STIMULATION OF REPARATION IN A LINEAR WOUND MODEL IN RATS BY BISCHOFIT GEL

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The aim of the article is to evaluate Bischofit gel reparative activity in a linear wound model in rats. Materials and Methods. The study was conducted on 36 male Wistar rats weighing from 193 to 218 grams. On the 8th day after modeling a linear wound defect 50±1 mm long, the reparative effect of bischofite, Actovegin and Contractubex in the gel compositions was evaluated. The evaluation was carried out using the following methods: 1) studying the physicomechanical characteristics of the wound defect (a wound-tearing machine Metrotest REM-0.2-1); 2) morphological examination of the skin graft taken from the wound area (stained with hematoxylin-eosin and Van Gieson’s solution); 3) determining the ratio of collagen types I and III in a polarizing microscope (the picrosirius was red); 4) colorimetric analysis of the hydroxyproline concentration in the wound surface tissues.

Results. On the 8th day, the wound defects sampled from the bischofite treated animals, were characterized by the most pronounced strength (the average force at the rupture moment was 13.70 N), which was significantly higher (p<0.01) than in the control group (11.76 N). Actovegin showed less influence on this parameter (12.60 N), and the use of Contractubex led to its decrease (8.10 N). The effect of the drugs on the morphological state of the skin tissue was similar. The hydroxyproline concentration in the studied groups’ samples was: Bischoft 13.23±1.68; Actovegin 15.89±1.37; Contractubex 17.61±0.67; the Control was 16.59±1.08. According to the impact on the ratio of collagen in types I and III, the studied drugs were arranged in the following sequence: Bischofit (0.73±0.023) > Actovegin (0.67±0.017) > Control (0.56±0.012) > Contractubex (0.38±0.020).

Conclusion. The carried out study showed that Bischofit has a pronounced ability to stimulate the regeneration of the skin wound defect. Hereby, the reference drug Actovegin showed less activity, and Contractubex worsened wound healing.

Keywords: bischofite, regeneration, Actovegin, Contractubex, hydroxyproline, collagen
INTRODUCTION

Despite the rapid development of streamlined synthesis, the emergence of highly selective drugs and biological therapy, simpler, multitarget compounds do not lose their relevance [1]. One of these tools is a gel based on Bischofit. Its natural mineral resource is presented in the territory of the Lower Volga region. For a long time, Bischofit has been used in clinical practice to treat a wide range of pathologies. The pharmacological activity of this mineral, including the gel form, has been studied in detail for several decades [2, 3]. Bischofit has proved to have anti-inflammatory and immunomodulatory activity, as well as accelerating regenerative processes [4–7].

MATERIALS AND METHODS

Animals

The study included 36 male Wistar rats weighing from 193 to 218 grams. The rats obtained from the mouse bank of “Stolbovaya” (Moscow region) were used as laboratory animals. All manipulations performed on the individuals were performed in accordance with international norms of experimental ethics (European Convention for the Protection of Vertebrate Animals used for experimental and other scientific purposes (Strasbourg, 22 June, 1998)) and with the requirements of good laboratory practices (GLP). The animals were placed in macrolon cells with lattice steel lids and a forage well. The litter material was non-coniferous sawdust. During the experiment all the rats were kept in standard vivarium conditions (humidity 65 ± 5%, temperature 22 ± 2ºC). Individuals were under natural light with free access to food and water. The cages, bedding and drinkers changed as they became soiled.

Study design

Under anesthesia (chloral hydrate 300 mg/kg) after preliminary depilation (80×45 mm) and treatment with an antiseptic (70% solution of ethyl alcohol) in the dorso area, a linear wound 50±1 mm long was modeled by cutting the skin along the paravertebral line with a blade with a depth limiter of 2 mm, after which the edges of the wound were brought together by imposing three sutures [8].

