Regeneration of Skin Surface by Multipotent Mesenchymal Stem Cells of Adipose Tissue in Laboratory Animals with Infected Wounds

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Abstract

This paper presents results of experimental studies in laboratory animals with a simulated infected wound, for which mesenchymal stem cells (MSCs) derived from adipose tissue were used in its treatment. The following peculiarities of MSCs for regeneration of skin defects are established: faster arrest of inflammation, accelerated wound healing processes, as well as observed stimulation of growth of skin appendages. The results of this study may serve the basis for further research from development to introduction into clinical practice of cellular technologies for the treatment of infected wound of various etiologies.

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Introduction

Infected wounds and trophic ulcers are among the major problems in surgical treatment that present special difficulty [1-5]. During the last decades, there are number of successes in solving problems in this aspect of surgery [4-7]. To date, a lot of methods and drugs aimed at accelerating reparative-regenerative processes in wounds and ulcers, as well as preventing their secondary infection are suggested [4,8,9]. However, general approbation of the majority of the suggested treatments showed that they were not effective enough [10-14]. Difficulties in treatments are compounded by the infection of wounds resulting in long-term healing process. Besides, non-healing wounds result in enormous health care expenditures, with the total cost estimated at more than $3 billion per year [2]. Therefore, the problem of treatment of this category of patients, in general, is still far from being solved.

Recently, considerable interest and promise in the treatment of non-healing and infected wounds using stem cell technology have been ignited [15-17]. Stem cells (SC) are immature cells capable of self-renewal and development to form specialized cells of the body. The ability to provide a wide variety of cell types makes SC an important reserve for the body in filling defects [18,20]. Pluri-and multipotency properties of stem cells make them ideal for use in transplantation [21,22]. It should be borne in mind that in addition to the fact that stem cells, which migrate to the zone of injury or damaged tissues of the body (homing) [18-22], also divide, and differentiate, forming new cell types in the tissue (local SC, fibroblasts, etc.), as if they were "central warehouse parts" – stromal cells of organs: adipose tissue (AT), red bone marrow etc.[21,22]

In recent years, one line of stem cell – mesenchymal stem cells (MSCs) have found promising use in various aspects of cell therapy, including filling of wound defects [22]. MSCs are versatile cells; they apparently are able to enter the bloodstream into the affected organ or tissue and locally influenced by several factors, give rise to specialized cells, which replace the dead cells. At present, sources of MSCs are adult red bone marrow, adipose tissue, blood, skin, etc. MSCs from adipose tissue have been described not so long ago. In 2002, American scientists first proposed the use of human adipose tissue as a source of multipotent stem cells [15,16]. Somewhat later, in 2003, there were reports from Japan about the prospects of clinical application of MSCs from adipose tissue [17]. Given the fact that fat tissue in humans is present in large quantity, a patient can be his own donor. This avoids immunological

incompatibility reaction [22]. Bone marrow tissue of humans and mammals is considered as one of the preferred sources of MSCs [22,23]. However, the clinical use of bone marrow as a source of MSCs is problematic, because the procedure is difficult, and as a result one can obtain only a small number of cells [22]. Data of several authors indicate that adipose tissue may serve as a source of cells that resemble bone marrow mesenchymal stem cells, which posses the morphology of fibroblasts, reproduce in vitro in standard medium, and are capable of multilineage differentiation [18-20,22,23]. Given that the method of obtaining MSCs from adipose tissue compared with MSCs from bone marrow or other tissues is easier and the number of cells obtained in the process is more, we can hope for good prospects, for their use in medicine in the recovery of damaged tissues.

The aim of this research was to study the peculiarities of regeneration of the skin of laboratory animals with stimulated infected wounds in the application of multipotent mesenchymal stromal cells of adipose tissue.

Materials and Methods

The ethics and bioethics committee of the Belarusian State Medical University for the use of animals in experimental research approved the study protocols.

Laboratory animals

In this study, adult albino rats of Vistar line weighing 160-200 g were used. All animals were kept on a standard diet in a vivarium with free access to food and water. All animals were divided into two groups: control (n = 10) and test (n = 10).

