# Morphofunctional Evaluation of the Effect of Transplantation of Allogeneic Fibroblasts on Healing of Burn Wounds in Albino Outbred Mice and Mutant $Hr^{hr}/Hr^{hr}$ Mice

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Morphofunctional evaluation of the effect of transplantation of allogeneic fibroblasts on skin regeneration after burn injury in albino outbred mice and mutant $Hr^{hr}/Hr^{hr}$ (nude) mice was carried out using the radioautographic method. The pronounced stimulating effect of allogeneic fibroblasts on healing of burn wound observed in mice of both groups can be attributed to the action of bioactive substances released from destructed allogeneic fibroblasts and stimulating proliferation of stem/progenitor cells in the wound (epithelial cells in skin appendages and dermal cells in microvessel walls) at the early stages of healing.

**Key Words:** *burn wound; allogeneic fibroblasts; $Hr^{hr}/Hr^{hr}$ nude mice; stem cell; light microscopic radioautography*

Transplantation of allogeneic fibroblasts (AF) for the treatment of burn wounds is long and successfully used in clinical practice [3,4,9]. However, the mechanisms of the stimulating effects of AF on reparative regeneration of the skin after burn injury is poorly studied [2,5]. This can be explained by objective difficulties such clinical studies. Experimental studies in this field are scanty [14]. Here we performed a comparative morphofunctional analysis of mechanisms of the stimulating effects of AF transplantation on healing of IIIA degree burn wounds in albino outbred mice and mutant mice carrying mutation in *Hairless* (*Hr*) gene. In mutant mice, hair follicles (HF) normally form during embryogenesis, but then regress, which leads to hairlessness [15]. Regeneration of skin epithelium after serious injuries (*e.g.* burn injury) is realized due to SC located in epithelial skin appendages: HF, sebaceous, and sweat glands [13].

Here we studied reparative regeneration of the skin in mutant mice with morphologically changed HF.

## MATERIALS AND METHODS

The experiments were carried out on albino outbred mice weighing 20-25 g and hairless mutant $Hr^{hr}/Hr^{hr}$ mice weighing 18-22 g. A total of 20 animals at the age of 3-5 months were used.

The animals were anesthetized with avertin in a dose of 0.25 mg/g body weight and a burn was inflicted by applying a 10-mm stamp heated to 95-100°C to the skin on the back (3% body surface, IIIA degree burn was modeled under morphological control). Polyvinylchloride rings were preliminary sutured to this skin area for preventing wound contraction. Two symmetrical burns were inflicted simultaneously: for spontaneous healing and for local treatment using AF transplantation. After burn modeling, the animals were housed individually.

For preparing bioactive dressing, skin fibroblast culture was prepared as described elsewhere [1] from the derma of outbred mice. Passage 7-10 fibroblasts

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were used. AF suspension was applied onto a hydrophobic perforated Carbosil-P organic-silica film on the bottom of a Petri dish and then applied onto the wound on the same film. The film was applied so that the cell-populated side faced the wound surface and fixed with a wet-to-dry gauze dressing. The film was removed after 1 day.

The material for morphological analysis was taken 6 h and 1, 3, 5, and 7 days after burn modeling.

For light microscopy, the wound tissue was fixed in 10% neutral formalin, the sections were stained with hematoxylin and eosin. For evaluation of functional (protein synthesis) and proliferative activity of wound cells at early terms (6 h and 1 day after burn) radioautography with RNA precursor $^3$H-uridine and DNA precursor $^3$H-thymidine was performed. Tissue samples were transferred into warm nutrient medium 199 and cut into 0.5-1-mm$^3$ fragments. The fragments were incubated in medium 199 containing 10 μCi/ml $^3$H-uridine (specific activity 26 Ci/mmol) or 2 μCi/ml $^3$H-thymidine (specific activity 21.6 Ci/mmol) at 37-38°C for 1.5 h. The material was fixed with 2.5% glutaraldehyde and 1% $OsO_4$ and embedded in araldite.

Radioautographs were routinely made on semithin sections (1.5-2 μ). The study was performed using an Axiostar plus light (Zeiss) and Eclipse 50i (Nikon) microscopes.

