Abstract

This article describes the results of experiments dealing with application of gel form of recombinant human angiogenin (Farmagen) used in the treatment of regular and suppurative tender wounds.

Key words: angiogenin, planar skin-muscle wound, linear wound, diabetes mellitus, complicated with trophic ulcer

INTRODUCTION

The treatment of long standing suppurative wounds and trophic ulcers appears to be of a high concern in modern medicine and is a medical-sociological problem of modern society. Therefore, the theoretical and experimental medicine is in a critical need to develop effective medical ointments.

At the moment, there is a variety of treatment methods of suppurative wounds. However, the topical treatment of suppurative wounds and ulcers remains the most recognized and widespread due to its simplicity, accessibility, low cost and effectiveness. Surgeons are equipped with a wide range of tools for topical medical treatment of suppurative wounds. These include antibacterial agents, proteolytic enzymes, multicomponent hydrophilic ointment based sorbents, biosynthetic and synthetic coatings, hydrocolloids, regeneration stimulators, etc. Most clinicians acknowledge that the most effective wound healing agents are multicomponent ointments with a hydrophilic base, as they have immersive actions (i.e. anti-microbial, absorbent, regenerating and analgesic effects at the same time). However, there are certain drawbacks, such as frequent allergic reactions, high cost, etc.

Despite the abundance of available topical suppurative wounds and trophic ulcer ointments in the modern pharmaceutical market, the desired degree of wound healing drug effect has not yet been reached. Existing standards of pharmacotherapy of purulent wounds and trophic ulcers are not satisfactory as they don’t achieve the final result.

OBJECTIVE

To research the potential of wound healing properties of recombinant human angiogenin ("Farmagen" gel for external use).

Based on the objective, the following specific objectives were planned:

1. To create experimental models of linear wounds, wounds plane and diabetes complicated by trophic ulcers.
2. To study the wound healing effect of recombinant human angiogenin on the model of linear wounds.
3. Examine the wound healing effect of recombinant human angiogenin model plane wounds.
MATERIALS AND METHODS

Recombinant human angiogenin

Angiogen is a main protein that induces in vivo blood vessel growth, and contains 123 amino acids with a molecular mass of about 14 kDa. Chemical-enzymatic synthesis of human angiogenin gene was implemented for the first time under the guidance of professor, academician of RANS (Russian Academy of Natural Sciences) N.P. Mertvetsova. Cloning of human angiogenin gene was carried out in several expression systems and as a result built-producing strain of angiogenin was constructed. The technology of cultivation of the producer strain in gas-vortex bioreactors, as well as separation and purification of the resulting recombinant human angiogenin were worked on. The strain of Escherichia coli BL (DE3) lpZZSA (Collection of Microorganisms, Interregional Center of human microcenosis, collection number MCKM B-127) is a producer of the recombinant fusion protein of human angiogenin.

Model building of planar skin-muscle wound

Wound models were built in male rats, weighing 250-300 g under ether anesthesia. Full flap skin wound was performed in the neck and the shoulder area by removing a skin graft and subcutaneous tissue of 3 square cm. In order to obtain the same size of the original wounds, small frames were sewed to the edges of all wounds. This is to minimize the stretching of the wounds, as the rats have extensive agility.

Frames were removed two days later. Prior to treatment, the wounds were infected with 0.1 ml of 15×10^9 cells/ml suspension of E. coli and St. aureus. Treatment started soon after the drying of the wound.

Model building of linear wound

Linear wound was prepared as follows: the wound (of 3 cm length) was performed until reach of fascia.

All models were built under deep ether anesthesia.

The studied drug was applied to the wound surface daily 1 time per day. For the purposes of taking material for morphological studies, the animals were removed from the experiment on the 5th day after the application of the injury. Materials were picked up at the border of healthy and damaged skin and were fixed in 10% formalin neutralized with chalk. The further processing and filling of material into paraffin was performed using the standard histological procedure. 5-7 microns thick cut slices were stained with hematoxylin and eosin. All the slides were examined using microscope of Axioskop-2 Carl Zeiss brand.

