Features of Skin Wound Repair Under Conditions of Stem Cell Secretome (Cytokines) Application

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Abstract. The goal of the study was to evaluate the effect of mesenchymal stem cell (MSC) secretome on wound healing both in vivo and in vitro. The in vitro study used a monolayer of epithelial-like cells, while the in vivo study used smooth-haired guinea pigs. The secretome used in the study contained cytokines that are known in literature to regulate inflammation, stimulate cell chemotaxis and the regenerative process, and induce angiogenesis (VEGF, GRO/KC, TGF-β1, TGF-β2, IL-6, MCP-1, IFN -γ, IP-10, IL-10). In the in vitro experiment, monolayer of epithelial-like cells was scratched and then placed in a culture medium, with MSC secretome being added to the experimental sample. The recovery rate between the two samples was compared, and showed increased cell proliferation activity in the cell monolayer of the experimental sample, which led to faster closing of the defect. In the in vivo experiment, the induced skin wound was treated with the MSC Secretome (in the form of drops) for 2 days. The results were analyzed using methods of macroscopic observation, planimetry, histology and microscopic morphometry. The use of MSC Secretome resulted in an earlier epithelization of the wound, an earlier onset of the remodeling phase of the regenerate, and a decrease in the formation of scar tissue. In addition, under the influence of the MSC Secretome, the regenerate acquired a structure that closely resembles organotypic skin.

Keywords: Mesenchymal stem cells · Secretome · Cytokines · Skin · Wound · Regenerative medicine

1 Introduction

The use of regenerative drugs is of great interest to veterinary medicine. A special place is occupied by drugs based on the regenerative potential of Mesenchymal Stem Cells (MSC). MSCs regulate the processes of inflammation, chemotaxis, and regeneration using a secreted complex of cytokines [1]. Secretomes are of particular interest, a new class of regenerative drugs that will accelerate tissue healing [2].

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Cytokines are known to positively affect repair due to the balance of pro- and anti-inflammatory cytokines [3–11]. Thus, with a secret, MSC regulates the growth and regression of capillaries, proliferation and maturation of fibroblasts, which prevents excessive sclerosis [11–13]. Despite the positive results of the use of cytokines in the experiment and clinic [14–18], the morphology of repair when used is described fragmentally.

This determines the need for further research. In 2017, the regenerative drug Reparin-Helper based on the MSC secretome was registered in the Russian Federation by T-Helper Cell Technologies LLC. It is a standardized cytokine complex. The effect of MSC secretome on cell and skin monolayer repair is presented in this paper.

2 Materials and Methods

The work was carried out at the Moscow State Academy of Veterinary Medicine and Biotechnology - MBA named after K. I. Scriabin together with T-Helper Cell Technologies LLC. Experiments included the use of the commercially available regenerative drug Reparin-Helper®. The active ingredient Reparin-Helper® is a secret with high concentrations of cytokines (VEGF, GRO/KC, TGF-β1, TGF-β2, IL-6, MCP-1, IFN-γ, IP-10, IL-10 and others).

The experiment involved 60 smooth-haired guinea pigs (weight 650–750 g, age 6 months, selected to be consistent). The animals were kept and cared for in accordance with the requirements of Orders of the Ministry of Health of the Russian Federation «Works using experimental animals» and the Order of the Ministry of Health of the USSR No. 48 dated 01/23/85 «On the control of work using experimental animals», International rules for the humane treatment of animals - Directive 2010/63/EU of the European Parliament and the Council of the European Union.

Animals were divided into experimental and control groups (Table 1).

Table 1. Characteristics of animal groups in the experiment

Time (days)	Total animals in each group	
	Experiment	Control
7	10	10
14	10	10
30	10	10

All animals were placed under neuroleptanalgesia (Meditin 1%) and local anesthesia (Novocaine 0.5%) followed by a 2.5 cm^2 excision from the thigh region on each side of each animal. In the control group, healing occurred without intervention (no drugs used), in the experimental group the MSC Secretome was applied 3 times a day for 2 days in drop form. The subjects underwent a clinical examination, as well as planimetry, light microscopy and micromorphometry evaluations at 7, 14 and 30 day intervals following

the operation. Planimetric studies were carried out according to L.N. Popova's method on the 7th, 14th and 30th days after the operation.

