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ORIGINAL ARTICLES

ORIGINAL ARTICLE

Features of apoptotic and proliferative processes in experimental infected radiation-induced skin ulcer under conditions of photodynamic therapy and the use of platelet-rich plasma

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ABSTRACT

Aim: The purpose of the study was to identify the features of apoptotic and proliferative processes in experimental Staphylococcus aureus-infected radiation skin ulcer under conditions of photodynamic therapy and the use of platelet-rich plasma.

Materials and Methods: An experimental study was conducted on 95 six-month-old male rats of the WAG population, which were divided into three groups. Group 1 included 25 animals that were simulated a radiation ulcer of the skin in the thigh area with subsequent application to its surface on the 7th day after irradiation with 0.2 ml of a suspension of the Staphylococcus aureus (ATCC 25923) reference strain (0.5 million microbial cells/cm^2). Group 2 included 25 animals with Staphylococcus aureus-infected radiation skin ulcer, which were subjected to photodynamic therapy a day after infection. Group 3 included 45 animals with Staphylococcus aureus-infected radiation skin ulcers, which, 1 day after infection, received photodynamic therapy in the first half of the day, and in the second half of the day the periphery of the wound defect was injected with platelet-rich plasma. The material for the study was skin with underlying soft tissues from the area of radiation exposure. Histological, immunohistochemical, morphometric and statistical methods were used.

Results: In cases of simultaneous use of photodynamic therapy and platelet-rich plasma, compared with photodynamic therapy alone, the processes of apoptosis and proliferation were more balanced, active, with a shift in the proliferative-apoptotic ratio towards proliferation processes and met the needs of the regenerative process. From the 10th to the 22nd day of the experiment these processes increased, which indicated active healing processes, that, during survey microscopy on the 22nd day, were manifested by the complete filling of the wound cavity with granulation and connective tissues with the presence of an epithelial layer on the surface of the regenerate. From the 22nd to the 45th day of the experiment, a decrease in the rate of regeneration was recorded, as evidenced by a decrease in the intensity of apoptotic and proliferative processes. The intensity of the latter was sufficient, which led to the healing of Staphylococcus aureus-infected radiation skin ulcer on the 45th day with complete restoration of the original structure of the skin.

Conclusions: Photodynamic therapy in combination with the use of platelet-rich plasma balancedly activates apoptotic and proliferative processes with a predominance of the latter in granulation and connective tissues filling the lumen of Staphylococcus aureus-infected radiation skin ulcer, which on the 45th day of the experiment leads to wound healing with complete restoration of the original structure of the skin.

KEY WORDS: apoptosis, proliferation, experiment, infected radiation skin ulcer, photodynamic therapy, platelet-rich plasma

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INTRODUCTION

Skin is a superficial and the largest organ in the human body that functions as both a barrier and a defender. It is the first tissue to be damaged [1, 2]. One of the factors affecting the skin may be radiation. The main causes of radiation-induced skin injuries include nuclear radiation accidents, occupational exposure, and tumor radiotherapy [3].

Radiotherapy is an important treatment method of patients with malignant tumors which has a killing effect on tumor cells and a powerful destructive effect on normal tissue cells in the irradiation field [3, 4]. It has been reported that 85-95% of cancer patients with radiotherapy still developed radiation-induced skin injuries [2], which may be acute and chronic [3]. Acute skin injuries (dry and wet desquamation, skin necrosis, ulcers, as well as bleeding) develop hours to weeks after the first exposure to the radiation. Chronic skin injuries (chronic ulcers, radiation-induced keratosis, telangiectasias, fibrosis, as well as skin cancer) may develop months, years or even decades after radiotherapy [5].

Radiation-induced skin injuries were classified into four grades according to Radiation Therapy Oncology Group where the ulceration is included in grade four [5]. Skin ulcers are a common and severe radiation-induced skin injury which are seriously impact quality of life, last for several months to years and are characterized by abnormal and delayed wound healing due to imbalanced

inflammatory response, oxidative stress response, lack of angiogenesis, high risk of bacterial infection etc. [4, 6].