Experimental wound modeling

In the experimental animals, simulations of round wound on the back was conducted according a well-established technique [24] with modifications. For this purpose, intraperitoneal anesthetics with 0.5-0.7 ml sodium thiopental (1%) was used. Thereafter, hair on the back was shaved, and then skin-fascial flap excised as a circle with a diameter of 1.50 cm (area of the wound about 1.77 cm^2). To obtain equal-sized defects the same standard and diameter was applied using a special mental. After treatment with antiseptics, the standard

mental device was applied to the operating field and a marker at the edges of the circle was applied as benchmarks against which skin incision was later made with a scalpel. Patches of the size and shape consisting of skin, subcutaneous tissue, and fascia to the muscle, similar to the ready-made standard mental were cut out, with Iris scissor and forceps (Figure 1).
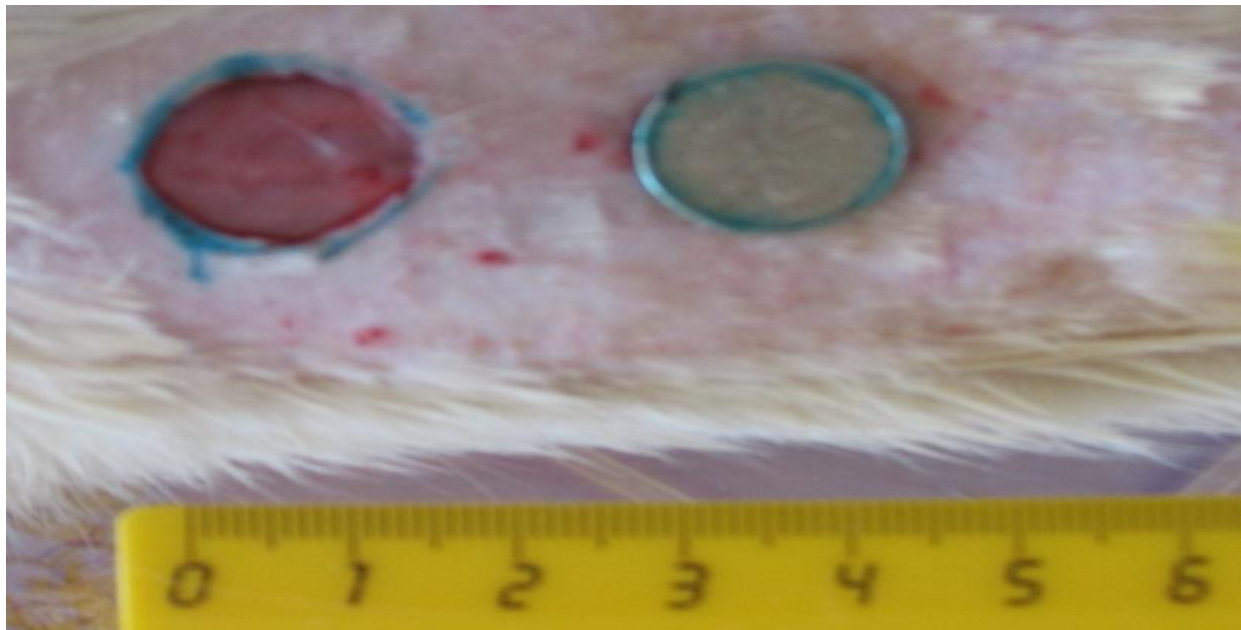

Figure 1. Simulation of wounds

Then the bottom and edges of the wound were infected by injecting a 24-hour monoculture reference strains of Staphylococcus aureus, suspended in 0.9% sodium chloride solution up to 1×10^9 CFU / ml (the concentration was determined by standard turbidity). The volume of injected suspension of bacteria was less than 2.0 ml. Purulent wounds were obtained two days later from the beginning of the simulation.

The inoculate for injection was prepared from pure 24-hour agar culture of Staphylococcus aureus ATCC 6538 (American Type Culture Collection, USA) with density of 1×10^9 CFU / ml, grown on the surface of a dense medium. For this purpose, 5-10 isolated colonies were suspended in the liquid medium of isotonic sodium chloride solution. The suspension or broth culture was diluted with isotonic sodium chloride solution until the turbidity of the optical standard according to SISCBP Tarasevich (Tarasevich State Institute of Standardization and Control of Biomedical Preparations, Moscow) was at 5 units. The inoculate was applied to the wound surface after preparation.

