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PATHOGENETIC CORRECTION OF MORPHOLOGICAL CHANGES IN SKIN AFTER THERMAL BURNS IN EXPERIMENT

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Abstract

The goal was to prove by complex morphological analysis the purposefulness of using a biocomposition based on silver nanoparticles stabilized in solution with sodium alginate and the inhibitor of proteases of aprotinin for influencing the course of the inflammatory process after thermal burn.

Using light and electron microscopy we studied morphological changes after thermal skin damage of II grade in rats using biocomposite based on silver nanoparticles stabilized in solution with sodium alginate and the protease inhibitor aprotinin to modify the course of the inflammatory process. Research was performed on 80 white male Wistar rats, 180-200g weight.

The early wound process (3rd day) did not differ in various groups and was characterized by the predominance of exudative-necrotic and dyscirculatory disorders over the regeneration processes. From the 7th day, the morphological picture began to differ depending on the applied medication therapy. There was noted the beginning of rejection of the scab, a decrease in the intensity of inflammation, in hemodynamic disturbances and the onset of epithelialization. On the 14th day, the epithelialization was observed, the inflammatory process was decreasing and the young scar was forming. The use of erythromycin facilitated the migration of keratinocytes and accelerated the restoring of connective tissue in dermis through synthetic activity of fibroblasts. The use of the silver nanoparticle composition had a moderate corrective effect. After proteinase inhibitors application, the dystrophic changes, both in the epidermis and in dermis, persisted longer than in other groups. At the same time, samples where burns were treated by combined way (the solution of nano-silver and an inhibitor of proteases of aprotinin) demonstrated morphological signs of the increase in the keratinocytes proliferative activity and functional activation of protein synthesis of fibroblasts. According to the results of light and electron microscopy, the best result was obtained after combined therapy; the morphological characteristic of wounds was somewhat worse after antibiotic or silver monotherapy. The monotherapy only with the proteinase inhibitor gave the worst results on the duration of healing and the severity of inflammation in the wound.

The local application of the silver nanoparticles stabilized in sodium alginate solution and aprotinin limited the destructive changes in the burnt skin, assisted in early repair processes, helped in the formation of granulation tissue, its maturation and epithelization.

Keywords: *thermal burn, skin morphology, therapy, silver nanoparticles, aprotinin.*

Introduction

The relevance of studying the pathogenesis and methods of correcting of thermal damage to the skin is caused by high burn injury rate, which is on the 2nd – 3rd place in the overall rate, after the road accidents: every year more than 500 thousand cases of burns of different stages of severity are

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recorded in Russia [*Alekseev A.A. , 2013, Eser T. et al., 2016*]. According to the World Health Organization, thermal injury rate is about 10-12% of all peacetime injuries [*Ciftçi I et al., 2013; Oseni O et al., 2017*]. Extensive deep burns are the most life-threatening conditions, followed by the development of severe burn disease, so traditionally this problem is of great attention in combustiology [*Alekseev AA, 2013, Warner P. et al., 2015*]; on the other hand, there is a progressive growth of local deep burns, in which the affected area does not exceed 10% of the body surface [*Gilmutdinova I.R., 2015*]. Regarding that, understanding of the pathogenetic mechanisms of burns transformations and improving of pharmacotherapy effectiveness is one of the urgent problems of fundamental medicine [*Nielson C.B.,2017*].

According to the latest data, a complex interaction of various pathogenetic mechanisms takes place in the focus of burn injury, which includes vascular thrombosis, impaired microcirculation, increased excretion of inflammatory mediators and pro-apoptotic influences [*Bohr S et al., 2013; Oskeritzian C, 2017*]. However, knowledge of those cellular mechanisms and a lot of studies did not allow to understand completely the multifactorial pathogenesis of the reparative process, as well as to improve significantly the clinical treatment of burn injuries. So, there is a need in further study the pathogenetic mechanisms of re-epithelialization and remodeling of burn wounds for their pharmacotherapy optimization [*Reddy A.S. et al.,2015, Makhatova B.G. et al., 2017*].

In modern conditions, the frequency of using the nano-silver products is increasing, and it becomes necessary to understand deeper the mechanisms of its influence, biological interaction and toxicity [*Ciloglu N.S. et al., 2014*]. Despite the advance in the study of the antimicrobial properties of the nano-silver, the data on its potential for anti-inflammatory and regenerative effects have recently emerged [*Jia Z. et al., 2016; Liu M. et al., 2017*]. To study the specific mechanisms of action and points of application, it is necessary to conduct an additional morphological study, and that was performed in our work.