The search for methods for treating radiation skin ulcers is a pressing issue today that requires complex clinical and experimental studies. Our previous experimental studies have proven that photodynamic therapy in combination with the use of platelet-rich plasma stimulated the healing process of radiation skin ulcers [7]. The processes of apoptosis and proliferation play an important role in wound healing [8]. At present, the processes of apoptosis and proliferation in an infected radiation-induced skin ulcer under conditions of simultaneous photodynamic therapy and the use of platelet-rich plasma remain unstudied which makes this research relevant.

AIM

The purpose of the study was to identify the features of apoptotic and proliferative processes in experimental Staphylococcus aureus-infected radiation skin ulcer under conditions of photodynamic therapy and the use of platelet-rich plasma.

MATERIALS AND METHODS

An experimental study was conducted on 95 six-month-old male rats of the WAG population, which were divided into three groups.

Group 1 included 25 animals that were simulated a radiation ulcer of the skin in the thigh area with subsequent application to its surface on the 7th day after irradiation with 0.2 ml of a suspension of the Staphylococcus aureus (ATCC 25923) reference strain (0.5 million microbial cells/cm^2). Group 2 included 25 animals with Staphylococcus aureus-infected radiation skin ulcer, which were subjected to photodynamic therapy a day after infection. Group 3 included 45 animals with Staphylococcus aureus-infected radiation skin ulcers, which, 1 day after infection, received photodynamic therapy in the first half of the day, and in the second half of the day the periphery of the wound defect was injected with platelet-rich plasma.

Radiation ulcer modeling was performed using the TUR-60 x-ray therapy device. The irradiation conditions were as follows: voltage – 50 kV, anode current – 10 mA, filter – 0.6 mm A1, dose rate – 33.5 Gy/min.

In groups 2 and 3, photodynamic therapy was performed using a photon device "Barva-LED/630" with a photosensitizer of 0.1% methylene blue solution. Energy exposure per session was 45 J/cm^3. The course of photodynamic therapy consisted of two sessions with an interval of 3 days [9].

In group 3, platelet-rich plasma was received according to the method of R. Dhurat et al. [10].

Animals of groups 1 and 2 were removed from the experiment on days 14, 21, 30, 37, 52 after irradiation (5 rats for each experimental period). Animals of group 3 were removed from the experiment on days 10, 13, 16, 19, 22, 25, 28, 31, 45 (5 rats for each experimental period).

The material for the study was skin with underlying soft tissues from the area of radiation exposure. The material was fixed in a 10% formalin solution. Compaction of tissues fixed in formalin was achieved by passing through alcohols of increasing concentrations, Nikiforov's liquid (96% alcohol and diethyl ether in a ratio of 1:1), chloroform and embedding in paraffin. Serial sections with a thickness of $4\text{-}5\times10^{-6}$ m were made from the prepared blocks for subsequent staining.

Immunohistochemical studies were carried out in accordance with the standardized protocols using rabbit monoclonal antibodies to the apoptosis marker p53 (clone SP5) and the proliferation marker Ki-67 (clone SP6). Monoclonal antibodies were manufactured by Thermo Fisher Scientific (USA). Primary antibodies were visualized using an UltraVision Quanto HRP detection system (Thermo Fisher Scientific, USA). Examination of the microslides was carried out using a laboratory microscope ZEISS Primostar 3 (Carl Zeiss, Germany) with a built-in color digital camera.

In a microscope field of view ×400, the absolute number of cells expressing p53 and Ki-67 was calculated. The immunohistochemical reaction was assessed in granulation and connective tissues located in the cavity of the wound defect.

The indicators were processed statistically using the PAST program (version 4.15, Natural History Museum, University of Oslo, Norway). Mean values of indicators in groups were compared using the Student's t-test and Mann-Whitney U-test. Differences were considered significant at $p<0.05$.