## RESULTS

Three-five-month-old $Hr^{hr}/Hr^{hr}$ mice were absolutely hairless. Instead of usual HF penetrating the whole thickness of the skin in outbred mice (Fir. 1, *a*), two types of structural epithelial formations were seen in mutant mice (Fig. 1, *b*). Structures of the first type were hyperkeratinized utricles connected with and sometimes open into the epidermis; they had deformed elongated acne-like shape. These utricles communicated with sebaceous gland ducts and often contained degrading hair-like filamentous structures. Structures of the second type were well-developed cysts or vesicles lying in rows in the derma. They had round or irregular shape and were lined with single-layer epithelium. They also often contained fragments of filamentous structures.

***Skin regeneration after burn injury in albino outbred mice.*** Six hours after burn infliction, pronounced destructive changes in the derma and subcutaneous fat with inflammatory reaction in the form of leukocytic infiltration and edema were observed, the epidermis was absent (Fig. 2, *a*). However, activation of epithelial cell proliferation was observed in preserved HF of the derma and subcutaneous fat.

In the wound treated with AF, more pronounced reaction of HF epithelium was observed against the background of destructive changes in the derma and leukocytic infiltration of deep layer of the wound. Epithelial cells of HF not only proliferated, but also actively migrated towards the wound surface, due to which HF stretched towards the wound. After reaching the wound surface, the epithelial cells of follicles crawled onto it (Fig. 2, *b*).

In spontaneously healing wounds, proliferation and migration of epithelial cells of HF was observed on day 1 after burn injury, 1-2 layers of epithelial cells covered the wound surface (Fig. 2, *c*). The cells were

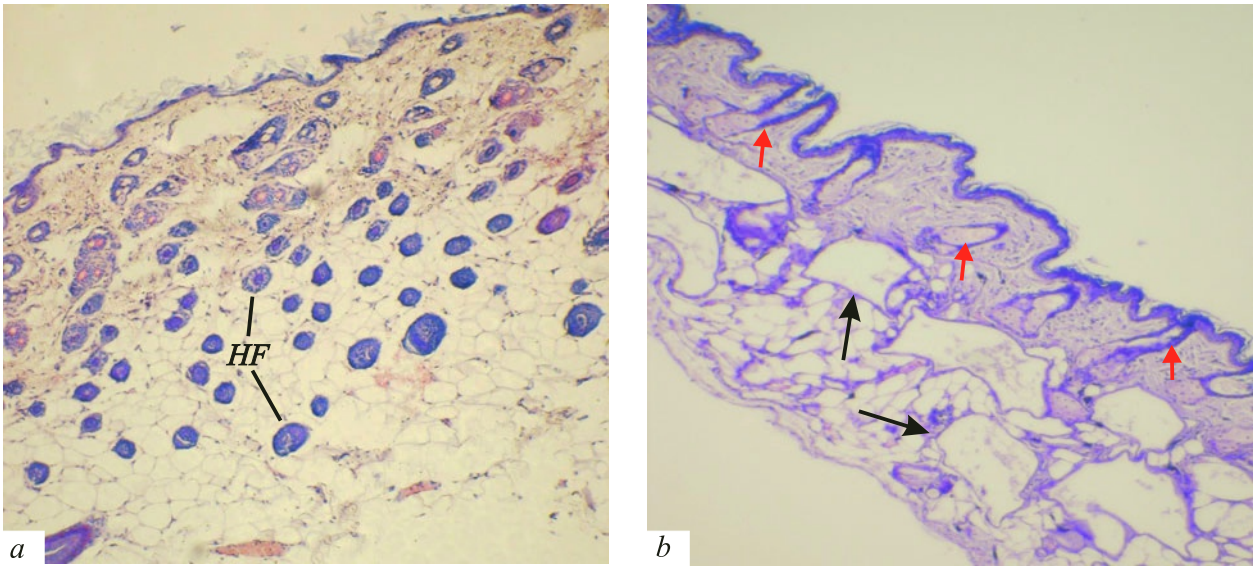


**Fig. 1.** Skin of intact albino outbred mice and mutant $Hr^{hr}/Hr^{hr}$ mice. Hematoxylin and eosin staining, ×100. *a*) thin surface epidermis in an albino mouse: numerous HF in the derma and subcutaneous fat tissue; *b*) derma of mutant $Hr^{hr}/Hr^{hr}$ mice, two types of epithelial structures: utricles (dark arrows) connected with the epidermis and vesicles of different size (red arrows).