Model of cell transplantation into experimental wounds

In the control group, starting from the second day of the experiment, for every day, standardized rehabilitation of wounds with antiseptics (3% hydrogen peroxide, 0.05% chlorhexidine, furacilin 0.02%) was performed locally. The test group consisted of experimental animals treated using autologous MSCs of AT in the second day of the experiment. In this group, after the reorganization of infected wounds, AT MSCs transplantation in and around the wound was carried out under aseptic conditions. The cellular biomaterial was delivered by injection in the quantity of 1x106 cells/ml. MSCs from AT were acquired according to a method previously reported [25].

Wound size measurements

Digital photographs of wounds were taken on each day including the first day after wound simulation. Wound photographs were shown on a computer monitor. For histological analysis of wound scars, the mice were euthanized on the 3rd, 5th, 7th, 10th, 14th, 30th days, and the whitish scars were excised, bisected, and fixed in 1% formalin. The samples underwent routine histological processing with hematoxylin and eosin.

Monitoring of the animals, as well as an objective examination of the wounds with dynamic photography (with Canon Power Shot A630) and subsequent computer planimetry were conducted daily. An image analysis program (Scion Image, USA) was used, and the border of unhealed area that was not re-epithelialized was traced manually. The size of the traced area was calculated automatically by the software.

Histological analysis

At the same time interval (i.e. 3rd, 5th, 7th, 10th, 14th and 30th day), pieces of tissue on the larger diameter of the wound edges and the underlying tissues were excised for histological examination. Paraffin sections were stained with a thickness of 4-5 microns with hematoxylin and eosin. Light microscopy of tissue sections of experimental wounds in different fields of view was performed.

Statistical analysis

Results are expressed as means±SEM (standard error of the mean). A Mann-Whitney U test was used for statistical analysis, and a value of $p < 0.05$ was considered statistically significant.

Results

Results on the wound size measurements from the second to the 14th day are shown in table 1.

Table 1: Results of wound area measurement (in mm^2, M±m) from the second to the fourteenth day of the experiment

Groups	Days					
	day 2	day 3	day 5	day 7	day 10	day 14
Control	177±0.00	144.40±7.98	127.10±13.65	106.40±6.92	99.40±5.86	84.40±2.54
Test	177±0.00	135.60±7.76	95.30±8.40[#*]	49.30±7.59[#*]	19.70±2.75[#*]	6.70±0.68[#*]

*P<0.01 in relation to the controls; [#]P<0.05 in relation to its own values on the previous day.

Before transplantation of AT MSCs, no difference in the character of wounds in both the test and control was observed. On the second day after infection, the wound in comparable groups was similar and characterized by hemorrhagic crusts with a moderate amount of serous-hemorrhagic fluid, and sometimes having purulent character. There was marked inflammatory infiltrate, redness, and swelling of surrounding tissues around the wound (Figure 2). Morphologically, in all animals of the control and test group, the surface of the wounds was

surrounded by purulent necrotic mass, reaching the adipose tissue. The whole surface scab wound was covered with a thin crust, consisting of protein mass and neutrophils.
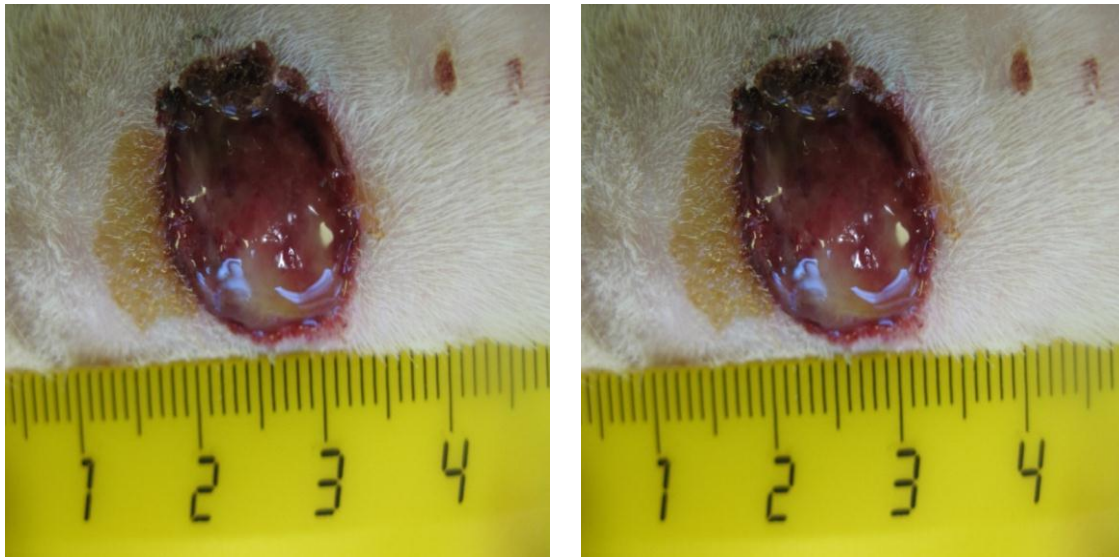

Figure 2. Macroscopic characterization of cutaneous wounds of the animals on the 2nd day after infection.