Modelling of diabetes mellitus, complicated with trophic ulcer

In order to create a model of diabetes mellitus complicated with trophic ulcer, the white lab rats (male and female, weighing 200-240 g.) were used. As per the design of the experiment, a model of alloxan diabetes was used. During the formation of this model, the reparative capabilities of animals are significantly reduced, thus giving opportunity to simulate chronic long standing wounds,. Diabetes was caused by intravenous injection of 45 mg / kg of alloxan into the tail lateral vein. The effectiveness of the model was evaluated by the level of serum glucose by standard clinical techniques. Long standing wound was caused by injecting 50 µl of 20% Potassium hydroxide into rear foot pad, after the diabetes mellitus is formed (5-7 days after injecting alloxan). Special features of this model include the complexity of the implementation, the reproducibility at 70%, high risk of death of the animals and need in special diets.
Cytological methods

Smears from the wound surface for cytological studies were prepared by "surface biopsy" method, which was proposed by Kamaeva. According to this method, the material for research is taken by light scraping the surface layer off the wound using surgical scalpel. The material obtained in this way was transferred onto a glass slide. In order to obtain a fixed drug, it was immersed into Nikiforov mixture (for 15 minutes), and then stained with Romanowsky-Giemsa method. Smear microscopy was performed under immersion.

Morphological methods

For the purposes of taking material for morphological studies, the animals were removed from the experiment by decapitation. Removing animal from experiment was conducted 3, 7, 10, 14 and 21 days after the formation of the model. Materials were collected at the border of healthy and damaged skin, and fixed in 4% buffered formalin. The further processing and filling material into paraffin was performed by the standard histological procedure. Cuttings of 5-7 microns thickness were stained with hematoxylin and eosin. All gisto-specimens were examined on the Axioskop-2 microscope branded Carl Zeiss.

Statistical methods

Statistical analysis of the results was carried out using nonparametric statistics methods, with evaluation of the significance of differences as per Student's distribution.

RESULTS AND DISCUSSION

Model building of planar skin-muscle wound

Experimental animals were divided into 3 groups:

1) control group (animals which have not been wounded);
2) test group (substance angiogenin 0.01%);
3) comparison group (Solkoseryl drug).

The dynamics of wound healing was assessed on the following parameters: time of divestiture of the primary wound scab, time of appearance of granulation and their quality, process of epithelialization according to visual inspection and morphology.

The study medication "Farmagen" was applied to the wound surface daily once per day. For the purpose of collecting materials for morphological studies, the animals were removed from the experiment on the 5th day after the wound was performed. Materials were collected at the border of healthy and damaged skin, and fixed in 10% formalin neutralized with chalk.

The further processing and filling material into paraffin was performed by the standard histological procedure. Cuttings of 5-7 microns thickness were stained with hematoxylin and eosin.

Morphology of skin-muscle wound during medical treatment

In the control study group on the 5th day of the experiment morphological picture of wound healing process was characterized by traumatic inflammation with ceroelastic and neutrophil exudation, and an abrupt vasodilatation and plethory. The edges of the wound were covered with a thin layer of epithelial, inflammatory infiltration in the underlying connective tissue beneath was closely associated with inflammatory changes of the dermis in the wound itself.
Figure 1. Wound, treated with the substance of angiogenin at a concentration of 0.01%, 5-th day. Stained with hematoxylin and eosin. Increased by 200

Comparative morphological study of the dynamics of wound healing on day 5 of the experiment in the first healing phase - the phase of traumatic inflammation - showed lesser degree of basophilia neutrophils white blood cells in the surface layer of the leukocyte-fibrinous wounds treated with the preparation "Farmagen". The These white blood cells are located in the gel-like mass of the therapeutic drug. Presumably, there has been a release of cationic proteins from leukocyte lysosomes, affecting, as well known, the development of initial and microcirculatory processes in inflammation. Probably, this fact should be taken into account in the study of mechanisms of tissue resistance formation and launch mechanisms of inflammation.

In addition to morphological studies, the wound healing during the day was evaluated. Duration of wound healing was recorded when scab was detached and the epithelium covered defect beneath the scab was present. As can be seen, both Farmagen and Solkoseryl accelerate the healing of skin wounds in rats, however the study drug Farmagen has the advantage by significant value compared to Solkoseryl.

Table 1. Effect of the angiogenin drug on periods of complete epithelialization of planar wounds

<table>
<thead>
<tr>
<th>Drug</th>
<th>Duration of healing, (M ± m, 24-hour period)</th>
<th>Difference of wound healing times compared to control group, ±24-hour period</th>
<th>Acceleration of healing times compared to control group, %</th>
<th>P&lt;</th>
<th>P1&lt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polyethylene</td>
<td>19,8 ± 0,63</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>(control group)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Angiogenin</td>
<td>12,1 ± 0,33</td>
<td>- 7,7</td>
<td>39,0</td>
<td>*</td>
<td>**</td>
</tr>
<tr>
<td>(experimental group)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Solkoseryl</td>
<td>15,1 ± 0,42</td>
<td>- 3,8</td>
<td>19,2</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>(comparison group)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
The results were statistically processed using the Student's t-test. The level of significance (P) values derived by comparison with the control, untreated: * p < 0.001. confidence level (P1) values derived by comparison with the control without treatment: ** P < 0.05

Modelling of the linear wound

Experimental animals were divided into 3 groups:
1) control group (animals which have not been wounded);
2) test group (substance Farmagen);
3) comparison group (Solkoseryl drug).