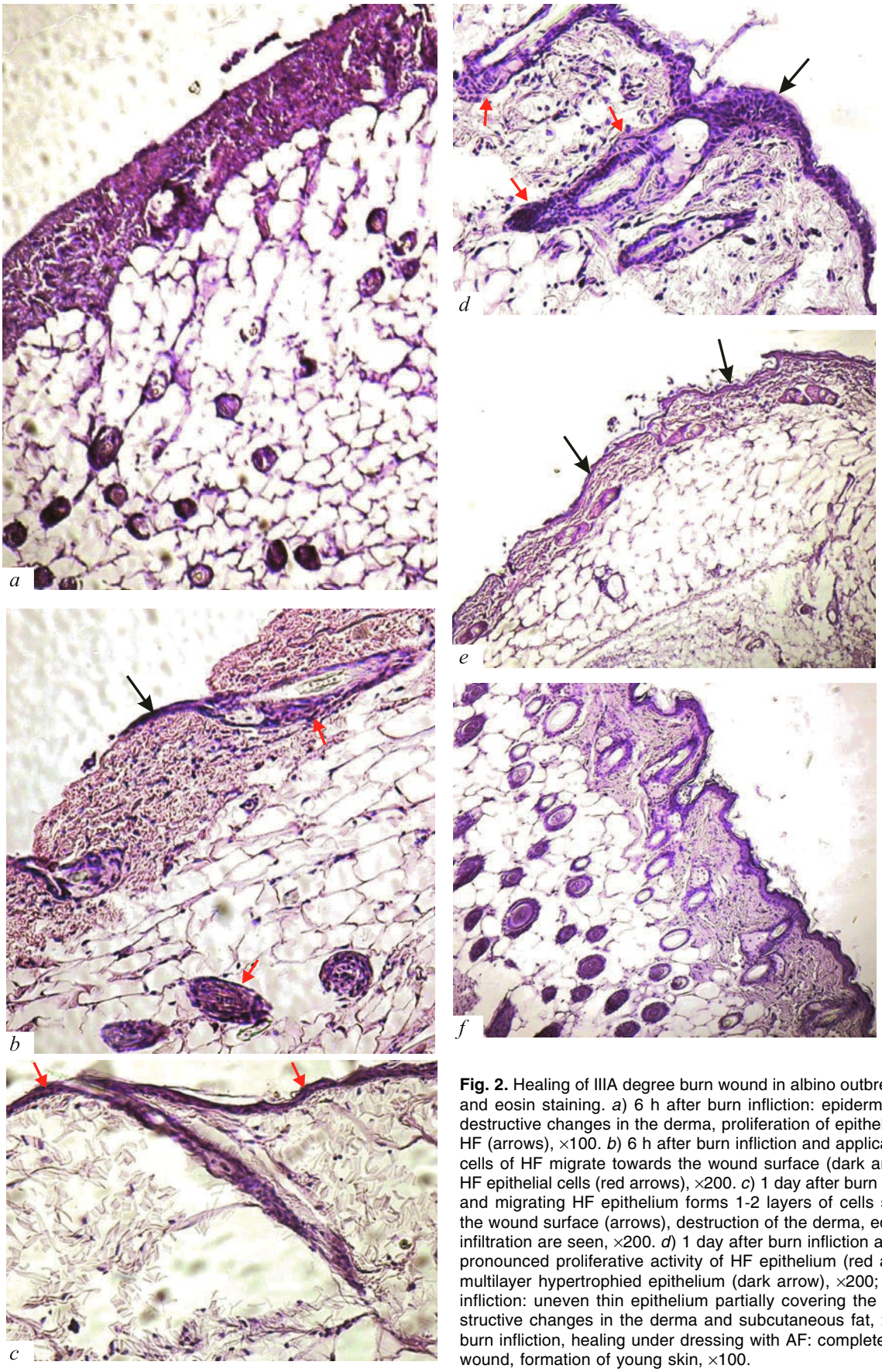


**Fig. 2.** Healing of IIIA degree burn wound in albino outbred mice. Hematoxylin and eosin staining. *a*) 6 h after burn infliction: epidermis is absent, edema, destructive changes in the derma, proliferation of epithelial cells in preserved HF (arrows), ×100. *b*) 6 h after burn infliction and application of AF: epithelial cells of HF migrate towards the wound surface (dark arrow); proliferation of HF epithelial cells (red arrows), ×200. *c*) 1 day after burn infliction: proliferating and migrating HF epithelium forms 1-2 layers of cells somewhere covering the wound surface (arrows), destruction of the derma, edema, and leukocytic infiltration are seen, ×200. *d*) 1 day after burn infliction and application of AF: pronounced proliferative activity of HF epithelium (red arrows), formation of multilayer hypertrophied epithelium (dark arrow), ×200; *e*) 3 days after burn infliction: uneven thin epithelium partially covering the wound (arrows), destructive changes in the derma and subcutaneous fat, ×100; *f*) 3 days after burn infliction, healing under dressing with AF: complete epithelization of the wound, formation of young skin, ×100.

poorly discernible in this thin epithelium, because the epithelium was not completely formed, but migrating, "crawling". Destruction, edema, and leukocytic infiltration were still seen in the derma. Sometimes, the epithelium covered sites of wound surface with pronounced destructive changes in the derma (destruction of cells, collagen fibers, and HF). Solitary fibroblasts against the background of fine fibrous reticulum were noted as soon as 6 h after burn in the depth of the wound at the level of subcutaneous fat. After 1 day, the number of fibroblasts migrating into the derma from the subcutaneous fat increased.

In the wound treated by AF application, epithelization of the greater part of the surface was observed after 1 day. The formed epithelium had uneven thickness. Sites with pronounced destruction of the derma were covered with thin singe-layer epithelium, but somewhere multilayer (3-4 layers) hypertrophied epithelium resulting from stacking of epithelial cells was seen (Fig. 2, *d*). This attested to high proliferative