Examination of the morphological picture of the wounds of the experimental animals showed that in the control group on the third day, the surface was covered with a thin crust all over the fatty tissue. There were small areas of necrotic tissue, which was saturated with protein in places located under the scab. Adipose tissue was infiltrated by lymphocytes, plasma cells and neutrophils. In the area of the wound edges, lobular proliferations of fibroblasts forming collagen fibers were identified. Following observation on the 5th day, in the controls the wound was still covered with a scab, which was separated from the underlying fat layer of necrotic tissue, and was saturated with proteins and erythrocytes. The underlying tissues were infiltrated by neutrophils and lymphocytes. Granulation tissue was formed in some places around the necrotic foci. Results for the 7th day did not show significant improvements in the wound size and healing (Table 1). On the 10th day of the study, the wound surface was covered with a thin layer of crust, which was located under the maturing granulation tissue with many fibroblasts and lymphocytes, and moderate amounts of collagen fibers. Visual observation on the 14th day of the experiment in the controls showed a small defect in the wound, covered with a thin layer of crust; hair on the periphery of the wound was absent (Figure 3a). Histological analysis of the sections collected on the 14th day, showed that the wound was covered by maturing granulation tissue with small blood vessels (meant that

inflammatory process remained), oriented along the wound and fibroblasts. In the deeper layers of the wound there was a mature connective tissue with accumulation of hemosiderophages, lymphocytes and plasma cells. The formation of sebaceous glands and hair follicles in the wound area was not observed. On the periphery of the wound defect there was small growth of stratified epithelium, although the defective epithelialization remained. Maturing granulation tissue was located under the stratified epithelium (Figure 3b).
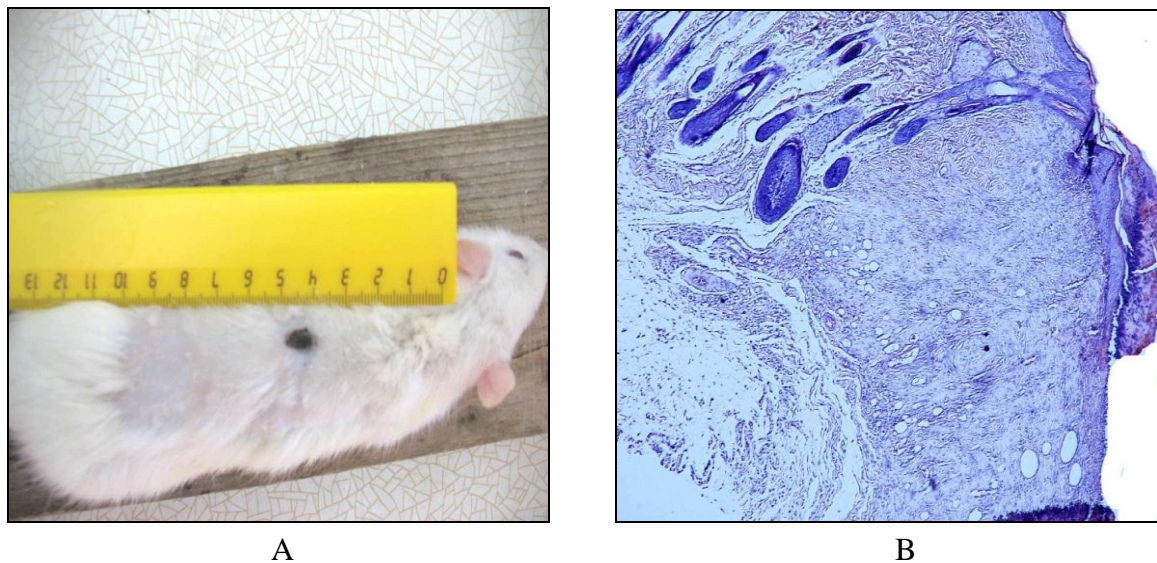
A   B

Figure 3. Macro and microscopic characterization of cutaneous wounds of animals of the control group on the 14th day of the experiment a) visual observation of skin wounds, and b) the periphery of the wound defect multilayered epithelium grows under the scab (1), there remains a defect of epithelialization; granulation tissue under the stratified epithelium (2). Staining technique: hematoxylin and eosin. Magnification: × 40.