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Then the animals were divided into 4 equal groups:
I – Control group – imitation of rubbing the drug on the shaved area 10 minutes after the wound modeling and for the next 6 days (once a day)
II – Bischofit – rubbing 500 mg of gel with Bischofit on the wound area and adjacent tissues 10 minutes after modeling the wound and for the next 6 days (once a day)
III – Actovegin – rubbing 500 mg of Actovegin gel on the wound area and adjacent tissues 10 minutes after modeling the wound and for the next 6 days (once a day)
IV – Contractubex – rubbing 500 mg of Cotractubex gel on the wound area and adjacent tissues 10 minutes after modeling the wound and for the next 6 days (once a day)

After natural predrying of the application area, the animals were placed in individual cages. In the next 6 days, in addition to applying the gels, the clinical condition, motor activity, feed and food consumption, as well as photographic images of the wound area were assessed. On the 8th day, the animals were removed from the experiment by the method of cranial dislocation under anesthesia, after which 4 skin grafts (the total surface was 25×45 mm) were sampled from the dorsal surface for research (Fig. 1).

To determine the HP concentration in the samples, a calorimetric method of detecting the reaction products of oxidized HP and Ehrlich reagent [10] was used. In the process of the sample preparation, round skin areas without underlying tissues with a diameter of 5 mm and including all the layers were taken from the euthanized animals using the Dermal Punch tool (USA). The samples were frozen in liquid nitrogen by immersion for 1–2 seconds and stored at minus 72°C in sealed Eppendorf tubes.

On the day of the study, the samples were thawed for 3–5 hours in the open air at the room temperature. The samples were weighed and cut so that the weight of one of the fragments was about 20 mg. Then hydrolysate was prepared from the samples. To determine HP, 1 ml of chloramine B was added to 1 ml of hydrochloric acid solution 36%, shaken and kept for 20 minutes at the room temperature. 1 ml of perchloric acid was added, shaken again and 1 ml of a 20% solution of Ehrlich reagent was

1. The study of physical and mechanical characteristics of the wound defect was performed using a wound-tearing machine. The cut skin fragment was fixed in a special installation with the help of threads and metal spokes. After launching the device, the force (discreteness=0.1 N), necessary for tearing tissues along the wound line, was monitored. The ultimate deformation data (stretching at the rupture moment) of the skin flap were also obtained. This parameter represents the elasticity of the wound defect.

2. Calorimetric analysis of hydroxyproline concentration in the wound defect tissues. To assess the degree of reparative reaction in the tissues, the concentration of hydroxyproline (HP) as the basic amino acid of collagen was determined. HP is formed as a result of the cotranslational hydroxylation of proline by the enzyme proline-hydroxylase, which occurs even before the synthesis of the polypeptide chain is completed [9].
added. The tubes were shaken again and placed into a water bath (60°C) for 20 minutes. Then the reaction was terminated by immersing the tubes into an ice bath and adding 5 ml of ethyl cellosolve. The optical density was determined at the wavelength of 557 nm. For the preparation of standards, crystal HP manufactured by Sigma-Aldrich (USA) was used.

3. Morphological investigation of the skin graft tissues taken from the wound area were carried out in a standard way. The samples were fixed with 10% buffered formalin. The cut-sections were stained with hematoxylin and eosin and Van Gieson’s solution. Staining with hematoxylin and eosin makes it possible to carry out a general assessment of the histological picture, and staining by Van Gieson’s solution makes it possible to carry out a detailed study of the connective tissue architectonics, differentiating between mature and immature collagen. Then the received preparations were assigned code names for an independent assessment by an expert commission consisting of 5 doctors of the pathoanatomical bureau from the Belgorod regional clinical hospital n. a. Saint Joseph (Russia, Belgorod). The assessment was made according to the specially developed scale (Table 1).