activity of epithelial cells most pronounced in HF and reflected the stimulating effect of the dressing with AF. Structural changes in the derma persisted, but inflammatory changes were minor. Numerous cells, primarily fibroblasts, were seen among collagen fibers. Appearance of capillaries and mitotic activity of their cells (endothelial cells and pericytes) were noted.

After 3 h, spontaneously healing wounds were not completely epithelized (Fig. 2, *e*) Uneven "crawling" of the thin epithelium on the wound surface was observed. Destructive changes and leukocytic infiltration persisted in the derma and subcutaneous fat, microbial cells were found. Complete epithelization of the wounds in these animals was observed on days 5-7. The wounds healing under dressing with AF were completely epithelized after 3 days: the formation of young skin was observed (Fig. 2, *f*). In regenerating derma, pronounced proliferative activity of epithelial cells of HF was noted.

***Skin regeneration after burn injury in mutant mice.*** In $Hr^{hr}/Hr^{hr}$ mice with IIIA degree burn, destruction of the epidermis and destructive changes in the derma similar to those observed in outbred albino mice were observed. The inflammatory reaction in the form of leukocytic infiltration and edema was noted in both the derma and subcutaneous fat. However, solitary epithelial utricles or their fragments and altered dermal cysts were present in the derma of these mice. Activation of cell reaction of preserved utricular epithelium was observed. The start of epithelization, in particular, epithelial cells migrating from proliferating epithelium of utricles, were noted in some sites of the wound surface (Fig. 3, *a*).

When dressing with AF was applied to the wound surface, the reaction of epithelial cells of HF derivates was more pronounced. Dermal cysts lined with thin single-layer epithelium came up approaching the wound surface and lied along it. Some of them could integrate into the surface, which was associated with proliferation and migration of their epithelium (Fig. 3, *b*). Active incorporation of epithelial utricles into the wound surface was noted: their proliferating and migrating cells started to cover the wound.