The goal of the study is to prove the advantage in application of biocomposition based on silver nanoparticles stabilized in solution with sodium alginate and an inhibitor of proteases aprotinin, to modify the course of the inflammatory process on the model of thermal damage in the experiment conducting complex morphological analysis.

Material and methods

The experiment was performed on 80 male Wistar rats weighing 180-200 g, which were kept in standard conditions. A series of works was carried out in the laboratories of the departments of general and clinical pathophysiology, pathological anatomy with a sectional course, as well as histology laboratory in the Center for Collective Use "Molecular Biology" of the Medical Academy named after S.I. Georgievsky. The experiment was approved by the Ethics Committee (Protocol No.2, September 11, 2015).

The modeling of the skin thermal burn of the II degree was performed after intravenous anesthesia with sodium thiopental of 20 *mg/kg*. The burn in the neck area was caused by application to the depilated area of skin, limited by a 1.5x1.0 *cm* frame, a thin-walled glass container with hot water at a temperature of 90 °C for 10 *sec* [*Surakova T.V. et al., 2012]*. This method allows to obtain a second degree burn of the standard size and depth. Throughout the experiment, the wound was open, each animal was kept alone. Animals were divided into two control groups (intact and having burn without treatment) and four groups with different therapeutic approaches: receiving monotherapy - 3-5 animals in group and complex therapy – 6 in group (Table 1) [*Yurkova I.N. et al., 2009*].

On the 3rd, 7th, 14th day, the rats were taken out of the experiment and the skin in the wound area was examined by the following methods: macroscopic description, histological examination with hematoxylin and eosin staining, transmission electron microscopy (TEM). Under thiopental anesthesia, euthanasia was performed and the samples of wound tissues were taken, with dimensions 1x1x0.5 cm. Samples preparation was performed by the standard method [*Korzhevsky D, Gilyarov A, 2010*]. For light microscopy the tissues were fixed in 10% neutral formalin; for electron microscopy - in glutaraldehyde. For paraffin embedding, a Logos histoprocessor (Mielstone, Italy), and Leica filling station EG 1150 H (Leica, Germany) were used. On a Leica RM 2255 automatic microtome (Leica, Ger-

TABLE 1.

Distribution of animals in groups

No	Group	Exposure	n
1	Intact	Control group of the intact animals	10
2	Burn without treatment	Burn without treatment, the animals were sacrificed in three different stages of healing – on the 3rd, 7th and 14th day, each stage - 10 species	30
3	Burn +antibiotic	For 7 days locally 1 *ml* 10% solution of erythromycine (0.5 *gr* of erythromycine made in powder, mixed with 10 *ml* 0.6% of Sodium alginate).	10
4	Burn + Ag	For 7 days locally water soluble composition of silver nano-particles (0.1%) in matrix of carbohydrate 0.6% of Sodium alginate.	10
5	Burn +Gordox	For 7 days locally the inhibitor of proteinase aprotinin (1 ml of 0.6% of Sodium alginate mixed with 1 *ml* of the product «Gordox», 10 000 *U* in 1 *ml*)	10
6	Burn+Ag+Gordox	For 7 days locally the combined solution of nano-silver with the inhibitor of proteinase aprotinin (1 *ml* of the product «Gordox», 10 000 *U* in 1 *m*l mixed with c 1 *m*l of silver nano-particles 0.1%)	10

many), 5 μm paraffin sections were made. Microphotos were obtained using a Leica DM2000 microscope and Aperio Leica ScanScope CS2 histoscaner (Leica, Germany). For TEM, samples preparation was carried out manually, strictly following the protocol [*Korzhevsky D, Gilyarov A, 2010*]; and PEM-125 transmission microscope (Sumy, Ukraine) was used for visualization.

