also proved. The less satisfactory results were obtained when applying Contractubex. Without having a significant impact on the physicomechanical properties of the wound, Contractubex increased the content of HP and reduced the content of mature collagen. Such results indicate that Contractubex reduced the reparative potential of tissues, increasing the growth of granulation tissue and slowing recovery. This conclusion is confirmed by the results of the histological study and can be explained using the available information on the pharmacodynamics of the drug.

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- Boris B. Sysuev, Doctor of Pharmaceutical Sciences, Department of Aesthetic Medicine, Faculty of Continuous Medical Education, Peoples' Friendship University of Russia. e-mail: boryssysuev@yandex.ru. Research design, consultation on research planning, quality assurance, animal randomization.

- Isita A. Khanaliyeva, Department of Aesthetic Medicine, Faculty of Continuous Medical Education, Peoples' Friendship University of Russia. e-mail: chanalieva@yandex.ru Linear wound modeling, photographing, sampling and description of histological material.

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- Anna V. Sokolova-Merkurieva, Ph.D. in Medicine, Department of Aesthetic Medicine, Faculty of Continuous Medical Education, Peoples' Friendship University of Russia. e-mail: sokolova-merkurjeva@yandex.ru Histological material sampling and studying the ratio of types I and III collagen