On the 30th day of observation in the control group, defect of epithelization still was present. The skin at the site of the wound was fixed and soldered to the surrounding tissues. Horn cyst formation was noted in some areas of multilayered epithelium. A forming scar tissue under the stratified epithelium was identified. Skin appendages in the fibrous tissue were also absent (Figure 4).
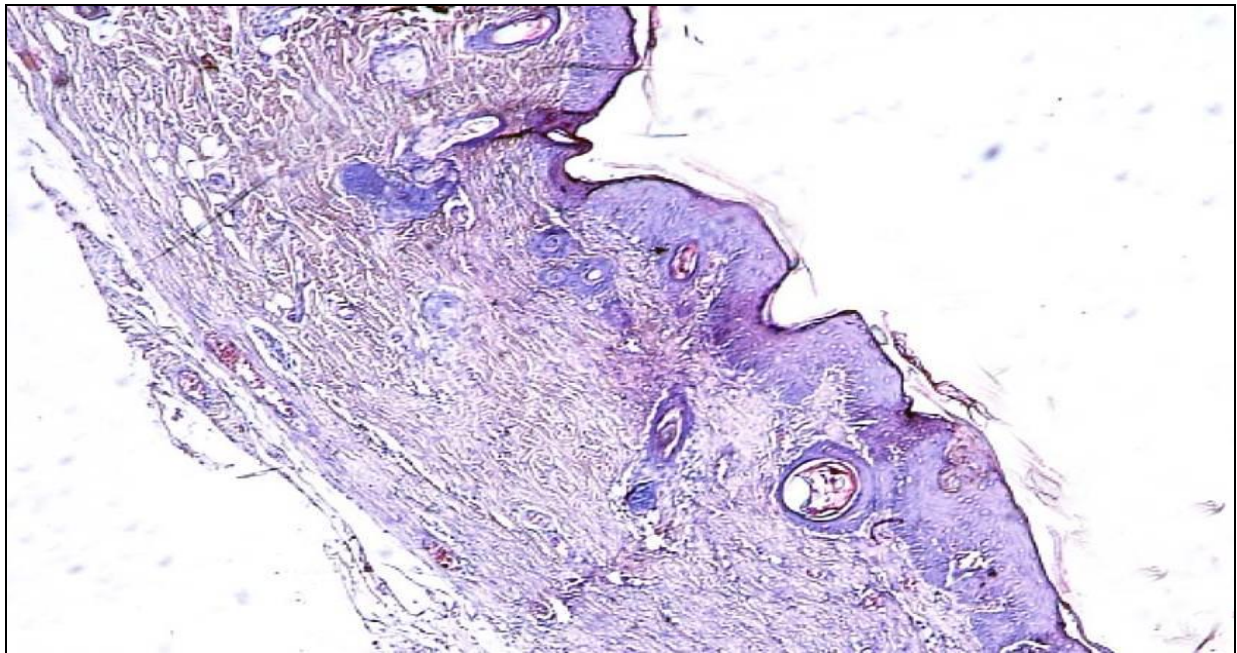
Figure 4. Microscopic characterization of cutaneous wounds of control animals on the 30th day of the experiment. The defect of epithelialization is absent. Scar tissue is forming under the multi-layered epithelium, indicated by the arrow is horn cyst. Skin appendages in the fibrous tissue are absent. Staining technique: hematoxylin and eosin. Magnification: × 60.