After 1 day, the greater part of the wound surface in mutant mice with spontaneous healing was not covered with the epithelium. Both marginal epithelization of the wound and proliferation and migration of the epithelium from epithelial utricles were observed (Fig. 3, *c*). Lifting of dermal cysts, swelling and proliferation of their single-layer epithelium were also noted. Fibroblasts predominated in the regenerating derma. Destructive changes, vascular thrombosis, and leukocytic infiltration were still seen in some sites of the derma.

After application of bioactive dressing with AF, epithelium formation was seen on the greater part of the wound surface 1 day after burn infliction. Its thickness was different: derma sites with pronounced destructive changes were covered by thin epithelium, while sites with active proliferation of epithelial cells in utricles and regenerating derma were covered by thickened (2-3 layers) epithelium (Fig. 3, *d*). In the derma, signs of inflammation were absent, fibroblasts and solitary leukocytes were seen among loose collagen fibers. The subcutaneous fat contained numerous blood capillaries. Active cell proliferation was noted around larger vessels. Among these cells, proliferating fibroblasts were present that probably migrated into the derma.

After 3 days, more than 50% wound surface in mutant mice with spontaneous healing was covered with the epithelium. However, focal microbial invasion of the formed epithelium was observed (Fig. 3, *e*). Microbes from the surface penetrated deep into the derma and destroyed it. Against the background of acute inflammatory reaction, dilatation of vessels, stasis, and erythrocyte lysis were observed in deep layers of the wounds. The wounds were completely epithelized by days 5-7.

The wounds healing under dressing with AF were completely epithelized after 3 days (Fig. 3, *f*). The formed epithelium was in some sites thickened (hypertrophied). Epithelial cells of dermal cysts exhibited pronounced proliferative activity. The derma was primarily presented by a loose network of thin collagen fibers containing capillaries and fibroblasts. However, the inflammatory reaction can persist in the subcutaneous fat, which was seen from the presence of leukocytes. Dilatation of large vessels and stasis of a large number of erythrocytes were also observed.