RESULT AND DISCUSSION

The major morphological changes manifested by the presence of exudative-necrotic foci and disorders of the microvasculature. In all groups of animals receiving the treatment, on the 3d day the histological picture was similar to the group without treatment. In the area of the wound - coagulation necrosis of all layers of the epidermis and partly of the upper parts of the dermis, the nuclei of fibroblasts were picnotic, bundles of connective tissue fibers in the superficial and partially in the deep-lying parts of the dermis had a pronounced oxyphilous color. In the adjacent tissues - the edema, minor hemorrhages, hyperemia were noticed. At the border with intact epidermis, under the formed scab, there was a slight epithelium hyperplasia and a zone of lymphocytic infiltrate. From the 7th day, the picture in groups began to differ depending on the type of therapy.

In group 3 (Burn + antibiotic), the microscopic picture indicated a pronounced reparative reaction, the degree of which was slightly lower than in the combined treatment group (Fig. 1A, B). On the 7th day, the scab was presented by a continuous, uneven layer. In a number of fields of view, the partial rejection with the formation of vesicles containing exudate was observed. Under the scab in the wound, the granulative tissue with many fibrils and active fibroblasts was significantly developed. Between the scab and the granulative tissue, a layer of tissue debris was visible in the wound, which had a maximal thickness in the center. Demarcation inflammation was represented by diffuse focal lymphatic macrophages infiltration with an admixture of leukocytes that spread to the underlying tissues. On the periphery of the zone of inflammation and in the tissues of the dermis there were the signs of spasm, edema, plethora, a small amount of hemorrhage, small perivascular lymphohistiocytic infiltrates. The epithelium at the edges of the wound was thickened, hyperplastic, with symptoms of acanthosis, hyper-and parakeratosis. Partial regional epithelialization, growth of granulation tissue and formation in young connective tissue were noted. On the 14th day, there was the rejection of the scab in large areas, the decrease in the wound surface due to the significant zone of epithelialization from the edges. The beginning of scar formation, the growth of granulation tissue with many vessels, functionally active spindle-shaped fibroblasts and a large number of collagen fibers oriented parallel to the surface of the dermis were noted in the burn zone. The phenomena of edema and plethora preserved. In the adjacent dermis, there were perivascular lymphoid infiltrates.

In group 4 (Burn + Ag) on the 7th day the scab was represented by a continuous, even thick layer,