RESULTS

In group 1, on the 30th day of the experiment, survey microscopy revealed granulation tissue in the wound cavity. Monoclonal antibodies to p53 and Ki-67 in granulation tissue were expressed by the cells of fibroblastic differon, immune cells and vascular endotheliocytes. Calculations of the absolute number of immunopositive cells, the results of which are shown in Table 1, revealed the predominance of apoptotic processes over proliferative ones.

On the 37th day of the experiment, in the granulation tissue from the regenerate, cellular elements showed apoptotic and proliferative potential similar to those on the 30th day, and the processes of apoptosis also prevailed over the processes of proliferation. Compared to the previous experimental period, in these animals the absolute number of p53-positive cells increased ($p<0.05$), and the absolute number of Ki-67-positive cells did not change ($p>0.05$).

On day 52 of the experiment, granulation tissue was also detected in the wound cavity, in which features of the expression of monoclonal antibodies to p53 and Ki-67, similar to days 37, were revealed (Fig. 1). Compared to the previous period of the experiment, the absolute number of Ki-67-positive cells did not change ($p>0.05$), while the absolute number of p53-positive cells increased ($p<0.05$). In these animals, the proliferative-apoptotic

Table 1. Absolute number of p53- and Ki-67-positive cells in granulation and connective tissues from the regenerate in groups 1 and 2

Group	Indicator	Experimental period, days				
		14	21	30	37	52
Group 1	Absolute number of p53-positive cells	-	-	15.7±0.6	18.6±0.7 [2]	19.3±0.8 [2]
	Absolute number of Ki-67-positive cells	-	-	5.5±0.7 [1]	5.9±0.6 [1]	6.4±0.6 [1]
Group 2	Absolute number of p53-positive cells	5.6±0.7	6.4±0.9 [2]	7.5±0.7 [2,3]	8.6±0.9 [2,3]	9.4±0.5 [2,3]
	Absolute number of Ki-67-positive cells	10.7±0.5 [1]	11.4±0.8 [1,2]	13.9±1.2 [1,2,3]	15.4±0.8 [1,2,3]	18.2±0.6 [1,2,3]

Note: [1] – significance of differences compared to the absolute number of p53-positive cells; [2] – significance of differences compared to the indicator of the previous experimental period; [3] – significance of differences compared to the indicator of group 1.

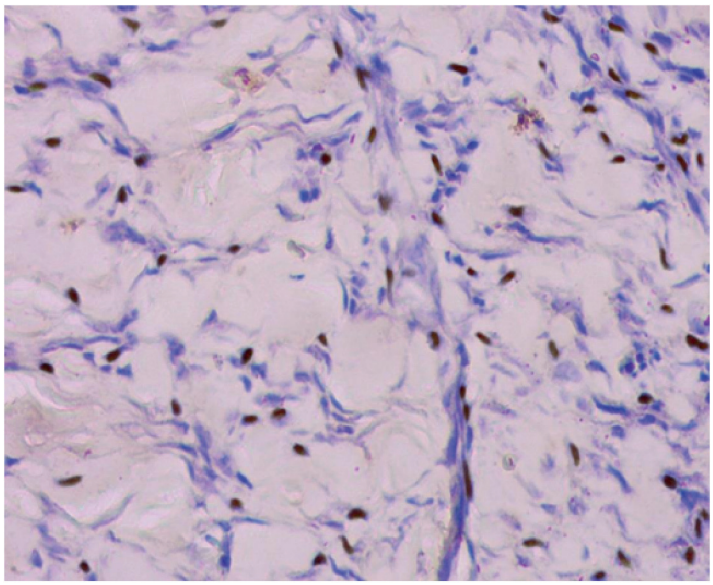

Fig. 1. Expression of p53 by cellular elements of granulation tissue. Immunohistochemical reaction with monoclonal antibody to p53, × 400.

ratio in granulation tissue also shifted towards apoptosis.