In the test group on the third day after transplantation of MSCs of AT, wound surface was covered by purulent fibrinous layers under which, formation of granulation tissue with isolated blood vessels was observed. Inflammatory infiltrate in the wound spread to fatty tissue and muscle. Necrotic areas, in contrast to the control group were absent. In the course of observation of wound regeneration on the 5th day, as opposed to the control group, the wound surface was covered with a dense crust, under which a diffusely young granulation tissue, infiltrated by lymphocytes and few neutrophils was located. Mostly leukocyte infiltration spread to fatty tissue, but it was much less pronounced in contrast to the control group. In the superficial parts of the granulation tissue, there were single walled vessels. Results of the 7th day showed improvement in contraction and single walled vessels increased (also see table 1 for significant wound size reduction). Observation on the 10th day in all animals was determined by the net surface of the wound, which was represented by maturing granulation tissue with the presence of collagen fibers. The area occupied by granulations in the test group was significantly higher compared with the control group (P<0.01) (also see table 1). In the study sections on the 14th day of observation there was no defect in epithelialization; visually, surface of the wound was covered with stratified

squamous epithelium, and hair in the wound was nearly restored (Figure 5a). New connective tissue was located under mature collagen fibers, and isolated fat cells were arranged within the stratified epithelium (Figure 5b).
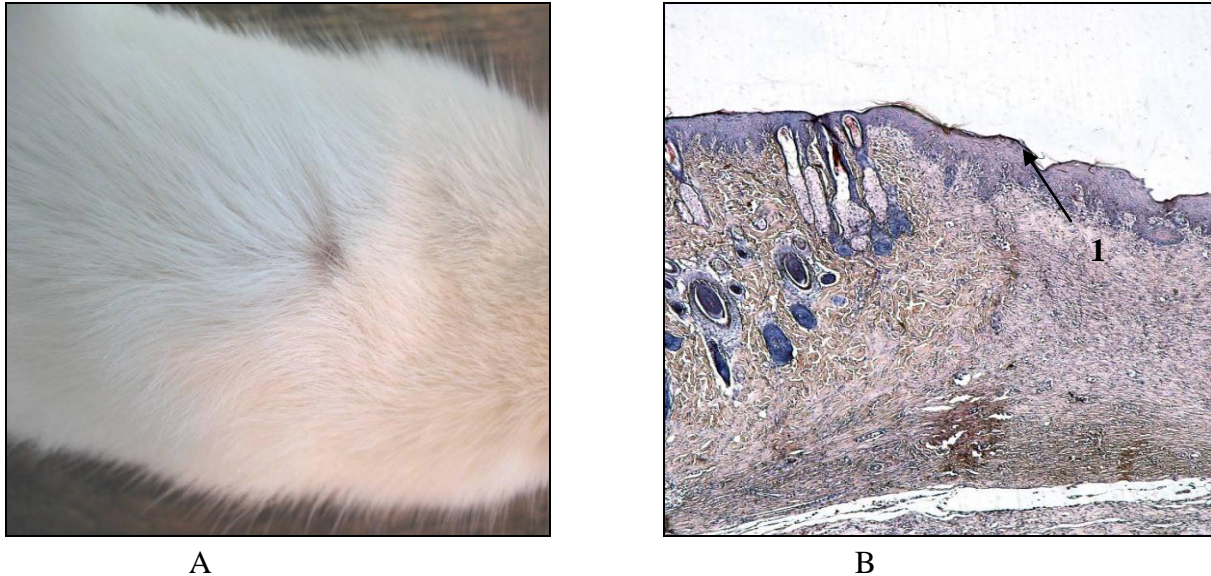

A   B

Figure 5. Macro and microscopic characterization of cutaneous wounds of the main group of animals on the 14th day of the experiment a) visual observation of skin wounds, and b) the defect of epithelialization is absent. Maturing granulation tissue is under the multi-layered epithelium (arrow) (1). Staining technique: hematoxylin and eosin. Magnification: × 40.