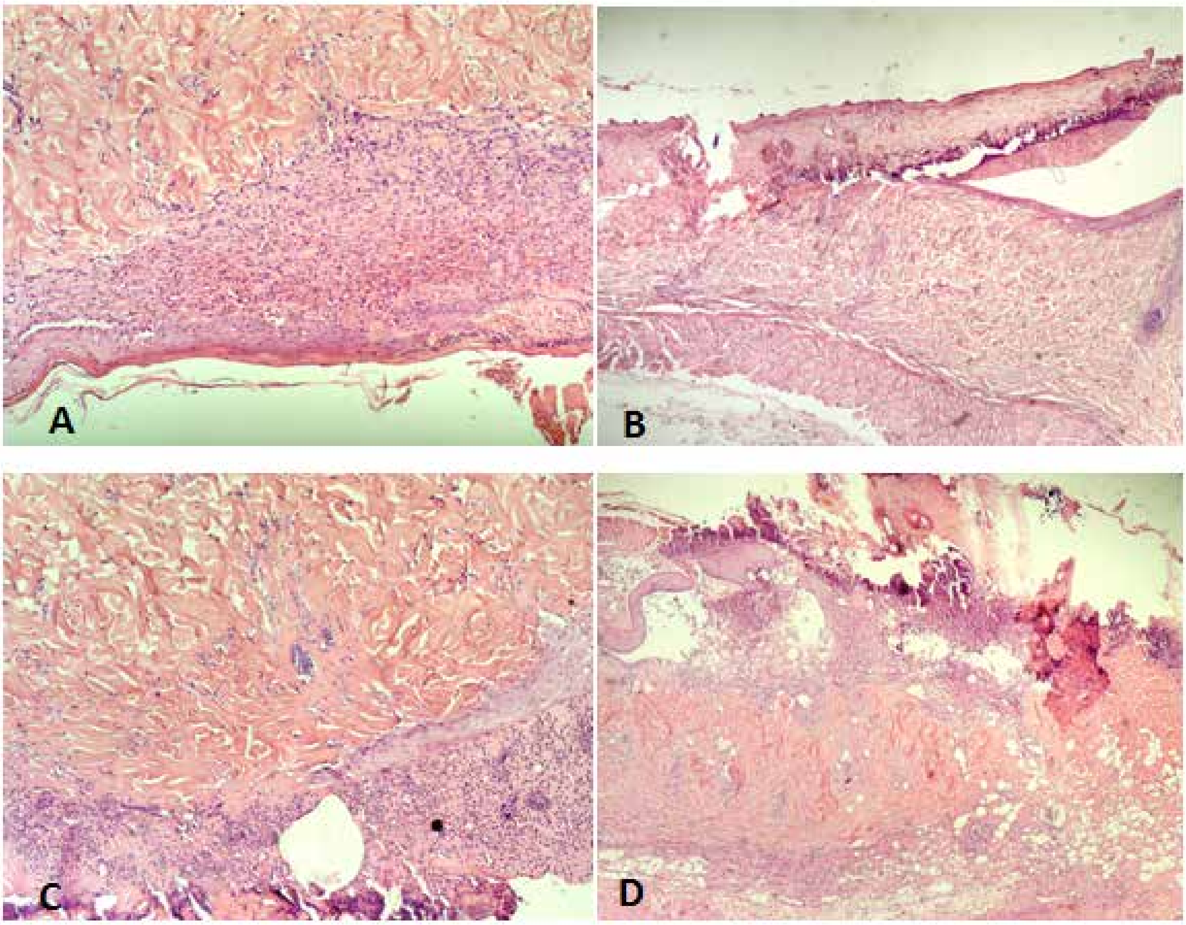

FIGURE 1. *Fragments of the skin of White rats after the thermal burns treated by various pathogenetically substantiated methods of correction. Paraffin sections, hematoxylin and eosin stain.* ***A****. Burn + Ag + Gordox, 100×.* ***B.*** *Burn + antibiotic, 40×.* ***C.*** *Burn + Ag, 100×.* ***D.*** *Burn + Gordox, 40×.*

which was rejected with the formation of vesicles containing exudate, or cell-tissue detritus was seen between the scab and the underlying tissues (Fig. 1 C). On the border of necrotic and microscopically intact tissue, there was the perifocal inflammation (lymphocytes and macrophages mixed with neutrophils), penetrating into the deeper parts of the dermis, also, the isolated microabscesses were noted. The epithelium at the edges of the wound was thickened, swollen, hyperplastic, with symptoms of acanthosis, hyper-and parakeratosis. Epithelization was represented by creeping of the epithelial layer under the scab in the marginal zone, with focal proliferation of the cells of the basal layer. The layer of granulative tissue, as well as the formation of connective tissue in the underlying tissues, is less evident compared to the groups that underwent complex therapy and antibiotic administration. The tissues below showed the symptoms of vasospasm, edema and plethora, visible small hemorrhages and perivascular scleral lymphohistiocytic infiltrates. On the 14th day of the phenomenon of edema, plethora, focal perivascular lymphoid infiltration in the tissues adjacent to the wound, persisted. There was a rejection of the scab in significant areas. Epithelization of the wound surface was less than in the groups with complex therapy and erythromycin administration. Granulation tissue with many capillary vessels of the type, perpendicular to the surface of the wound and collagen fibers oriented parallel to the surface of the dermis, with a moderate number of active fibroblasts.

In group 5 (Burn + Gordox) on the 7th day, the scab was represented by a thick continuous layer consisting of necrotic underlying tissues and a stratified squamous epithelium (Fig. 1 D). In some areas, the rejection with the formation of vesicles containing exudate with significant number of inflammatory cells, was observed. At the edges and bottom of the burn wound, the pronounced inflammatory changes took place in the form of diffuse focal lymphocytic infiltration, partially penetrating into the deep layers of the dermis, forming accumulations of neutrophils like microabscess in separate fields of view. The epithelium of the skin at the edges of the wound was thickened, hyperplastic, with symptoms of acanthosis, hyper- and parakeratosis. The epithelization of the wound was negligible and was presented in the form of small foci of proliferating basal cells of the epidermis, located in the marginal zone. The granulation tissue was moderately developed, it contained small foci of necrosis. Perifocal inflammation (lymphocytes and macrophages with neutrophils admixture) was expressed on the border of the affected and intact tissue, penetrating into the deeper parts of the dermis. In the underlying tissues, there was a plethora of the vascular bed with symptoms of moderate edema, a small number of petechial hemorrhages and perivascular scleroid lymphoid accumulations. On the 14th day the scab was of irregular thickness; there was rejection in large areas. Epithelialization occured due to creeping of the epithelial layer from the edges of the wound; there was no complete epithelialization. The degree of development of granulative tissue is higher compared with other groups after treatment, and was represented by a set of full-blooded capillaries, a moderate number of fibroblasts and a large number of young collagen fibers, oriented parallel to the surface of the dermis. The phenomena of edema, plethora, perivascular lymphoid infiltration persisted.