Biopsies for histological analysis were taken on the 7th, 14th, and 30th days after the operation. The material was fixed in a 10% formalin solution, and after washing and dehydrating in alcohols of ascending concentration, it was poured into paraffin according to generally accepted methods. The sections were prepared on a rotary automated microtome HM-325 (Microm international GmbH, Germany) and stained with hematoxylin and eosin, as well as according to Van Gieson. Histological sections and microphotography were analyzed using a Jenamed-2 light microscope (CarlZeiss, Jena, Germany) combined with the ImageScope C digital microscopy system (Systems for Microscopy and Analysis LLC), which was also used for microscopic morphometry of digital material.

3 Results

3.1 Results of Macroscopic Studies (in Vivo)

The macroscopic appearance of the wound was used to establish qualitative differences in the wound healing processes between the experimental and control groups.

In the experimental group In the experimental group, the wound size decreased more markedly after 7 days than in the control group (Fig. 2A). The wound was covered with scab, signs of marginal epithelialization are visible. After 14 days, cicatricial regeneration in the center was observed in the defect area, the peripheral part is similar to the surrounding skin (Fig. 2B). On day 30, only a small scar was observed in the center of the regenerate, the peripheral part being similar to intact skin (Fig. 2C). In the control group after 7 days, the wound was covered with a scab, had dry, uneven edges and signs of marginal epithelialization (Fig. 2D). After 14 days, the central part of the regenerate was not yet completely epithelialized and was still covered with a scab (Fig. 2E), in contrast to the experiment. On day 30, a depigmented scar without hair growth was seen in the area of the defect (Fig. 2F). The data obtained from the macroscopic observation of wound healing are supported by the results of planimetric studies.

3.2 Results of Planimetric Studies

Differences in the size of the wound area were observed at day 1, 7, 14 and 30 (Table 2).

In the experimental group, by the 7th day, the area of the remaining wound was 50% of the area of the original wound, that is, an epithelialized regenerate was already formed on 50% of the area of the defect. By the 14th day the area of the defect was completely closed, exhibiting a small scar, with the total scar area of 26%, decreasing to 12% on the 30th day (relative to the area of the original wound). In the control group, by the 7th day, the wound area decreased by 30%. By the 14th day, the defect area was not yet completely epithelialized, with a total defect area of 44% relative to the original wound. By the 30th day, scar tissue formed in the area of the defect with the total size of the scar tissue being 24% of the area of the original wound. The data show that the reduction of the wound area under the influence of MSC Secretome occurs faster than in the control, and by the 30th day. The area of scarred skin in the experimental group is half as much as in the control.

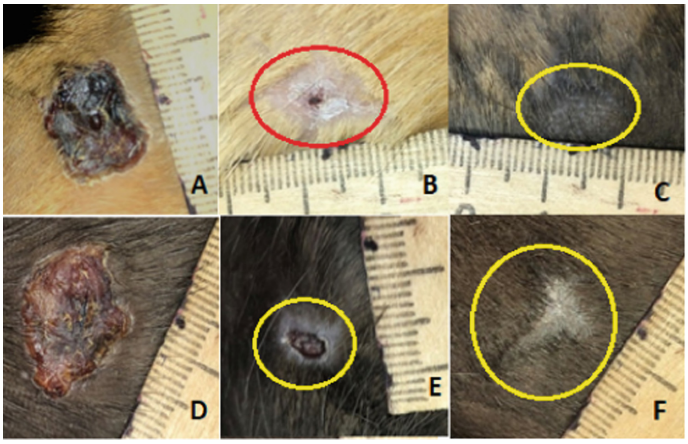

Fig. 2. The appearance of the defect area at varying time periods of the experiment. Experimental group: A - 7 days, B - 14 days, C - 30 days. Control group: D - 7 days, E - 14 days, F - 30 days.

Table 2. Planimetric characteristics of healing area, cm^2

Time (days)	Groups	
	Experiment	Control
1	2.5 ± 0.4 (wound)	2.5 ± 0.3 (wound)
7	1.25 ± 0.15 (wound)	1.75 ± 0.5 (wound
14	0.65 ± 0.15 (scar)	1.1 ± 0.12 (wound)
30	0.25 ± 0.04 (scar)	0.64 ± 0.02 (scar)

3.3 Microscopic Study Results

Studies showed that granulation tissue was formed in the experimental group on day 7, in which there were more hemocapillaries and fibroblasts than in the control group (Tables 3 and 4).

In the experimental group, the granulation tissue begins to mature earlier than the control tissue. Signs of regenerate remodeling are visible by days 14: tissue with histotypic features prevails in the regenerate structure; scar tissue covers a relatively small area. In the control group, scar tissue prevails in the regenerate structure, and the central portion of the regenerate is not yet fully epithelized, as observed and macroscopically.

