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FEATURES OF MORPHOLOGICAL CHANGES IN THE SKIN DURING WOUND HEALING

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Abstract

Concomitant diseases can cause a violation of the orderly and timely course of the wound healing process, which dramatically increases the risk of chronic wounds. Currently, there are only a small number of studies studying the relationship between chronic kidney disease and impaired wound healing. The aim of our work was to study the peculiarities of the morphological changes of the skin during the healing of wounds complicated by chronic kidney disease in the experiment.

Studies were performed on 48 white rats. Animals were divided into two groups: control and experimental (rats with chronic kidney disease (CKD)). A model of a chronic wound was reproduced in animals of both groups. On the 7th, 15th and 28th days after the application of wounds, 6 rats from each group were removed from the experiment. Histological examination were subject to skin samples of animals of both groups. To assess the dynamics of wound area reduction, digital macro photography of the wound surface was performed.

The results of histological studies indicate an increase in the time course of the phases of inflammation, as well as the imposition of phases on each other. Measurement of the area of the wound surface revealed a slowdown in the process of reducing the area of wounds at all stages of healing in animals with CKD compared with intact animals. Differences in the structure of the tissue are detected even after the completion of the wound healing process in both groups.

The results of our study confirm the significant effect of CKD on wound healing. A prolongation of the inflammatory stage of healing is observed, which leads to disruption of the normal epithelialization of the wound and slower maturation of granulation tissue in experimental animals.

Key words: renal insufficiency, chronic, wound healing, inflammation, cytokines, granulation tissue, regeneration, rats.

Introduction

Currently, the treatment of patients with wounds continues to be a difficult issue for medicine. Lack of knowledge about the factors influencing the course of healing is an obstacle to the optimization of wound healing methods. Wound healing occurs in four highly coordinated phases, namely hemostasis, inflammation, cell proliferation and remodeling [1]. In the process of healing of injuries, as a result of strictly coordinated processes in space and time, the structures and functions of tissues are restored. Disruptions in an orderly and timely reparative process caused, for example, by comorbidities, can lead to the formation of chronic wounds due to reduced vascularization, an increase in the duration of the inflammatory phase and delayed wound closure. Thus, patients suffering from chronic kidney disease (CKD) show impaired wound healing [2]. And given the high global prevalence of CKD (about 13%) [3], such violations are quite common.

Although there are a number of works on CKD and wound healing, there are only a small number of studies that examine the relationship between CKD and impaired wound healing [2, 4, 5, 6].

Thus, the study of the pathogenesis of wound healing complicated by chronic diseases, and their clinical manifestations, is a necessary step in developing methods to improve the effectiveness and improve the results of diagnosis and treatment of patients with chronic wounds.

Objective: To study the characteristics of morphological changes in the skin during healing of wounds complicated by chronic kidney disease in the experiment.

Materials and methods

Studies were performed on 48 white rats weighing 250 ± 30g at the age of 9 months. The experiments were carried out in accordance with the principles of the European Convention for the

Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (Strasbourg, 1986) and the General Principles of Animal Experiments, approved by the First National Congress on Bioethics (Kiev, 2001).

Animals were divided into two groups: control (n = 24) and experimental (n = 24). The animals of the experimental group were rats with pre-formed CKD, which developed 8 weeks after a single injection of 50% aqueous glycerol solution at a dose of 10 ml/kg animal body weight [7]. Intact animals of the control group in a similar way were injected with saline. The development of CKD was controlled in accordance with the method [8]. For the study of the functional state of the kidneys from each group, 15 rats were randomly selected for urine samples, and 6 rats for blood samples. Blood for the study was taken from the heart. In urine samples, levels of protein and creatinine were determined, and their daily excretion was calculated. The blood samples were measured for creatinine and urea. The content of analytes was determined using DAK-SpectroMed (Moldova) kits. By the clearance of endogenous creatinine, glomerular filtration rate (GFR) was calculated.

The remaining rats of the control (n = 18) and experimental (n = 18) groups reproduced the chronic wound model in the form of a circle with a diameter of 20 mm in the interscapular region [9].

On the 7th, 15th and 28th days after the application of wounds, 6 rats from each group were removed from the experiment. Then the animals were sampled skin flap with a wound area.