The study medication was applied to the wound surface daily once per day. For the purpose of collecting materials for morphological studies, the animals were removed from the experiment on the 5th day after the wound was performed.

Morphological picture of the wounds on day 5 in the control group of the study was characterized by the inflammatory phase of wound healing process. The defect tissue was filled with necrotic masses and covered with fibrinopurulent leukocyte layer of varying thicknesses along with numerous neutrophils and pyocyte. The inflammatory infiltrate had moderate severity and captured the muscle bundles located in the papillary dermis. The reticular dermis was showed edema and plethory of the blood vessels. There was a thin layer of epithelial regeneration along the edges of the wound, which crawled under the necrotic mass. The border between the wounded surface area with intact skin surface is well observed.

On the fifth day of the experiment during the treatment of wounds with substance angiogenin 0.01%, the surface fibrinopurulent leukocyte layer was uniformly permeated with gel (Figure 2). Granulation tissue was presented as a thin layer and was characterized by moderate level of maturity. There were small hemorrhages. Along the edges of the wound the enhanced tumor of small blood vessels is well observed.

![Figure 2](image-url). The wound treated with angiogenin 0.01% on the 5th day, growth of small blood vessels. Stained with hematoxylin and eosin. Increased by 200.

On the 5th day a massive fibrinous-necrotic film all along the wound surface remained in a group treated with Solkoseryl. Inflammatory infiltration was expressed much less in comparison to control
group. Young tissue, in one case, was characterized by the start of the granulation tissue with the appearance of fibroblasts and small oval shaped blood vessels, and in another case lacked such characteristics.

Thus, taking into account the polymorphism of morphological picture of early wound healing processes in individuals from different groups, the most favorable trend towards healing wounds was found to be treatment with angiogenin.

Table 2. Dynamics of the structure after modelling of linear wound and application of angionenin, 24-hour day (M ± m) (absolute number in the standard view)

<table>
<thead>
<tr>
<th>Indicator</th>
<th>1</th>
<th>3</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interstitial tissue area  мкм³ × 10³</td>
<td>313±19*</td>
<td>220±14*+</td>
<td>192±11+</td>
</tr>
<tr>
<td>Bloodstream capacity, мкм³×10³</td>
<td>*</td>
<td>0,37*+</td>
<td>0,77±0,03*+</td>
</tr>
<tr>
<td>Total cellularity</td>
<td>1064±41*+</td>
<td>1070±80*+</td>
<td>1010±80*+</td>
</tr>
<tr>
<td>Interstitial tissue area  мкм² × 10³</td>
<td>313±19*</td>
<td>220±14*+</td>
<td>192±11+</td>
</tr>
<tr>
<td>Total cellularity</td>
<td>1064±41*+</td>
<td>1070±80*+</td>
<td>1010±80*+</td>
</tr>
<tr>
<td>Fibroblasts</td>
<td>197±12*+</td>
<td>394±23*</td>
<td>450±31*</td>
</tr>
<tr>
<td>Macrophages</td>
<td>45±3*+</td>
<td>57±6*+</td>
<td>58±5*+</td>
</tr>
<tr>
<td>Labrocytes</td>
<td>33±2*+</td>
<td>46±5*+</td>
<td>50±4*+</td>
</tr>
<tr>
<td>Extravascular erythrocytes</td>
<td>180±10*</td>
<td>107±8*</td>
<td>48±4*+</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>41±2*+</td>
<td>74±7*</td>
<td>74±7*</td>
</tr>
<tr>
<td>Polymorphonuclear leukocytes</td>
<td>189±13*+</td>
<td>133±10*+</td>
<td>93±8*+</td>
</tr>
</tbody>
</table>

* Significant difference from the norm; + Significant difference from the group without treatment.