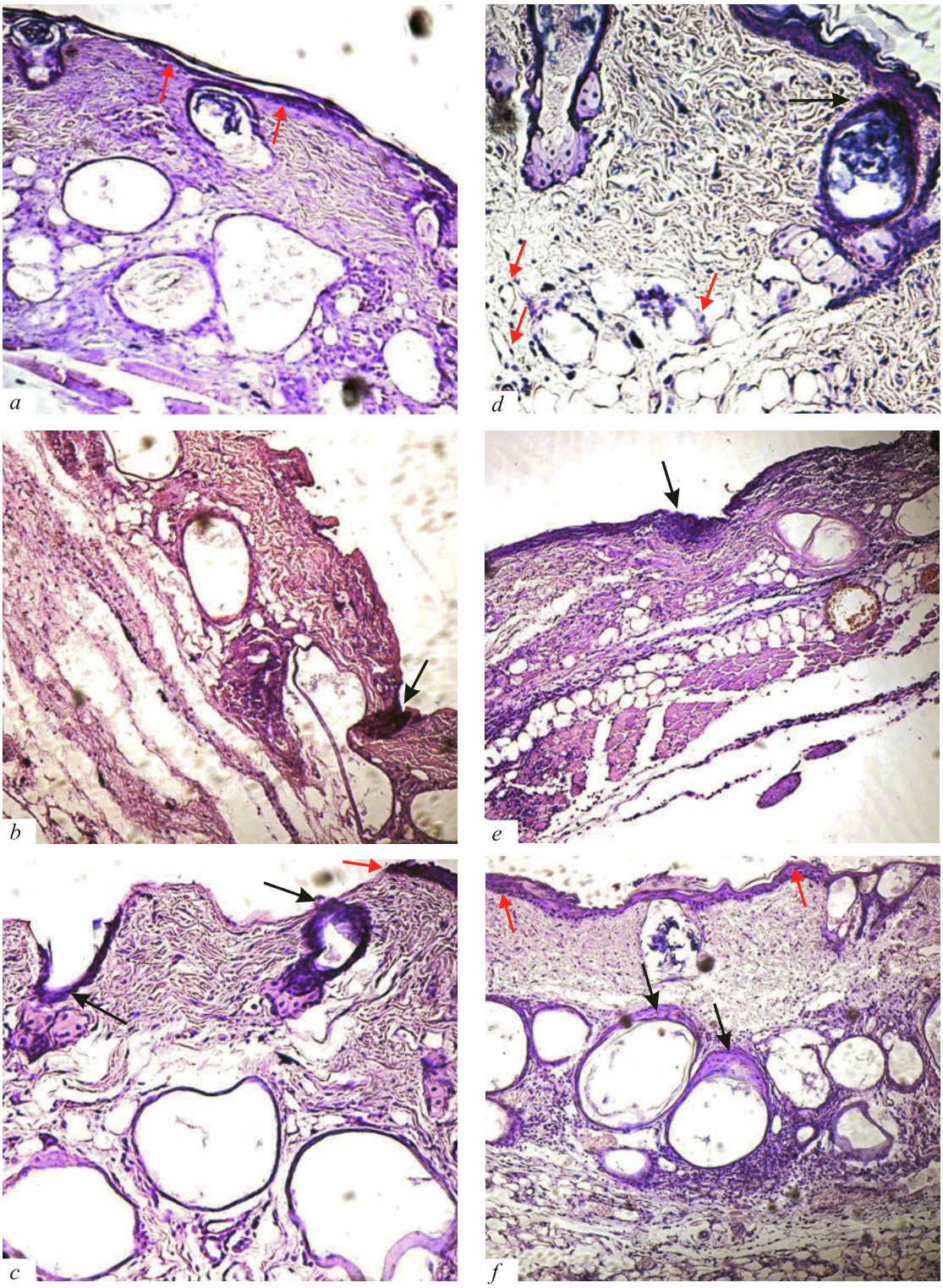


**Fig. 3.** Healing of IIIA degree burn wound in mutant mice. Hematoxylin and eosin staining. *a*) 6 h after burn infliction: absence of the epithelium and pronounced destructive changes in the derma, migration of epithelial cells from proliferating epithelium of the utricle (arrows), ×100; *b*) 6 h after burn infliction and application of AF-containing dressing: growth and proliferation of dermal cysts located along the wound surface (arrow), ×100; *c*) 1 day after burn infliction: epithelium migration from the wound edges on its surface (red arrow), its proliferation and migration from epithelial utricles (dark arrows), ×200; *d*) 1 day after burn infliction and application of AF: thickened epithelium in sites with active proliferation of epithelial cells from utricles (dark arrow), regenerating derma with loose collagen fibers and fibroblasts, microvessels in the derma and subcutaneous fat (red arrows), ×200; *e*) 3 days after burn infliction: focal microbial invasion of formed epithelium (arrow) destruction of the derma, thrombosis of microvessels, inflammation, ×100; *f*) the wound under AF-containing dressing on day 3 is covered with epithelium, somewhere thickened (red arrows); proliferative activity of epithelial cells of dermal cysts (dark arrows), ×100.