Table 1. Scale for assessing the reparative activity of the studied drugs using the histological picture of the wound defect area

<table>
<thead>
<tr>
<th>Qualitative character</th>
<th>Points and their characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytoarchitectonics disruption</td>
<td>0 – None</td>
</tr>
<tr>
<td>Architectonics disruption of intracellular matrix</td>
<td>1 – Low-grade</td>
</tr>
<tr>
<td>Hemorrhage, enlarged vessels</td>
<td>2 – Obvious</td>
</tr>
<tr>
<td>Violation of epithelialization</td>
<td>3 – Florid</td>
</tr>
<tr>
<td>Leukocyte infiltration</td>
<td>0 – None</td>
</tr>
<tr>
<td></td>
<td>1 – Low-grade</td>
</tr>
<tr>
<td></td>
<td>2 – Obvious</td>
</tr>
<tr>
<td></td>
<td>3 – Florid</td>
</tr>
</tbody>
</table>

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

To assess the viability of the reparative process, the ratio in collagen types I and III was determined, since the predominance of mature (I) collagen over the immature one (III) indicates a normal regeneration of the wound. To quantify the ratio of mature (I) and immature (III) types of collagen, the sections were stained with picrosirius red, then microscoped in a polarization microscope and photographed. For each cut-section, 10 fields of view were photographed at x400 magnification. The color ratio of the differential coloration was established by automatically analyzing color histograms for each of the microphotographs using the image J program and subsequent statistical processing. A lower ratio indicates a higher proportion of immature type III collagen [11].

Statistical processing of the obtained data was performed using STATISTICA 10.0 software. Descriptive statistics was applied to all the data. The normality of distribution was determined using Shapiro-Wilk and Kolmogorov-Smirnov criteria. The statistical significance of the differences was carried out using Newman-Keuls test depending on the nature of the data was carried out using the Student’s and Mann-Whitney tests with the Bonferroni correction. The differences were recognized statistically significant at p≤0.05.

RESULTS AND DISCUSSION

After recovery 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 and other unwished effects. By day 7, the greatest visual differences had been observed between the animals treated with Bischofit gel and the Control group (Fig. 2).

![Figure 2. General view of the animals immediately before euthanasia](image)

Note:
A – a group of animals treated with Actovegin gel;
B – a group of animals treated with Contractubex gel;
C – a group of animals treated with Bischofit gel
Determination of physicomechanical characteristics of the wound defect

When determining the force at the rupture point using a wound-tearing machine (Metrotest REM-0.2-1, Russia), it was found out that the average force required to rupture a skin flap along the wound defect in the control group was 11.76±0.71 N. The greatest strength of the wound defect can be positioned as follows (in descending order): gel with Bischofit (13.70±0.76 N, p<0.01); Actovegin (12.60±0.63 N, p<0.05); Contractubex (9.65±0.59 H, p<0.01) (Fig. 3).

![Figure 3. The results of determining physicomechanical characteristics of the wound defect. The force at the rupture point (N) in assessing the strength of a wound defect using a mechanical wound breaker (M±m)](image)

When analyzing the ultimate deformation of the skin flap, it was detected that the increase in the length of the skin flap in the Control group at the rupture point was 8.0±1.7%. According to the effect of the preparations on the elasticity of the wound defect, they can be arranged as follows (in descending order): gel with Bischofit (10.7±2.3%); Actovegin (8.2±1.9%); Contractubex (8.1±1.7%). The statistical processing showed that this parameter (p<0.05) reliably differs from the Control group only in the group that received Bischofit gel (Fig. 4).

![Figure 4. Results of determining physicomechanical characteristics of the wound defect. Elastic limit deformation (%) when evaluating elasticity of a wound defect using a wound-tearing machine (M±m)](image)
Colorimetric analysis of hydroxyproline concentration in the tissues of the wound defect

In colorimetric analysis it was found out that the highest concentration of hydroxyproline was in the tissues of wound defects in the animals treated with Contractubex. However, there was no statistically significant difference with the Control group. In comparison with the control concentration of HP (p<0.05), the tissues of the modeled wounds in the animals treated with Bischofit gel (79.7% of the control) contained significantly lower concentration of HP (Table 2).

<table>
<thead>
<tr>
<th>Group</th>
<th>Control</th>
<th>Bischofit</th>
<th>Actovegin</th>
<th>Contractubex</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration of HP, mg/g</td>
<td>16.59±1.08</td>
<td>13.23±1.68</td>
<td>15.89±1.37</td>
<td>17.61±0.67</td>
</tr>
</tbody>
</table>

Note: * – the presence of statistical significant differences when compared with the control group upon Mann-Whitney criterion (p≤0.05)

Taking into account the data obtained in determining the physicomechanical characteristics of the wound defect, the probable cause of an increase in the concentration of HP in the tissues of the animals treated with Contractubex is safekeeping of the inflammatory reaction, the prolongation of the remodeling processes of the newly formed connective tissue and the growth of granulation tissue.