In the 6th group that received the combined treatment (Burn + Ag + Gordox), on the 7th day there were thinning and rejection of the scab over large areas, epithelialization of the wound surface was expressed to a large extent and spread almost to the wound center (Fig. 1A). One could see a significant amount of granulative tissue with a large number of thin connective tissue fibrils and active fibroblasts. Instead of the chaotic direction of the fibers, we observed their predominantly horizontal orientation parallel to the surface of the epidermis. The layer of granulative tissue in the center was thicker, and as it approached the edges, it became thinner. The phenomena of plethora, edema reduced, as well as inflammatory infiltration in the zone of damage, the presence of perivascular lymphohistiocytic infiltrates in the dermis was observed. On the 14th day, there was rejection of the scab and epithelization on the entire surface of the wound with the appearance of mature, multi-layered squamous horny epithelium and the formation of a tender scar. Superficial epithelial cells had the signs of keratinization, and in the deeper layers there was parakeratosis. Under epithelium there was proliferation of granulations with significant number of active fibroblasts producing collagen fibers, the increase in the number and thickness of connective tissue fibers. The phenomena of edema and plethora were expressed slightly. Single, small perivascular lymphoid infiltrates were found in deeper regions of the dermis.

When conducting TEM, similar dynamics was revealed at the ultrastructural level. After the reatment of burn with erythromycin in group 3 (Burn + antibiotic), there was better preservation of all skin structures compared with other groups of animals

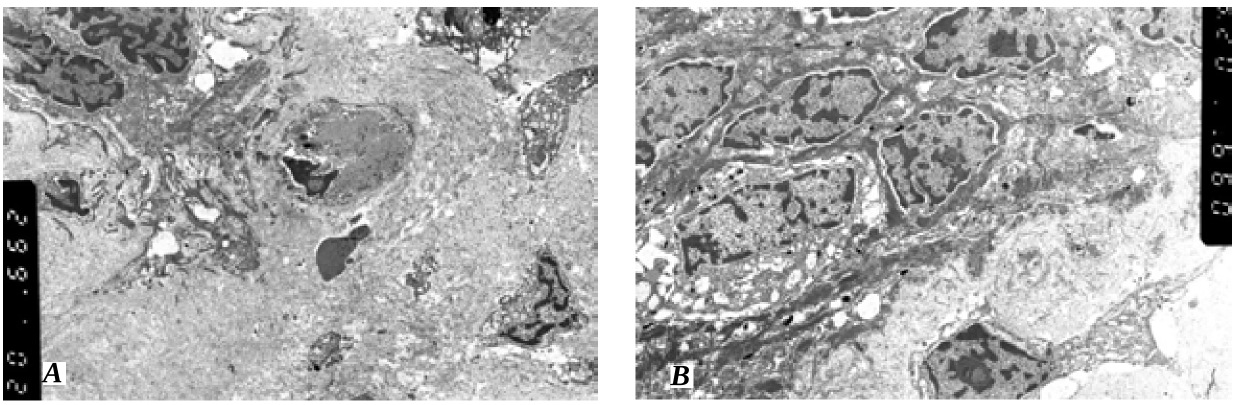

*FIGURE 2. A fragment of the epidermis and dermis (**A**, 5000x) and of the dermis (**B**, 4000x) of the skin of a White rat after the correction of the burn injury with erythromycin solution for 7 days. TEM. Keratinocytes and fibroblasts are dominating with the signs of functional activation.*