Histological examination was subject to skin samples of experimental animals. The material was fixed in 10% neutral formalin. Dehydrated in ethanol solutions of increasing concentration: 50°, 70° and 96° (twice). Then the material was carried out in alcohol with chloroform, chloroform and embedded in paraffin [10]. The sections, 5–7 μm thick, were stained with hematoxylin and eosin, with pikrofuksin according to Van Gieson. The preparations were analyzed and photographed using a PrimoStar microscope (Zeiss) and a Microocular digital camera.

To assess the dynamics of wound area reduction, digital macro photography of the wound surface was performed. The area of the wound surface was measured in photographs using the ImageJ program (NIH, USA). The relative area of the wounds (S) was calculated by the following formula:

$S = St / So \times 100\%$, where So is the area of the wound immediately after its application, and St is the area of the wound surface at a given healing period.

In all experimental groups, wound healing occurred under a scab to form granulation tissue.

Statistical processing of the results was performed using Statistica 6.0 statistical analysis package. To describe the results obtained, the data were presented as M ± SE, where M is the arithmetic mean, SE is the standard error of the arithmetic mean. The significance of differences between groups (statistical significance) was determined using the non-parametric Kruskal-Wallis ANOVA test for independent samples. Differences were considered statistically significant at $p<0,05$.

Results

Considering the risk of developing chronic kidney disease after an episode of acute kidney damage [11], a glycerol model of acute renal failure was created. 8 weeks after the glycerol injection in the experimental model, CKD was formed, the basis of which is interstitial nephritis and nephrosclerosis [12]. Histological examination of the kidney of experimental rats in our previous studies [13] revealed significant structural changes associated with the violation of the excretory function of the body. Our studies have shown a significant decrease in GFR in rats of the group with chronic bronchitis in comparison with intact animals, which indicates the development of CKD, which is associated with damage to the glomerular apparatus of the kidney .

Indicators	Groups	
	Control	CKD group
GFR, ml/min	0,167 ± 0,023	0,089 ± 0,006*
Blood urea, mmol/l	4,961 ± 0,333	10,008 ± 0,944*
Protein excretion, mg/100 g * day	1,131 ± 0,166	2,352 ± 0,484*

Note: * - $p<0,05$ compared with the control group

Table.1 Indicators of the functional state of the kidneys in groups (M ± SE)

In addition, in animals with CKD, there was a significant increase in blood urea levels compared to rats in the control group. Urea is one of the final products of protein metabolism containing nitrogen. The concentration of urea in the blood reflects the balance between the rate of its synthesis in the liver and the rate of excretion by the kidneys. With a decrease in the filtration capacity of the kidneys, urea and other nitrogenous products are retained in the blood, which reflect the increased levels of urea in rats with CKD.

The protein excretion rate in animals of the experimental group increases by 2,1 times compared with intact animals, which may be due to an increase in the permeability of the renal filter.

Thus, our studies have shown that in animals of the group with CKD a chronic impairment of the excretory function of the kidneys was formed (Table 1).

Histological examination of skin samples with experimental wounds in the control group on the 7th day observed the formation of immature granulation tissue. Its upper layer consisted mainly of fibrin and leukocyte deposits at different stages of necrobiosis. The cellular component of the granulation tissue was made up of fibroblasts, lymphocytes and leukocytes, macrophages, plasma cells, and giant cells of foreign bodies were also noted. Between the cells were loosely packed bundles of collagen fibers. In the zones adjacent to the bottom of the defect, they acquired a horizontal orientation (parallel to the wound surface in accordance with the mechanical load) (Figure 1a). Minor swelling was noted in some areas. A large number of newly formed capillaries were observed in the granulation tissue. In the upper layers they had a horizontal stroke, in the deeper ones they were vertical (Figure 1a, 1b). In the majority of animals in the control group, on the skin areas adjacent to the wound defect, proliferation of epithelial cells and the “creeping” of the newly formed epithelial layer onto the wound edges were noted (Figure 1c).