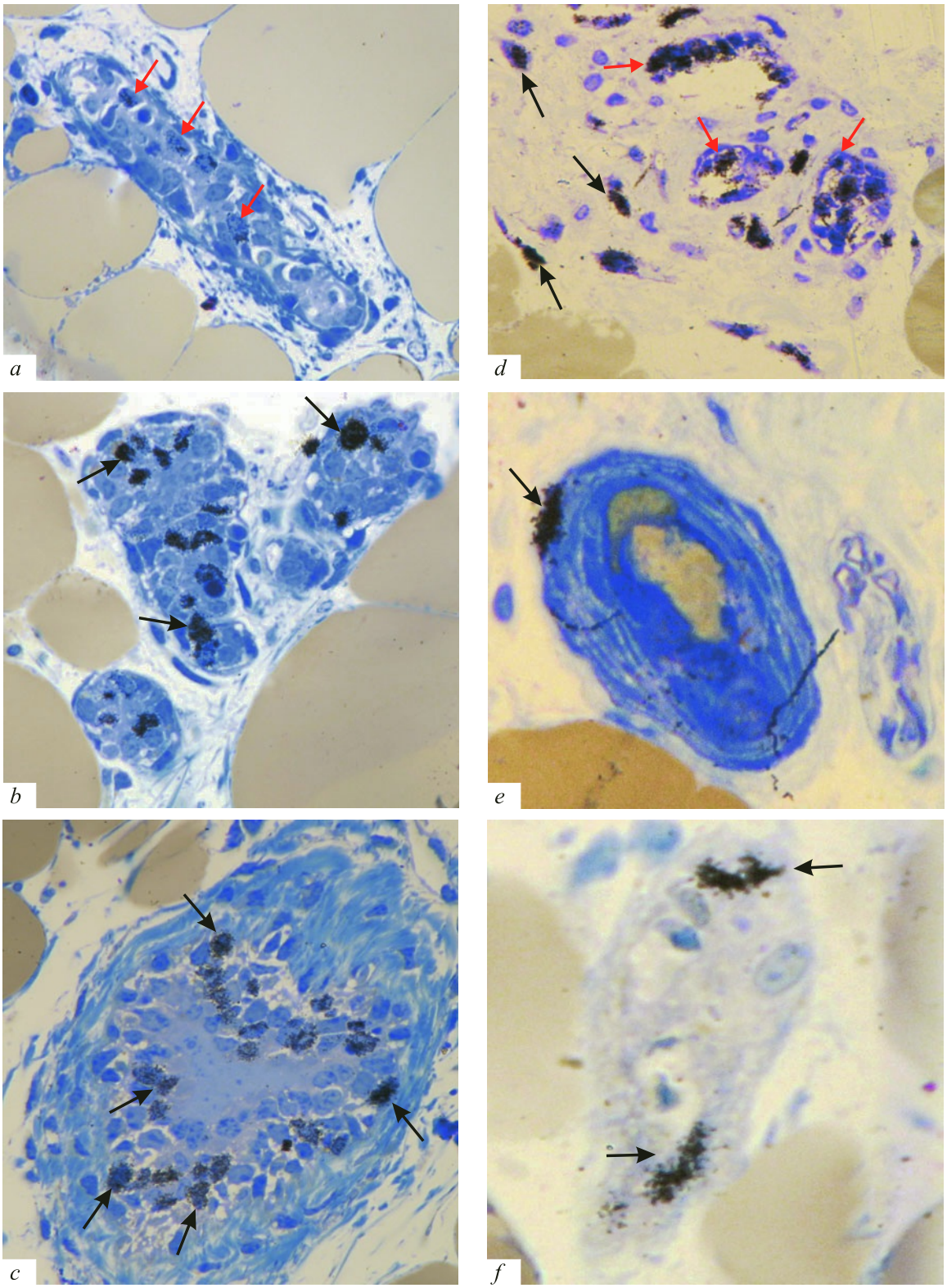


**Fig. 4.** Biosynthetic activity of cells during burn wound healing. Semithin sections. Toluidine blue staining, ×630. *a*) 6 h after burn infliction in outbred mice: DNA synthesis (black silver grains) in epithelial cells of sweat glands (arrows); *b*) 6 h after burn infliction and application of AF in outbred mice: intensive DNA synthesis (black silver grains) in epithelial cells of sweat glands (arrows); *c*) 6 h after burn infliction and application of AF in outbred mice: intensive DNA synthesis (black silver grains) in epithelial cells of HF (arrows); *d*) 6 h after burn infliction in mutant mice: RNA synthesis (black silver grains) in epithelial cells of sweat glands (red arrows) and fibroblasts (dark arrows); *e*) 6 h after burn infliction and application of AF in mutant mice: DNA synthesis (black silver grains) in epithelial cells of dermal cyst (arrow); *f*) 1 day after burn infliction and application of AF in mutant mice: DNA synthesis (black silver grains) in vascular endothelial cells (arrows).

Thus, reaction of skin appendages, primarily HF or their derivates (in mutant mice) in the form of swelling, proliferation, and migration of epithelial cells was observed 6 h after burn injury in both outbred and mutant mice against the background of destructive changes in skin tissues. This reaction was more pronounced after application of dressing with AF.