treated with monotherapy (Fig. 2). The nuclei of keratinocytes had shallow invaginations of the karyolemma. The perinuclear space of most cells was moderately widened and continued to the cistern of the granular endoplasmic reticulum (GEPS) surrounding the nucleus (Fig. 2A). The nucleoli were small, located in the peripheral part of the karyoplasm. Cytoplasm contained ribosomes, polysomes, and fragments of GEPS cisterns in the perinuclear zone. Mitochondria were numerous, small, most of them contained dark matrix and the significant number of cristae. Along with the indicated type of keratinocytes in the epidermis, there were cells that had the lobated nucleus, small and medium-sized vacuoles, formed as the result of expansion of the GEPS cisterns and the Golgi complex (Fig. 2A). In cytoplasm, there was significant number of mitochondria of the round or oval shape with the clearing of the matrix and the destruction of the cristae. It can be assumed that the revealed structure of keratinocytes reflects the active synthetic activity of cells. The capillaries of the dermis had the thickened basal membrane, endotheliocytes were intact. The thickened layer of connective tissue formed by circular fibers was arranged around the vessels (Fig. 2B). Fibroblasts were characterized by structure variability. Some cells had the normal ultrastructure of the nucleus and cytoplasmic organelles. In the other part of fibroblasts, hydropic changes were noted with the formation of vacuoles due to the swelling of mitochondria, the expansion of the GEPS cistern and the Golgi complex.

In group 4 (Burn + Ag), the use of silver nanoparticles caused moderately pronounced changes in keratinocytes and connective tissue cells as compared with the group of animals that received the antibiotic and the Gordox product (Fig. 3).

Keratinocytes had clear boundaries, even contours (Fig. 3 A). In the nuclei, small invariants of the karyolemma were detected, the perinuclear space was narrow, uniform, extended into parallel tanks of the GEPS. In a number of cells it was moderately expanded. The outer nuclear membrane contained many ribosomes. Mitochondria were found in significant number, they were clearly structured, contained dark matrix and densely located laminar cristae. The restoration of intercellular contacts and complexes of tonofilaments with desmosomes was observed, the content of tonofilament bundles increased. The fibroblast polymorphism was well pronounced: functionally active fibroblasts were found among the undifferentiated cells (Fig. 3 B). Correction by the preparation of nano-silver contributes to proper preservation of the vascular bed. Endotheliocytes lining the capillaries had flattened nuclei, heterochromatin was represented by medium-sized clumps along the karyolemma. Their cytoplasm contained ribosomes, polysomes, fragments of GEPS, pinocytotic vesicles. The basal membrane was clear. The elements of connective tissue loosened around the capillaries. The collagen fibrils were compact in some fibers, loose in the others, and had transverse striation.

In group 5 (Burn + Gordox), the use of proteinase inhibitors contributed to less damage to both epidermis and dermis: fibroblasts, macrophages, lymphocytes, the vascular bed. The vacuolization of the cytoplasm was reduced in comparison with a

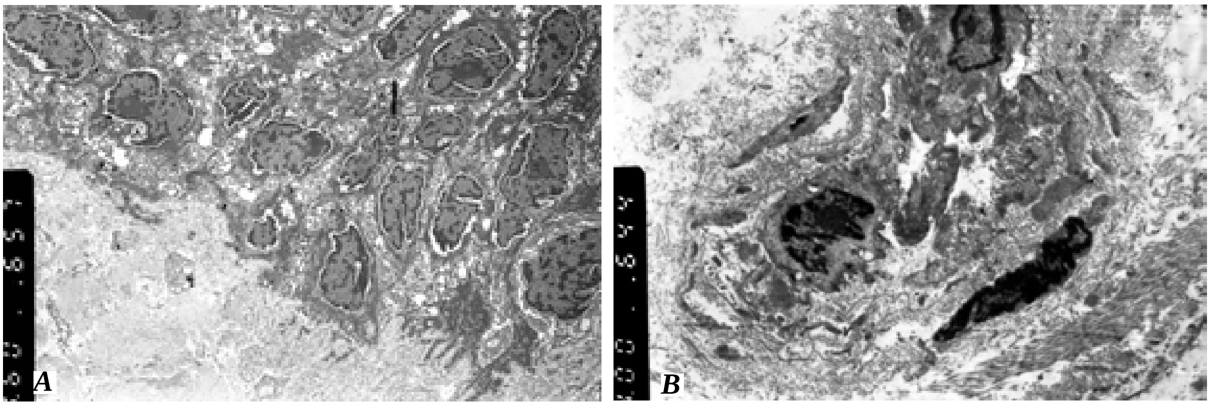

Figure 3. *A fragment of epidermis and dermis (A) and a fragment of dermis (B) of the skin of White rat after correction of the burn injury with the solution of nano-silver (Burn + Ag) for 7 days. TEM enlargement 4000x. Formation of intercellular contacts in the epidermis. Fibroblasts polymorphism.*

burn without therapy, while the high degree of dystrophic changes in keratinocytes of various degrees of maturity and connective tissue cells was maintained (Fig. 4).