By day 30, in the experimental group, most of the regenerate had an organotypic structure (restoration of structural zones, hair follicles, sebaceous glands). A small central part is formed by a scar (Fig. 3 A, B). In the control group, significant scar changes of the skin persisted more during this period (Fig. 3 C, D). All this indicates an acceleration of regenerate remodeling under the influence of cytokines. The histology data suggests an accelerated regenerative process in the experimental group and correlates well with the results in the macroscopic and planimetric studies.

The data of microscopic studies are supplemented by the results of micromorphometry quantifying the differences in tissue composition between experimental and control groups.

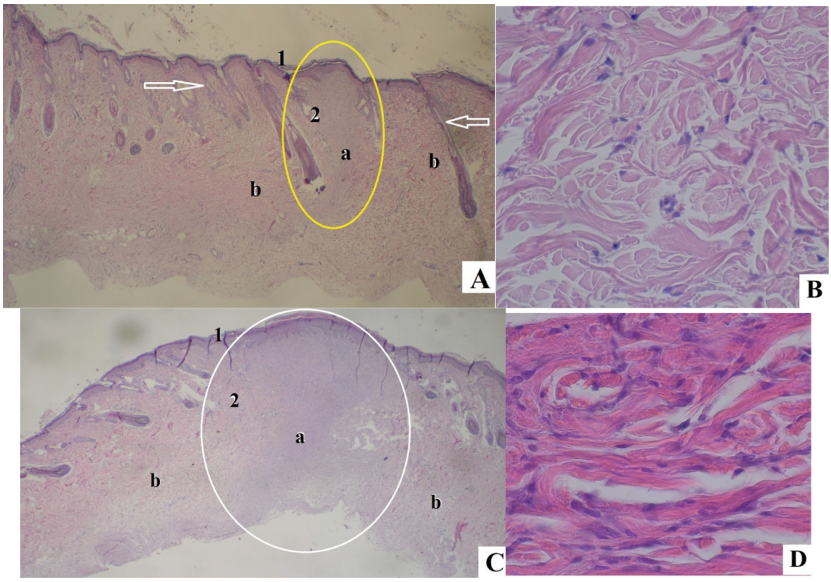

Fig. 3. Structure of skin regenerate. 30 days.

A - experiment: structure of the regenerate, a small part with scar structure (a, circled) and the peripheral part (b), which has an organotypic structure. 1 - epidermis, 2 - connective tissue part of regenerate. Arrows show hair follicles. Hematoxylin and eosin, x40.

B - experiment: the structure of the regenerate connective tissue is similar to the structure of the dermis, bundles of collagen fibers are thin, multi-directional, with pronounced inter-fiber spaces. Fibroblasts are visible between them, few blood capillaries observed. Hematoxylin and eosin, x400.

C - control: the regenerate has a partial scar structure (a, circled) that is larger than in the experimental group. (b) peripheral part of the tissue with observed sclerosis phenomena. 1 - epidermis, 2 - connective tissue part of regenerate. Hematoxylin and eosin, x40.

D - control: scar tissue characterized by thick, tightly arranged bundles of collagen fibers, with numerous fibroblasts visible. Hematoxylin and eosin, x400.

3.4 Results of Micromorphometric Studies of Skin Regenerative Tissue

Micromorphometric studies of regenerative tissue can help elucidate the mechanistic differences in the regeneration process. The following factors were evaluated: the number of hemocapillaries, the total fibroblast count and the thickness of collagen fibers bundles.

Differences between groups were also observed in the dynamics of capillary formation (Table 3). Differences between groups were also observed over time in hemocapillar counts (Table 3). In the control group, the total number of capillaries gradually decreased from day 7 to day 30 and corresponded to scar tissue (1–2 in the field of vision). In the experimental group, the number of hemocapillars on days 7 and 14 was twice that of the control. On the 30th day, it was also more than in control. Better regenerate vascularization provides better trophic supply and oxygenation of regenerate tissue. This can lead to faster remodeling.

Table 3. Hemocapillary dynamics in regenerate connective tissue

Time (days)	Total number of counted capillaries in field of view (x400)	
	Experiment	Control
7	12 ± 1	6.5 ± 1.2
14	8 ± 1	4.5 ± 1
30	5.5 ± 1.5 in regenerate surface layers 3.5 ± 1 in regenerate deep layers	2.5 ± 0.5

Differences between groups were also noted in the total number of regenerate tissue fibroblasts (Table 4). In the control group, the maximum number of fibroblasts in the granulation tissue was recorded on day 7, decreasing slightly by day 14 and remaining significant until day 30 of the experiment. This is a sign of ongoing collagenogenesis and incomplete remodeling of scar tissue. In the experimental group, significantly higher fibroblast counts are observed on day 7, which is significantly reduced to match the control group by day 14, followed by a rapid decrease in fibroblast counts by day 30. On day 30, the number of fibroblasts in the experimental sample was lower than in the control sample, which may indicate an earlier completion of the scar tissue remodeling process.