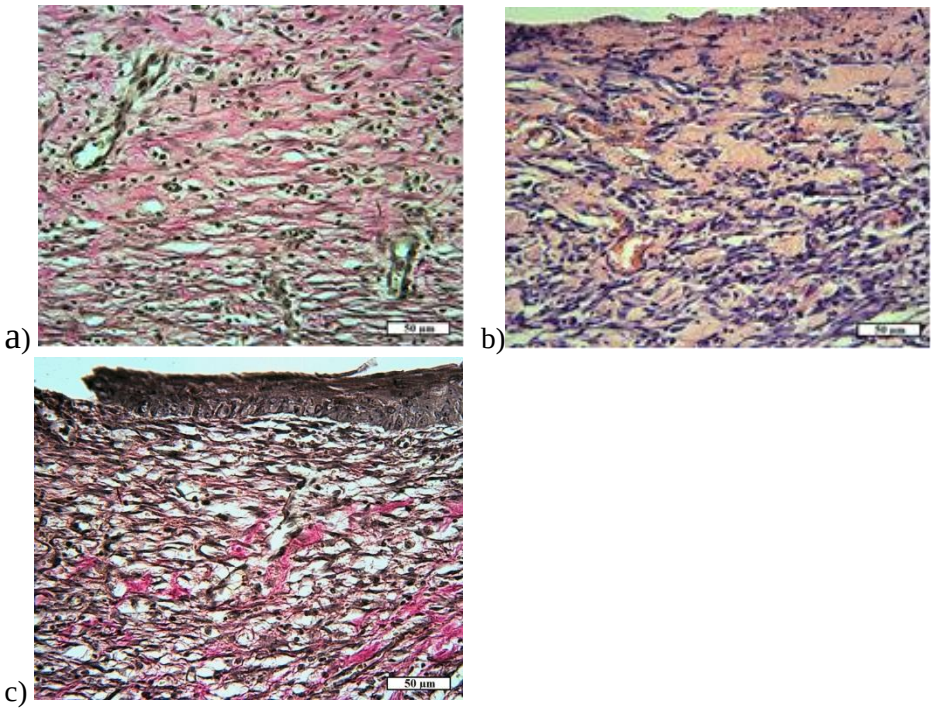

Figure 1. Zone of wound defect in the skin of a rat of the control group on the 7th day. a) A section of a deep layer of granulation tissue: fibroblast proliferation, a small number of white blood cells, vertically arranged capillaries, bundles of collagen fibers of horizontal orientation. Van Gieson. b) A section of the upper layer of granulation tissue: fibroblast proliferation, leukocytes, a large number of capillaries. Hematoxylin and eosin. c) The area adjacent to the edge of the wound: "crawling" of the newly formed epithelium, bundles of collagen fibers. Van Gieson.

In the group of animals with experimental CKD on the 7th day, the granulation tissue had the following features. The cellular component is represented mainly by cells of the inflammatory series – leukocytes, macrophages, as well as single fibroblasts. Zones with polymorphoncellular leukocyte

infiltration and hemorrhages were noted. The vessels were expanded, they often observed the division of blood into regular elements and plasma, as well as erythrostasis, which is a manifestation of circulatory disorders and rheological properties of blood (Figure 2a, 2b). In all histopreparations of this group, signs of edema were also recorded. When stained with pikrofuksin by Van Gieson, isolated, thin and randomly located bundles of collagen fibers were detected (Figure 2c), which may indicate a slowdown of the regeneration process.