<table>
<thead>
<tr>
<th>№</th>
<th>Mass, g</th>
<th>The concentration of glucose, mM, before alloxan injections</th>
<th>The concentration of glucose, mM, after alloxan injections</th>
<th>№</th>
<th>Mass, g</th>
<th>The concentration of glucose, mM, before alloxan injections</th>
<th>The concentration of glucose, mM, after alloxan injections</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>215</td>
<td>4,5</td>
<td>16,5</td>
<td>1</td>
<td>201</td>
<td>3,6</td>
<td>17,6</td>
</tr>
<tr>
<td>2</td>
<td>210</td>
<td>3,8</td>
<td>16,8</td>
<td>2</td>
<td>219</td>
<td>5,3</td>
<td>lethal</td>
</tr>
<tr>
<td>3</td>
<td>232</td>
<td>5,1</td>
<td>lethal</td>
<td>3</td>
<td>202</td>
<td>3,6</td>
<td>12,6</td>
</tr>
<tr>
<td>4</td>
<td>207</td>
<td>3,7</td>
<td>7,7*</td>
<td>4</td>
<td>238</td>
<td>4,8</td>
<td>19,8</td>
</tr>
<tr>
<td>5</td>
<td>237</td>
<td>5</td>
<td>20</td>
<td>5</td>
<td>218</td>
<td>4,1</td>
<td>17,1</td>
</tr>
<tr>
<td>6</td>
<td>202</td>
<td>4,3</td>
<td>14,3</td>
<td>6</td>
<td>205</td>
<td>3,1</td>
<td>5,9*</td>
</tr>
<tr>
<td>7</td>
<td>212</td>
<td>3,2</td>
<td>17,7</td>
<td>7</td>
<td>220</td>
<td>4,6</td>
<td>19,6</td>
</tr>
<tr>
<td>8</td>
<td>202</td>
<td>5,2</td>
<td>19,2</td>
<td>8</td>
<td>213</td>
<td>3,5</td>
<td>lethal</td>
</tr>
</tbody>
</table>
Modeling of diabetes mellitus, complicated with trophic ulcer

In order to study the healing process, alloxan diabetes was modeled in rats according to the described method (figure 3).

Thus, in modeling of alloxan diabetes in experimental animals, there are 3 lethal cases recorded out of 20 animals, also 2 animals did not develop diabetes within two weeks. All rats, which have been successfully implemented the model of diabetes, were injected 50 µl of 20% Potassium hydroxide into the rear foot pad. At the time of formation of the wound (about a week) the active treatment with test preparations throughout the observation period were conducted.

The results of the histological study of chronic wounds during alloxan diabetes.

Morphological picture of the wound before the treatment (that is, at the time of the formation of the model) is characterized by deep dermal damages, abundance of pyocyte and minor fibrinous layer with signs of necrosis. The presence of neutrophils is noted. Wound channel was laid by fibrinoid masses, covered with a layer of leukocytes. There were lots plethoric vessels and signs of inflammatory infiltration, hemorrhage areas.
The surface at the time of the end of treatment was epithelialized. Tabs of hair follicles were seen in the underlying connecting tissues. However, complete healing of the wound has not occurred, it is noted that the complete formation of the dermis has not happened yet.

Due to angiogenin treatment, chronic wound fully epithelialized along with full restoration of skin structure on the 21st day of observation.
Table 4. Duration of healing times during angiogenin treatment

<table>
<thead>
<tr>
<th>Group</th>
<th>Duration of healing, 24 hr period</th>
<th>Difference compared to control group, ± 24 hr period</th>
<th>Acceleration of wound healing compared to control group, %</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>27,8±0,92 -</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Comparison group treated with Solkoseryl</td>
<td>24,7± 3,1</td>
<td>3,1</td>
<td>11,2</td>
<td>&gt; 0,05</td>
</tr>
<tr>
<td>Angiogenin</td>
<td>19,8 ± 0,63</td>
<td>8,0</td>
<td>28,8</td>
<td>&lt; 0,05</td>
</tr>
</tbody>
</table>

Note: the level of significance compared to control

Thus, angiogenin drug has beneficial effects on the healing of chronic wounds.

CONCLUSION

Safety assessment and effectiveness of angiogenin substance was evaluated. To assess the effectiveness, the modelling of skin-muscle and linear wounds were performed and treatment of these two with the substance of angiogenin was performed. According to the results, angiogenin has showed a far better wound-healing activity when compared to Solkoseryl. The experiment on the model of skin and muscle and linear wounds has showed that recombinant human angiogenin has a wound-healing effect, accelerates the process of tissue repair, and stimulates angiogenesis.

An assessment of the effectiveness of angiogenin on the model of long-term healing wounds in alloxan diabetes was conducted. Wound healing properties have been expressed fully in morphology and histology of wounds, when compared to the control and experimental groups. A rapid melting of necrotic masses and moving to the edges of the wound epithelialization are noted.

REFERENCES

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