Autoradiography showed active proliferation ($^3$H-thymidine incorporation indicating DNA synthesis) of epithelial cells of HF and sweat glands in outbred mice 6 h after burn infliction (Fig. 4, *a*), which was more pronounced after application of dressing with AF (Fig. 4, *b, c*). In $Hr^{hr}/Hr^{hr}$ mice, protein synthesizing activity ($^3$H-uridine incorporation indicating RNA synthesis) epithelial cells of sweat glands, microvascular wall cells (endothelial cells and pericytes), and fibroblasts was noted during spontaneous healing (Fig. 4, *d*). In wounds treated by application of dressing with AF, DNA synthesis in epithelial cells of cysts, HF derivates, was noted (Fig. 4, *e*)

One day after burn infliction, the above described reaction of wound cells was more pronounced. Proliferating and migrating epithelium started to cover the wound with different rates in different groups. Similar pattern of wound epithelization was noted in mice of both groups: combination of marginal and patchy epithelization (the latter is due to proliferation of skin appendage epithelium). Epithelium covered greater (application of AF) or lower (spontaneous healing) part of the wound surface. At the same time, signs of regeneration were observed in the derma against the background of destructive changes and inflammatory reaction (neutrophilic infiltration). In all groups, the number of fibroblasts in the derma increased, probably due to their migration from the subcutaneous fat. When AF-containing dressing was applied onto the wound, pronounced reaction of blood vessels was observed in the derma and subcutaneous fat in both outbred and mutant mice: the number of capillaries and cells around blood vessels (primarily fibroblasts) increased, mitotic activity of endothelial cells and pericytes of microvessels and adjacent cells was noted. Radioautography showed that proliferative activity was most pronounced in vascular endothelial cells of mutant $Hr^{hr}/Hr^{hr}$ mice (Fig. 4, *f*).

Epithelization of the wounds under dressing with AF was completed on day 3 in both mutant and outbred mice. In spontaneous healing, epithelization was observed after 5-7 days.

Wound healing after IIIA degree burn consists in re-epithelization of the burned surface and restoration of the derma. Under conditions of derma destruction, the formation of primary extracellular matrix is required for migration of keratinocytes from the preserved epithelium. Migrating keratinocytes can only partially restore the epidermis [11]. Complete recovery can be attained at the expense of SC from skin appendages, primarily, HF.

Intensive proliferation of epithelial cells of HF and sweat glands in outbred mice and epithelium of deep dermal cysts in mutant mice (DNA synthesis) detected 6 h after burn infliction and application of AF probably attests to stimulation of stem/progenitor cells in skin appendages of outbred mice and HF derivates of mutant mice. In the latter, the outgrowth of the outer root sheath of HF (tissue niche of SC in skin epithelium) after disintegration of HF was retained in deeply located dermal cysts [15].

An important role in derma regeneration is played by microvascular wall cells. Microvessels are not only responsible for tissue metabolism, but also represent centers of cell proliferation and differentiation [7]. Microvascular wall is a niche of resident SC providing tissue regeneration [12,16]. Microvascular wall cells (endothelial cells and pericytes) are the main sources of new cells, in particular, fibroblasts, in the tissue. Microvessels also supply the wound with bone marrow SC essential for derma regeneration [10].

Thus, biological dressing containing cultured AF exhibits a pronounced stimulating effect on healing of IIIA degree burn wound. We believe that this effect can be explained as follows. First, AF as the main component of the dressing with collagen produced by them during culturing play a role of primary exogenous derma temporarily (at the early stage of healing) replacing the destroyed derma and providing conditions for migration of survived keratinocytes. Second, great contribution into the stimulating effect is made by growth factors and cytokines released from transplanted AF after their degradation [5]. We believe that these bioactive substances activate proliferation of stem/progenitor cells of the epithelium (skin appendages) and derma (endothelial cells and pericytes of microvessels) at the early stage of healing.

These findings and the results of our previous studies [6] suggest that reparative regeneration of the skin in hairless mice carrying mutation in *Hr* gene has the same regularities as skin regeneration in outbred albino mice. No deviations from the normal were revealed during reparative regeneration of the derma in mutant mice. Despite considerable changes in HF morphology and disturbances in their main function, hair formation, they still represents the main source of epithelial SC, because the tissue niche of epithelial SC is retained in HF derivates (dermal cysts).

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