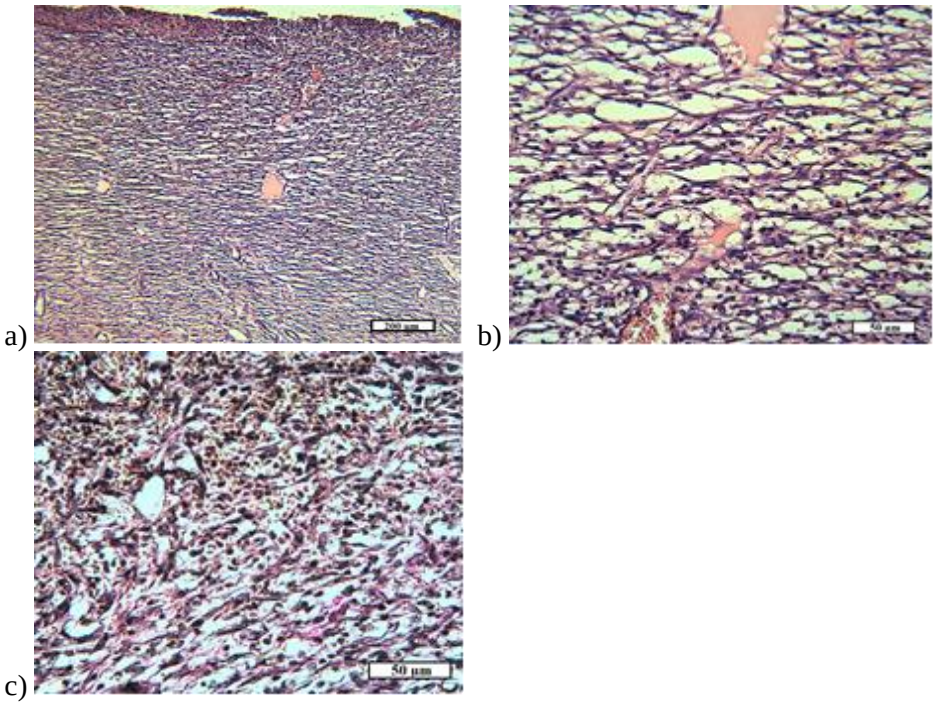

Figure 2. The zone of wound defect in the skin of a rat group with CKD on the 7th day. a) A section of granulation tissue: dilated vessels, signs of edema. Hematoxylin and eosin. b) A section of granulation tissue: dilated vessels, blood distribution to the formed elements and plasma, signs of edema, elements of inflammation. Hematoxylin and eosin. c) A section of granulation tissue: hemorrhages, thin chaotic bundles of collagen fibers. Van Gieson.

Microscopic examination of skin samples of rats of the control group after 15 days revealed an almost complete epithelialization of wounds, with the exception of the central areas. In the marginal parts of the wounds, the epidermis was multi-layered; the rest of the area consisted of 3-6 layers of cells (Figure 3a). The defect area was filled with ripening granulation tissue with a moderate number of capillary type vessels, tightly packed bundles of collagen fibers arranged parallel to the wound surface. Its cellular component was composed of fibroblasts and fibroblasts, white blood cells in a small amount (Figure 3b).

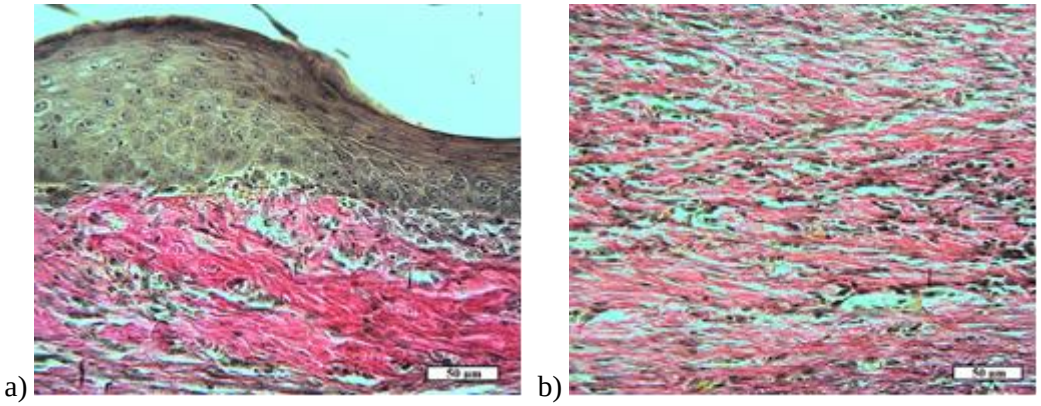

Figure 3. Zone of wound defect in the skin of a rat of the control group for 15 days. a) The area adjacent to the wound edge is a multi-layered epithelium, tightly packed bundles of collagen fibers of ripening granulation tissue. Van Gieson. b) Central wound site: tightly packed bundles of collagen fibers of maturing granulation tissue, a small number of capillaries, fibroblasts and fibroblasts. Van Gieson.