Epidermal outgrowths became narrower and shorter, the basal membrane was clear, without gaps. In the epidermis, the intercellular contacts were modified. Wide spaces remained between some keratinocytes, which indicated the persistence of intercellular edema (Fig. 4 A). The desmosmes were represented by dense formations with central enlightenment in the intercellular space, which indicates their destruction. The keratinocytes contained the insignificant amount of melanin granules, some granules of different electron density were located in expanded intercellular spaces. In the cytoplasm of keratinocytes the dystrophic changes were expressed. Mitochondria swelled, cristae were only fragmented. Some mitochondria were damaged, their matrix was poured into the cavity of the cytoplasm vacuoles (Fig. 4A). Both in the dermis, and in the epidermis, the changes in cells were variable (Fig. 4 B). Some fibroblasts had signs of active protein synthesis: the bladed nucleus, large nucleoli adjacent to the karyolemme, the predominance of euchromatin, the large number of nuclear pores, moderately widened GEPS tanks, the significant number of ribosomes and polysomes. Other cells of the fibroblastic differon reflected dystrophic-destructive processes inside. The phenomena of intercellular edema persisted. Tissue basophils contained granules of uniform structure, there were no degranulation.

In group 6, the combined treatment (Burn + Ag + Ggordox) contributes to better preservation of the skin structures: keratinocytes, fibroblasts, dermal vessels (Fig. 5).

Epithelial cells tightly adjoin each other, intercellular contacts and complexes of tonofilaments with desmosomes were restored (Fig. 5 A). Although vacuolization is preserved, its degree was much lower than in the previous groups

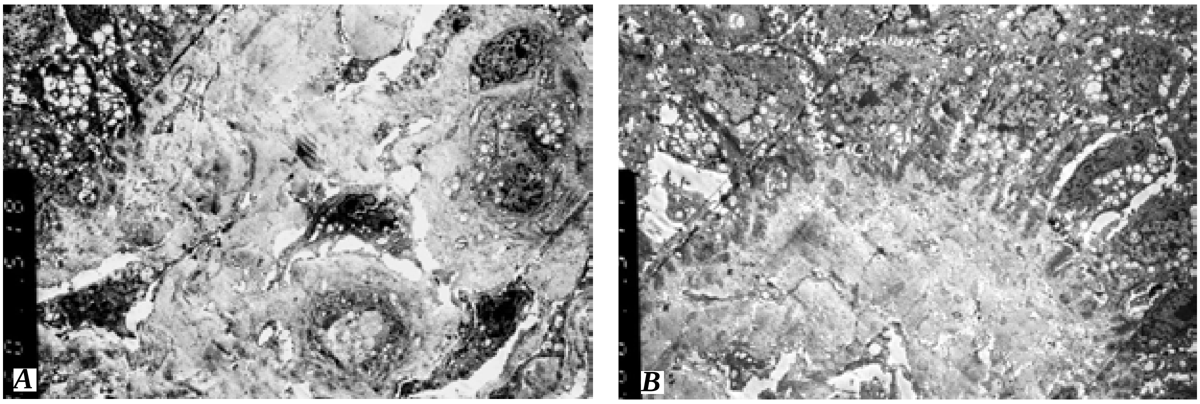

*FIGURE 4. A fragment of the epidermis and dermis (**A**) and a fragment of the dermis (**B**) of the skin of the White rat after the therapy of the burn injury with proteinase inhibitor (Burn + Gordox) for 7 days. TEM. 4000x. Vacuolising of cytoplasm in keratinocytes, intercellular edema and destruction of desmosomes.*