Table 4. Change in fibroblast count in the connective tissue portion of the regenerate in control and experimental groups

Time (days)	Total count of fibroblasts in field of view (x400)	
	Experiment	Control
7	34 ± 4	23.5 ± 1.6
14	18 ± 2	17.5 ± 1.4
30	12 ± 2	16 ± 1.5

As shown in Table 5, the thickness of the collagen fiber bundles in the experimental and control groups is not different. In the experimental group, however, the collagen fiber bundles are multidirectional and less tightly packed, giving them similarities to dermal

fibroarchitectonics. At the same time, both groups demonstrate a typical dynamics of the thickness of the collagen fiber bundle: the thickness of the bundles is minimal on the 7th day of the experiment, on the 14th day the indicator has intermediate values reaching maximum values on the 30th day.

Table 5. Thickness of collagen fiber bundles in the connective tissue part of the regenerate. RC – center of regenerate, P – peripheral part of regenerate.

Time (days)	Thickness of collagen bundle thickness, μm	
	Experiment	Control
7	1.9 ± 0.3	2.5 ± 0.5
14	2.1 ± 0.6	4.2 ± 0.8
30	2.3 ± 0.7 – RC 3.9 ± 0.9 – P	4 ± 0.8 – RC 6.5 ± 0.7 – P

The obtained data shows that, as a whole, the dynamics of morphometric indicators correspond to the typical course of the reparative regeneration process. However, the experimental group showed signs of more active vascularization of granulations and their earlier maturation, as well as an earlier start to the of scar remodeling stage. By the 30th day the structure of the regenerate formed in the experimental group was similar to that of organotypic skin.

4 Discussion

The results of our studies are consistent with the data on the positive effect of stem cell secretome (cytokines) on the repair of skin wounds [1–11]. We have shown an increase in the proliferative activity of epithelium-like cells by the influence of MSC Secretome in vitro, which led to a faster closure of the defect. This finding is supported by in vivo studies, where, under the influence of MSC Secretome, the wound epithelized faster and regenerate remodeling began earlier. As a result, the area of scar skin changes in the experimental group was half that of the control group. The quality of the scar tissue was also different: in the experimental group it was soft, and the surface level coincided with the level of the surrounding skin, pigmentation remained and hair growth was observed. In the control group, the scar was dense, depigmented, hair growth was not observed. This is supported by the results of microscopic and micromorphometric studies showing signs of earlier regenerate maturation.

The results of the studies are also consistent with literature [11–13] that describes how cytokines enhance capillary growth in the healing area and prevent the formation of skin scarring. The results also show that the MSC Secretome optimizes the process of reparative skin regeneration by reducing the scar remodeling phase and leads to the formation of a regenerate that is close in structure to the organotypic one. We attribute these effects to the presence of a balanced complex of cytokines in the MSC Secretome, including VEGF, GRO/KC, TGF-β1, TGF-β2, IL-6, MCP-1, IFN-γ, IP-10 and IL-10.

The cytokines stimulate the internal reparative potencies of skin tissues, including the growth of hemocapillaries, and activation of progenitor cells and fibroblasts. The data obtained add to the understanding of the mechanisms of tissue healing under the influence of cytokines.

5 Conclusion

A number of conclusions can be reached, based on the results of the studies described above.

The MSC Secretome is useful for improving the healing of skin wounds. Applying the MSC Secretome results in earlier epithelialization of the defect, progressive reduction of the wound and scar area and formation of a regenerate similar in structure to the surrounding skin. The scar-like defect in the healing area is 2 times smaller than in the control samples.

Under the influence of the MSC Secretome, the granulation tissue exhibits an increased quantity of hemocapillaries and fibroblasts. This granulation tissue matures faster than in the control group and undergoes remodeling earlier, resulting in an organotypic regenerate structure. The addition of MSC Secretome to the damaged epithelial-like cell monolayer in vitro enhances the proliferative activity of the cells and leads to earlier closure of the defect.

The findings reveal some of the biological mechanisms of action of the cytokines in the MSC Secretome during reparative regeneration and provide a practical basis for further research in regenerative biology and medicine.

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