After 15 days in the group of animals with CKD, epithelization of the areas adjacent to the wound edges was noted. The newly formed epithelial layer consisted of 2-4 layers of cells, there were cracks, shadow cells, vacuolation epithelial cells (Figure 4a). The granulation tissue in the defect area was predominantly immature, with signs of edema and inflammation. Focal and diffuse leukocyte infiltration, dilated full-blooded capillaries and hemorrhages were detected (Figure 4b). Bundles of collagen fibers in areas lost orientation parallel to the wound surface, were stratified and fragmented. Between the bundles of fibers due to swelling, gaps were formed (sometimes of considerable size). Fibroblasts were present in large numbers, but some of them were at different stages of necrobiosis (Figure 4c).

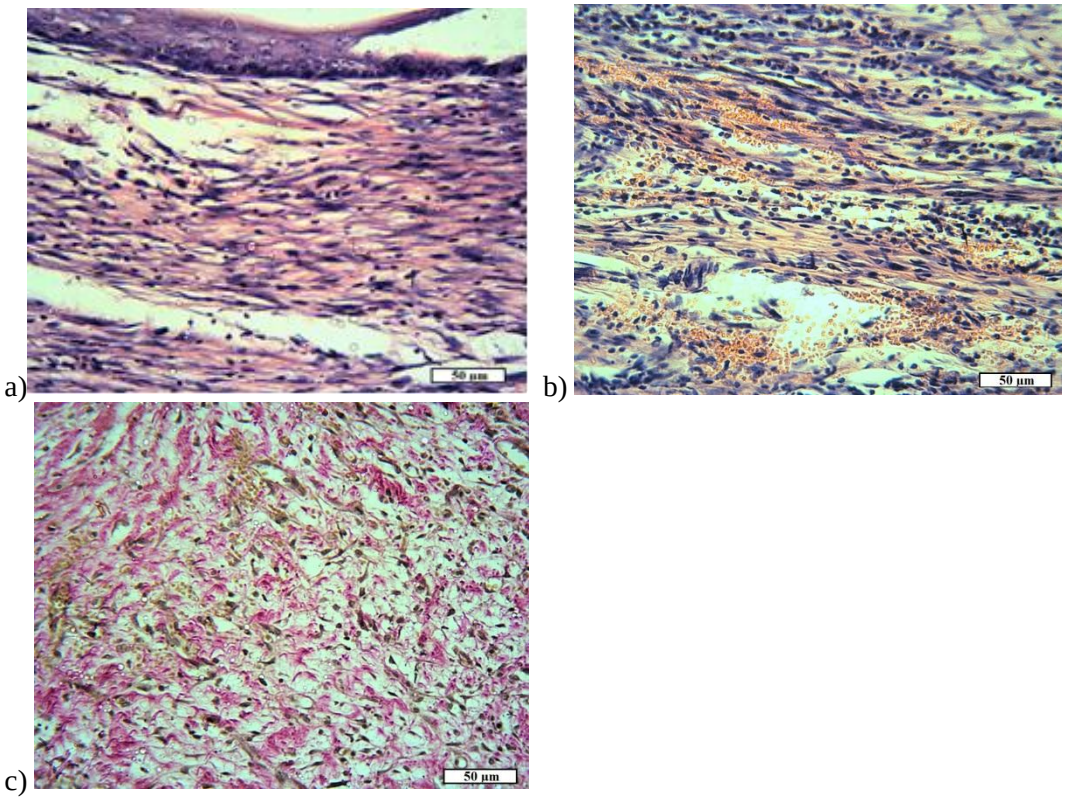

Figure 4. The zone of wound defect in the skin of a rat from the group with CKD after 15 days. a) The area adjacent to the edge of the wound: a crack in the newly formed epithelium, shadow cells, vacuolation epithelial cells, edema of granulation tissue. Hematoxylin and eosin. b) Severe diffuse leukocyte infiltration and hemorrhage in granulation tissue. Hematoxylin and eosin. c) Fragmentation of collagen fibers, hemorrhages, fibroblasts with signs of necrobiosis. Van Gieson.