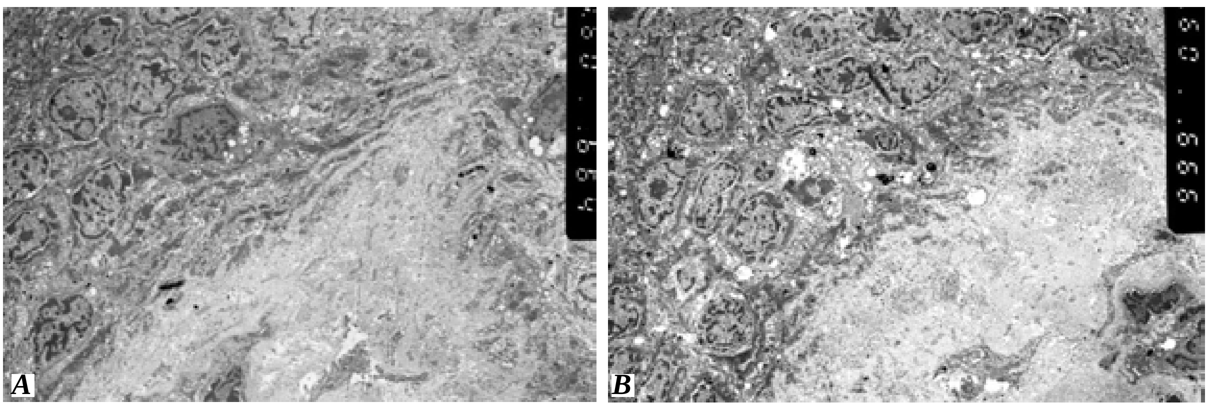

*FIGURE 5. Fragments of the epidermis and dermis (**A**) and the area of pronounced proliferation of the epidermis (**B**) of the skin of White rat with burn injury after correction with the combined method (Burn + Ag + Gordox) for 7 days.TEM. 4000x.*

(Fig. 5 B). Ultrastructure was preserved in keratinocytes (Fig. 5 A). The nuclei contained numerous invaginations. Euchromatin was predominant, and heterochromatin was equally represented by small-sized karyosomes and marginal heterochromatin. The perinuclear space of most cells was not expanded and got into the GEPS cistern. The significant number of polisomes was found in the hyaloplasm. The cytoplasm of cells contained the significant number of mitochondria with a preserved structure, dark matrix, great amount of cristae. In the growth layers of the epidermis, there were single young cells with large lobar nucleus (Fig. 5B). Their cytoplasm was moderately clear, contained small mitochondria of normal structure; some depletion of organelles is noted. The appearance of such keratinocytes could be considered as a result of the activation of cell regeneration. In the vessels of the microvasculature of the dermis significant changes were not detected. The thickening and loosening of the basal membrane was found out, the number of organelles in the endothelial cells was moderately reduced. In the papillary layer of the dermis there were the groups of differentiated fibroblasts with signs of functional activation of synthetic processes.

Conclusion

1. The wound process for up to 3 days after the burn injury was represented by purulent-necrotic phenomena, local hemodynamic disturbances, coagulation necrosis of the epidermis and underlying tissues, with the formation of the scab. During this period, the morphological picture did not differ in groups and was characterized by the predominance of exudative-necrotic and dyscirculatory disorders, and from the 7th day the picture began to differ depending on the therapy type.

2. On the 7th day, the beginning of the scab rejection, subsiding of the inflammatory processes and hemodynamic disturbances, tas well as the onset of epithelialization, more pronounced in the group with complex treatment, was noted. On the 14th day in that group there is a complete epithelialization of the wound and the formation of the young scar. In animals treated with monotherapy, manifestations of inflammation, epithelialization and the formation of young connective tissue were less pronounced due to inflammation in the wound.

3. At the ultrastructural level, a number of pathogenetic patterns of application of different types of therapy have been identified. Thus, the use of erythromycin for burns enhanced keratinocyte migration and accelerated the recovery of connective tissue in the dermis through the synthetic activity of fibroblasts. The use of silver nano-particles had a moderate corrective effect. After using the product “Gordox” for local treatment, the significant degree of dystrophic changes preserved, both in the epidermis and in the dermis. At the same time, the samples where burn injuries were treated by combined method (the solution of nano-silver with Gordox) demonstrated the morphological signs of the increase in the proliferative activity of keratinocytes and activation of protein synthesis by fibroblasts.

4. The best result was obtained after the use of combined therapy, bit worse morphological characteristic of wounds was noted after antibiotic or silver monotherapy. The treatment only with proteinase inhibitor as monotherapy gave the worst results on the duration of healing and the severity of inflammation in the wound.

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