Defect in epithelization in all test animals was not observed on the 30th day of dynamic observation. The coat was completely restored. The skin in the wound site was mobile and not soldered to the surrounding tissues. Sebaceous glands and hair follicles in the fibrous tissue under the stratified epithelium was formed (Figure 6).
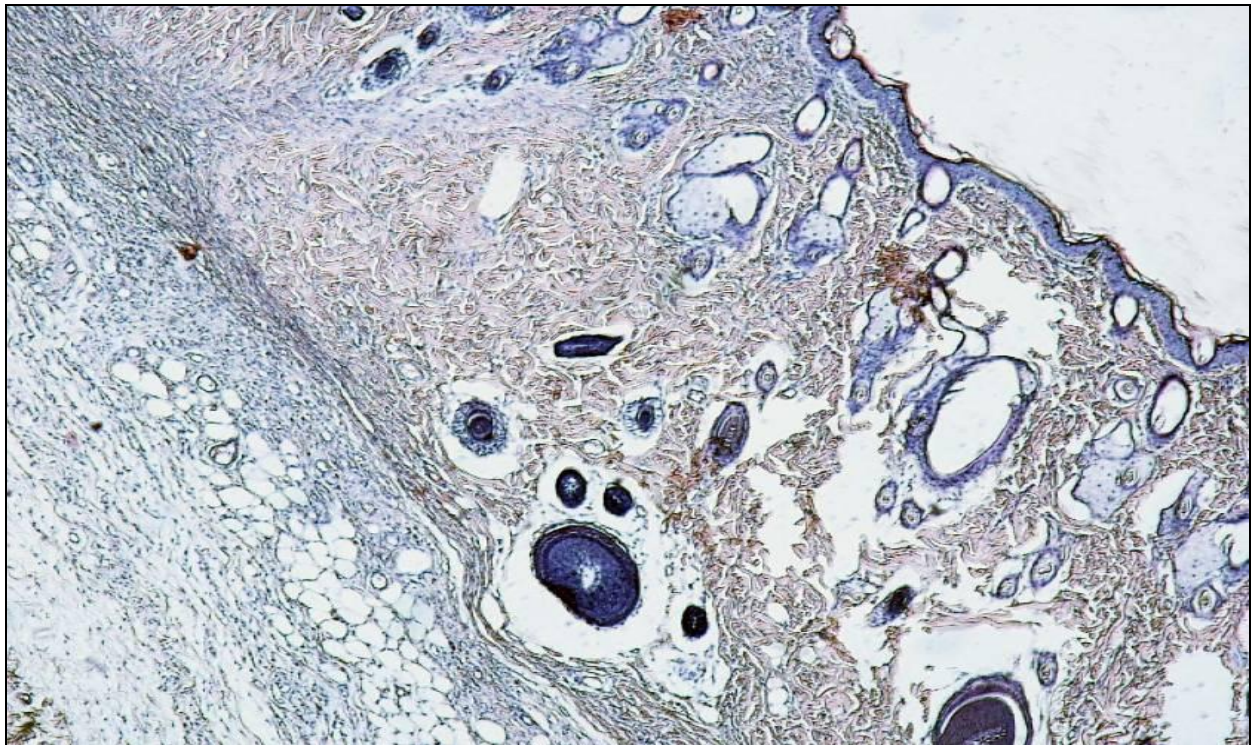
Figure 6. Microscopic characterization of cutaneous wounds of the main group of animals on the 30th day of the experiment. Defect of epithelialization is absent. In the fibrous tissue (1) sebaceous glands (2) and hair follicles (3) are been formed under stratified epithelium (indicated by arrows). Staining technique: hematoxylin and eosin. Magnification: × 40

Discussion

This study provides important findings to deeper research using MSCs of AT in the complex and effective treatment of non-healing wounds in surgical practice.

Surgical wounds in normal, healthy individuals heal through an orderly sequence of physiologic events that include inflammation, epithelialisation, fibroplasia, and maturation [Prem Rathore]. Although, many factors (age, sex, diabetes, obesity) affect wound healing, complications involving infections present special difficulty [3]. Prevalence of infected wound as reported by several authors for different kind of wounds may vary and from country to country – about 14% [3] to 99.4% [26]. The most frequent etiological agents of wound infection are Staphylococcus aureus, Proteus mirabilis, Pseudomonas aeruginosa, Klebsiella aerogenes, Escherichia coli, Staphylococcus epidermidis, Streptococcus pyogenes and

Streptococcus faecalis, Candida albicans and Candida tropicalis [26]. Therefore, infections at the site of wound are a major etiological factor for non-healing wounds. The use of Staphylococcus aureus as the preferred ethiological agent for infection of the experimental wounds in this study is supported by its high prevalence in non-healing wounds [26], as well as difficulty in treatment outcome [26,27]. In addition, a retrospective analysis from 2006 to 2010 conducted by our team in the emergency care hospital in Minsk revealed that Staphylococcus aureus was the etiological agent of roughly all non-healing infected wounds [28].