Histological examination of the skin samples of rats of the control group after 28 days showed complete epithelization of the wound. The layer of the epithelium was multi-layered, with well-differentiated cells that made up the basal, prickly, granular and horny layers (Figure 5a). Under the newly formed epidermis, the defect area was filled with coarse-fibrous connective tissue, which was based on bundles of collagen fibers of sufficient thickness, oriented parallel to the wound surface (Figure 5b). Between them, repeating the shape of the fibers, fibroblasts were located and in a smaller number fibroblasts. The latter were found mainly in the surface layers of the central area of the wound, which may indicate the continuation of the process of maturation of granulation tissue in these areas. In the connective tissue evenly located capillaries, in the deep layers, filling the bottom of the wound,

the vessels were differentiated into arteries and veins. The formation of hair follicles and sebaceous glands was observed in the marginal wounds (Figure 5b).

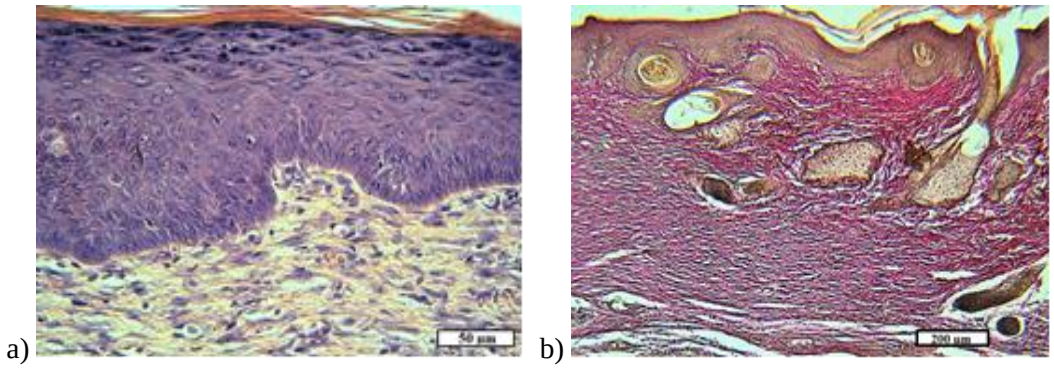

Figure 5. The area of the wound defect of the skin of the rat control group after 28 days. a) Multi-layered epithelium with well-differentiated cells. Hematoxylin and eosin. b) Multi-layered epithelium, horizontally arranged dense bundles of collagen fibers, newly formed hair follicles and sebaceous glands. Van Gieson.

In the group of rats with CKD after 28 days, complete epithelization of wounds also occurred. However, the epidermis was uneven in thickness, in many areas it was very thin without a clear differentiation of cells into layers (Figure 6a). Individual cells of the basal layer had pycnotic nuclei, and spinous cells had vacuolar dystrophy, which reflects the course of destructive processes in the newly formed epidermis (Figure 6b). The vessels of the wound defect zone were mainly enlarged, and perivascular hemorrhages were noted (Figure 6c). Proliferation of fibroblasts and a large number of newly formed capillaries in many areas may indicate a slowdown in the maturation process of granulation tissue that fills wounds (Figure 6c, 6d). This slowdown may be due to edema and inflammatory processes in the connective tissue from the area of the wound defect, which were observed in all animals of the group.