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

Morphological study of the tissues of the skin flap taken from the wound area

Control group. In the Control group, a newly-formed connective tissue scar occupies a wide area, and the areas of uneven maturation of the connective tissue are visualized. The regenerated epidermis covering the wound is 3–4 times thicker than the epidermis of the intact skin lying next to it (Fig. 5A).

Figure 5. Photomicrograph of the skin cut in the wound area in the group of control observations

Note: stained by Van Gieson’s solution × 100 (A); × 200 (B)

In the thickness of the epidermis against the background of mitotic dividing cells of the basal layer, epithelial cells with pycnomorphic nuclei and phenomena of karyolysis have been visualized. The heterogeneity of the structure of the connective tissue scar should also be notified (Fig. 5B). The fibrous component in the scar area is represented by thin multidirectional collagen fibers. The cellular component prevails over the fibrous one. It should be notified that in the area of the scar there are no hair follicles and sebaceous glands Fig. 6).
**Bischofit.** On histological cut-sections of the skin of the animals treated with Bischofit gel, a thin connective tissue scar is visualized in the wound area. A complete regeneration of the epidermis is determined. It is several times larger than in the adjacent wound of the epidermis. In the scar zone, no derivatives have been detected (Fig. 7A). Directly under the epidermis, a wide band of connective tissue containing blood-filled vessels with local hemorrhages into the surrounding tissue is visualized. (Fig. 7B, C).

Regarding the spatial organization of the newly formed connective tissue scar, the violation of the layered structure of the skin should be notified. On the part of the newly formed connective tissue, germination occurs in the underlying hypodermis and muscle tissue (Fig. 8A).
Actovegin. When treated with preparations, there is a fully formed connective tissue scar of a wedge-shaped form. A complete closure of the wound defect with a stratified squamous epithelium is observed (Fig. 8B). A large number of mitoses are visualized in the basal and spinous layers of epidermis (Fig. 9A).

The cellular component prevails over the fibrous one.

At the base of the wedge-shaped connective tissue scar, the cellular component predominates over the fibrous one. In the field of view fibroblasts and lymphocytes, single fibrocytes prevail. He absence of a well-formed papillary layer should be notified. In sight, mature brightly oxyphilic collagen fibers placed randomly, dominate.

Hereby, in the areas adjacent from the scar, dermis, the fibers are thick structured and have all the normal functional criteria for dense unformed connective tissue (Fig. 9B).

Contractubex. In the study of the skin preparations of the animals treated with Contractubex, a wide area of the scar and complete filling of the wound defect with granulation tissue are visualized (Fig. 10A).

Over the entire surface of the scar there is a detachment of the newly formed thickened epithelium. The presence of heterogeneity in the spatial organization of the connective scar draws the attention. Local areas with a high degree of maturity of the newly formed connective tissue take place. Hereby, collagen fibers located chaotically, not tightly, alternate with portions of single-positioned fat cells with different diameters that are not prone to fusion (Fig. 10B).

At the base of the newly formed connective tissue scar, which continues in the deep layers of the dermis, the hypodermis and the muscular layer of the skin, a large number of dilated blood vessels with a tendency to hemorrhage into the surrounding tissue are found (Fig. 10C).
Figure 10. Photomicrograph of the skin cut in the wound area in the group of observations with the use of Contractubex

Note:
A – complete filling of the wound defect with granulation tissue. Stained by Van Gieson’s solution; × 100;
B – an alternation of areas with a high degree of maturity of the newly formed connective tissue with areas of single fat cells with different diameters that are not prone to fusion. Stained by Van Gieson’s solution; × 100;
C – in the deep layers of the dermis, hypodermis and muscular layer of the skin, a large number of dilated blood vessels with a tendency to hemorrhage into the surrounding tissue are detected. Stained by hematoxylin and eosin; × 200

Comparative quantitative assessment
In the questionnaire survey by the expert committee, the average score was determined in each group (Table 3). A lower score indicates a more consistent histological pattern of specimens obtained from the groups.