Wound healing involve complex mechanisms at both the cellular and subcellular levels [12,29]. These complex cellular and subcellular processes that occur during wound healing can be divided into at least three continuous and overlapping processes: an inflammatory reaction, a proliferative process leading to tissue restoration, and, eventually, tissue remodeling [30]. Although some authors distinguish four stages, with the first stage being hemostasis, highlighting the importance of vascular regeneration [9]. For a review of the different stages/modern definition of wound healing responses see a recent article by Jie Li and colleagues [30]. In our study, following inflammation, the various stages of wound healing observed were rather overlapped. Vascular regeneration was more pronounced for the test group as it was already noticed starting from the third day, while on the 5th day distinct blood vessels could be spotted. This was an important development for the fast tissue regeneration (wound healing) in the test animals. Although antiseptics were used in the control group, vascular regeneration delayed, and hence, wound regeneration was significantly slowed.

Complex mechanisms involving several processes are the result of the positive transplantation effect of MSCs [31,32]. The activities of cells such as platelets, macrophages, leukocytes, fibroblasts, endothelial cells, and keratinocytes particularly during the inflammatory and proliferation stages of healing are much better known. Molecules such as interferon, integrins, proteoglycans and glycosaminoglycans, matrix metalloproteinases, and other regulatory cytokines play a critical role in the regulation of healing mechanisms [29]. According to the cell contraction and cell traction theories, fibroblast too play significant role in wound healing [33]. But, cytokines and growth factors released by autocrine signaling are most responsible for wound healing [14,30]. Cytokines and growth factors that enhance the regeneration of tissue following MSCs transplantation are reported [23,34,35]. Cells that fasten regeneration,

such as keratinocytes, collagen fibers, fat cells were of large numbers in the test animals. Since in the controls, inflammatory processes prolonged up to the 14th day, wound regeneration was significantly slowed, compared with the test animals.

The regenerative ability of MSCs transplantation observed in this study is apparently, due to the fact that the cells of the mononuclear fraction of adipose tissue produce several cytokines and growth factors to stimulate granulation tissue formation and regeneration of skin appendages. In addition, the use of MSCs can accelerate the process of regeneration through their inherent ability to differentiate into the various elements of the skin tissue. In spite of the use of antiseptics in the control group, wound healing was significantly slowed. It therefore follows that the faster healing and regeneration of wounds in the test animals was not due to the aseptic condition provided, but mainly the transplantation effect of MSCs of AT. It is pertinent to note that, in contrast to the controls, there was complete healing of the wound surfaces in the test animals.

MSCs play a significant role in tissue repair and homeostasis. An emerging body of evidence shows that in addition to being a progenitor cell population with self-renewing and multipotent differentiation capabilities, MSCs have unique immunomodulatory properties, making them even more attractive for tissue regeneration. Emerging discoveries in stem cell biology have revealed a multitude of mechanisms through which MSC could potentially augment the current techniques in aesthetic surgery [22]. The antioxidant property of MSCs of AT might also account for a faster wound healing [31,32]. The results received in our study about the faster skin surface regeneration in the test animals confirm the useful cosmetic properties of MSCs, specifically of adipose origin. Bioengineering using stem cells for chronic wound treatments show positive result according to a recent study [13].

Faster wound healing with MSCs of AT is also due to its ability to neovascularize damaged tissue [36,37]. MSCs of AT have been shown to improve vascularization in mouse model of ischemia [37] and enhance nerve differentiation at the site of transplantation [34]. MSCs of AT have been recently shown to improve metabolic functions [38].

The use of allogenic stem cells in our study avoids immunocompatibility reactions and immunosuppression therapy, which actually result in longer period of wound healing [39].

MSCs of adipose tissue transplantation has shown great promise in other areas including oncology [40], hepathology [21], orthopedic surgery [35], stroke and neurological diseases, cardiovascular disease, urology [23].

This present study provides key findings buttressing a recent review by G.U. Gurudutta, and colleagues (2012) [41]; provides the basis for further research and the development of novel and futuristic treatment modality for disaster injuries [41], including burns [42]. The development of MSCs of AT for transplantation into non-healing/long-term healing wounds in clinical practice would also save the huge amount of finance and time needed for the treatment and rehabilitation of this group of patients.

Conclusions

The use of MSCs of AT in the treatment of infected wounds allows to quickly restore the integrity of the skin, compared to traditional methods of treatment. The regeneration of wound is greatly accelerated due to the ability of stem cells to strengthen anti-inflammatory and regenerative processes. MSCs stimulate the development epidermis, accelerate wound healing, and hence may improve cosmetic outcome of treatment. The use of MSCs of adipose tissue for transplantation may improve the treatment outcome of non-healing wounds in surgical practice.

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