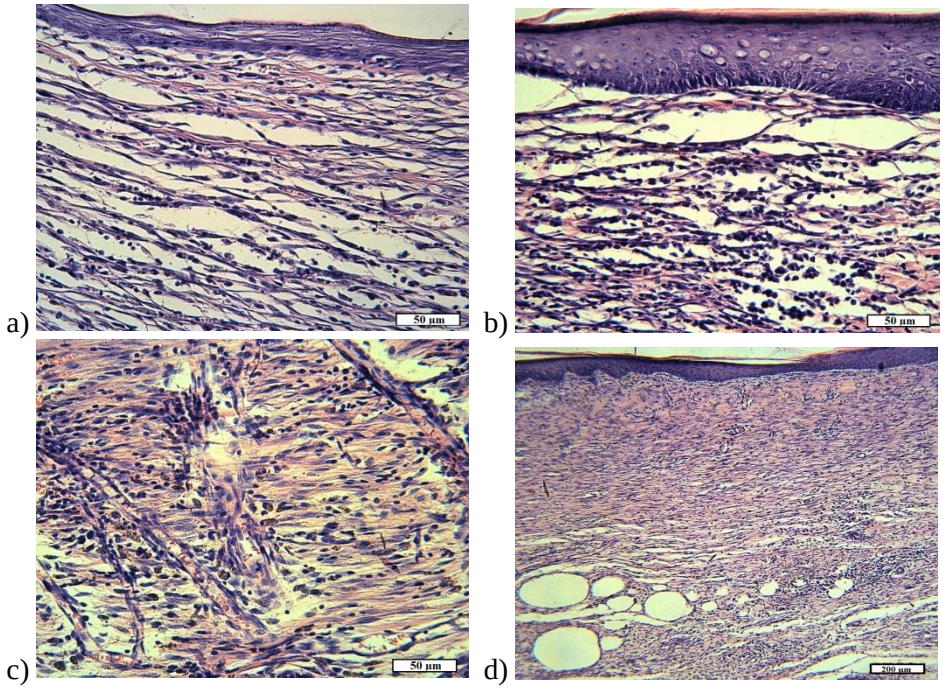

Figure 6. Zone of wound defect in the skin of a rat group with CKD after 28 days. a) Thin epithelial layer without differentiation into layers. Edema and elements of inflammation in the connective tissue. Hematoxylin and eosin. b) Epidermal cells of the basal layer with pycnotic nuclei, prickly – with vacuolar dystrophy. Edema and elements of inflammation in the connective tissue.

Hematoxylin and eosin. c) Proliferation of fibroblasts and a large number of newly formed capillaries at the site, perivascular hemorrhages. Hematoxylin and eosin. d) Proliferation of fibroblasts and a large number of capillaries in the upper layers. Polymorphous cell leukocyte infiltration in the hypodermis. Hematoxylin and eosin.

Measurement of the relative area of the wound surface revealed a slowdown in the process of reducing the area of wounds at all stages of healing in animals with CKD compared with intact animals (Figure 7).

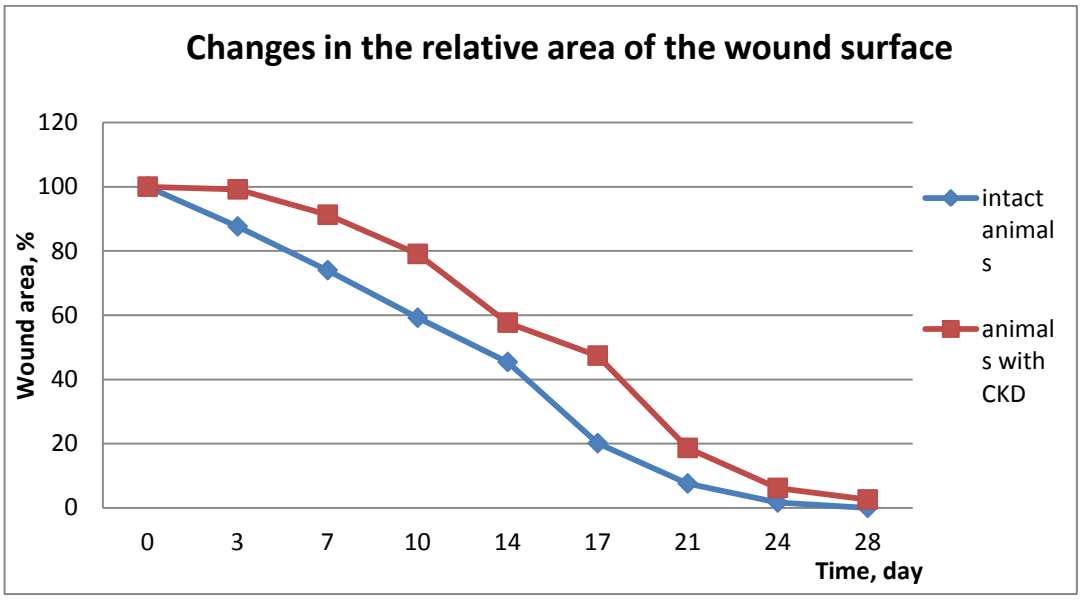

Figure 7. Changes in the relative area of the wound surface

Discussion

Wound healing is a complex, multifactorial process that is realized through the dynamic interaction of cells and regulators. Each of the stages of inflammation is characterized by the predominance of different types of cells in the focus of inflammation that carry out the inflammatory response: leukocytes, macrophages and fibroblasts. In addition, each of the stages of the inflammation process prepares and starts the next stage. In chronic inflammation, the macrophage is the central figure, and therefore the duration of the course of this phase of inflammation is the decisive criterion for the transition of acute inflammation to chronic [14].