Table 3. Results of the scoring microscopic skin samples by the expert committee (M±m)

<table>
<thead>
<tr>
<th>Qualitative character</th>
<th>Control</th>
<th>Bischofit</th>
<th>Actovegin</th>
<th>Contractubex</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytoarchitectonics disruption</td>
<td>1.71±0.18</td>
<td>1.34±0.21</td>
<td>1.49±0.15</td>
<td>1.49±0.15</td>
</tr>
<tr>
<td>Intracellular matrix architectonics disruption</td>
<td>1.32±0.21</td>
<td>1.12±0.09</td>
<td>1.21±0.10</td>
<td>1.31±0.11</td>
</tr>
<tr>
<td>Hemorrhage, enlarged vessels</td>
<td>1.91±0.19</td>
<td>1.24±0.11</td>
<td>1.54±0.16</td>
<td>1.39±0.15</td>
</tr>
<tr>
<td>Violation of epithelialization</td>
<td>1.72±0.21</td>
<td>1.52±0.15</td>
<td>1.51±0.19</td>
<td>1.79±0.21</td>
</tr>
<tr>
<td>Leukocyte infiltration</td>
<td>1.84±0.23</td>
<td>1.32±0.31</td>
<td>1.29±0.12</td>
<td>2.0±0.21</td>
</tr>
<tr>
<td>Average score</td>
<td>1.70±0.20</td>
<td>1.31±0.21*</td>
<td>1.41±0.15*</td>
<td>1.60±0.16</td>
</tr>
</tbody>
</table>

Note:
a lower score indicates a more consistent histological pattern;
* – p <0.05 when compared with the Control

From the data presented in Table 3 it can be seen that less pronounced morphological changes are observed in the groups treated with Bischofite gel and gel with Actovegın.

Evaluation of the ratio in collagen types I and III in a polarizing microscope
When assessing the ratio in collagen types I and III in the tissues of the wound defect when dyeing with picrosirius red, it was established that, by the number of mature collagen fibers, the studied groups can be arranged in the following sequence (descending): Bischofit > Actovegin > Control > Contractubex (Table 4, fig. 11).
Table 4. Ratio in collagen types I and III in tissue samples of modeled wounds received on day 8 after starting the experiment (M±m)

<table>
<thead>
<tr>
<th>Group</th>
<th>Control</th>
<th>Bishofite</th>
<th>Actovegin</th>
<th>Contractubex</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ratio in collagen types I and III</td>
<td>0.56±0.012</td>
<td>0.73±0.023*</td>
<td>0.67±0.017</td>
<td>0.38±0.02*</td>
</tr>
</tbody>
</table>

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

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

CONCLUSION

The study showed that the best results had been obtained when using Bischofite gel. The wound defect in this group was characterized by the greatest strength, elasticity, a good histological pattern. Judging by the low concentration of hydroxyproline and collagen type III, it is less prone to scar formation. Actovegin has a less significant, but pronounced reparative effect on this model. Actovegin gel showed a positive effect on the macro- and microscopic picture of the wound defect, as well as the strength of the wound and the preventive effect on the excessive formation of the scar tissue. Less satisfactory results were obtained when applying Contractubex. Without having a significant impact on the physicomechanical characteristics of the wound, Contractubex increased the content of HP and reduced the content of mature collagen (type III). The similar results show that Contractubex has reduced the reparative potential of tissues, increasing the growth of granulation tissue and slowing down its recovery. This conclusion is confirmed by the results of histological examination of the animals treated with Contractubex and can be explained in terms of the available information on the pharmacodynamics of this drug.

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Figure 11. Microscopic picture of the modeled skin wound area

Note:
polarization microscopy. Sirius Redstain. × 400;
Control group (A);
Bishofit (B);
Contractubex (C)


Conflict of interest
The authors declare no conflict of interest.

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