The results of the histological study showed that in the experimental group at all periods of healing, some areas of wounds were simultaneously in different phases of healing, and the transition from one phase to another was disconnected, which indicates the transition of the normal wound healing process to chronic [15]. Thus, in the experimental group, on day 7, the cellular component was represented mainly by cells of the inflammatory series and individual fibroblasts, the formation of the capillary system sharply lagged behind the control (Figure 2). These data indicate an increase in the time course of the phases of inflammation, as well as the imposition of phases on each other. It can be assumed that the cause of impaired healing and chronization of the wound was a change in the regulation of the expression of inflammatory mediators at various stages of the healing process. The release of cytokines (interleukin-1 (IL-1), interleukin-6 (IL-6), tumor necrosis factor (TNF-α)) and growth factors (platelet growth factor (TGF), transforming growth factor β (TGF β), epidermal growth factor (EGF), etc.) occurs already at the initial stage of inflammation during platelet degranulation [16], which are key cells participating in the initiation of inflammation [17]. These proteins initiate the process of wound healing by attracting leukocytes, fibroblasts, endothelial cells and macrophages, and also stimulate cell division and collagen synthesis. Leukocytes phagocytic bacteria and degradation products of tissues, destroying them with lysosomal enzymes. Following the leukocytes in the focus of inflammation accumulate macrophages. Macrophages are the most important cells of the inflammation

phase, because in addition to their bactericidal function, they are able to secrete cytokines and growth factors necessary for the proliferative healing phase [18]. Growth factors stimulate the development of the epithelium of the skin and vascular endothelium, collagen synthesis. Granulation tissue develops, in the construction of which fibroblasts play a crucial role. Our study showed a decrease in the amount of collagen produced by fibroblasts in the experimental group on day 7. Collagen fibrils in histopreparations of animals of this group were located chaotically, without signs of their structurization (Figure 2c), in contrast to bundles of collagen fibers of horizontal orientation in the control group (Figure 1a). These data indicate a violation of the functions of fibroblasts, which is characteristic of chronic wounds [19]. Fibroblasts are stimulated by cytokines and growth factors that release neutrophils and macrophages in the early stages of inflammation [20]. These same growth factors trigger the process of angiogenesis, which promotes the passage of fibroblasts into the wound and plays a key role in the formation of granulation tissue [21].

On the 15th and 28th day, wounds in the experimental group were also characterized by a longer phase of inflammation, a decrease in the activity of inflammatory cells and a slowing down of the maturation process of granulation tissue. So, after 15 days, an immature granulation tissue with signs of edema and inflammation was observed in a group of animals with CKD (Figure 4). Focal and diffuse leukocyte infiltration was detected. Unlike tightly packed bundles of collagen fibers in the histopreparations of animals in the control group, the collagen fibers of wounds of CKD rats were stratified and fragmented (Figure 4c). After 28 days, both groups showed complete epithelialization of wounds. However, in the histopreparations of animals with CKD, the epidermis was uneven in thickness, without a clear differentiation of cells into layers. Proliferation of fibroblasts and a large number of newly formed capillaries were observed (Figure 6). During this phase of wound healing, the accumulation of collagen in granulation tissue should reduce the density of blood vessels [22]. However, this is not observed in the wounds of animals with CKD. All these data indicate a slowdown in the process of regeneration and maturation of granulation tissue that fills wounds, which leads to its chronicity.

Measurement of the relative area of the wound surface revealed a slowing down of the wound area reduction process at all stages of healing in animals with CKD compared with intact animals, despite the completion of the wound healing process in both groups (Figure 7). This result confirms our findings on delayed wound healing due to the prolongation of the inflammatory stage and the delayed formation of granulation tissue.

Conclusion

Thus, the results of our study confirm the significant effect of CKD on wound healing. CKD leads to disruption of the normal process of epithelialization of the wound and slowing down the maturation process of granulation tissue in experimental animals due to prolongation of the inflammatory stage.

Further detailed study of the mechanisms of reparation and the factors affecting them will greatly assist in finding ways to treat patients with chronic wound defects and improve the therapeutic prognosis in this group of patients.

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