In group 2, during survey microscopy on the 14th day of the experiment, granulation tissue was noted in the wound cavity, in which the cells of fibroblastic differon, immune cells, and vascular endothelial cells expressed monoclonal antibodies to p53 and Ki-67. An analysis of the absolute number of p53- and Ki-67-positive cells revealed a shift in the proliferative-apoptotic ratio towards proliferation processes (Table 1).

On days 21 and 30, granulation tissue was also determined in the wound cavity, in which features of the expression of monoclonal antibodies to p53 and Ki-67, similar to days 14, were revealed. In the wound cavity, positive expression of monoclonal antibodies to Ki-67 and p53 was detected on the 37th day of the experiment in the granulation and connective tissues, and on the 52nd day – in the connective tissue. In the latter, cells of fibroblastic differon, immune cells, and vascular endothelial cells expressed markers of apoptosis and proliferation. In these animals, proliferation processes prevailed over apoptotic processes.

In group 2, from 14 to 52 days, the absolute number of p53- and Ki-67-positive cells increased ($p<0.05$), which indicated an increase in the apoptotic and proliferative potential of the cellular elements of granulation and connective tissues of the regenerate.

An intergroup comparative analysis of the indicators revealed that in group 2 compared to group 1, the absolute number of p53-positive cells was lower ($p<0.05$), and the absolute number of Ki-67-positive cells was greater ($p<0.05$) (Table 1).

In group 3, from 10 to 22 days of the experiment, granulation and connective tissues were determined in the regenerate filling the wound cavity, and from 25 to 45 days, only connective tissue. In this group, the proliferation marker Ki-67 and the apoptosis marker p53 expressed

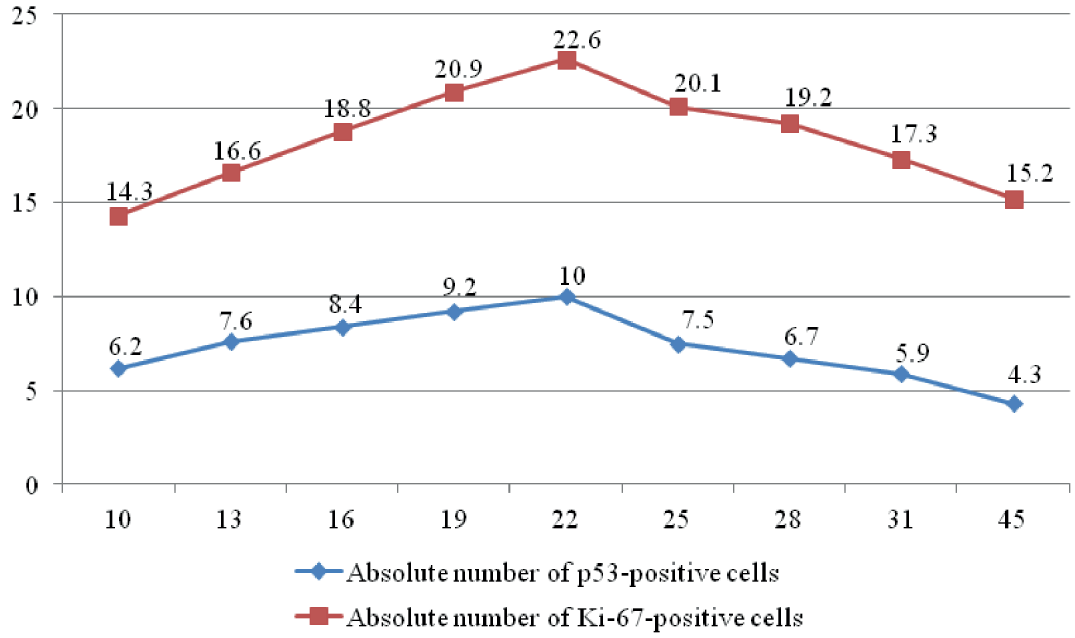

Fig. 2. Absolute number of p53- and Ki-67-positive cells in granulation and connective tissues from the regenerate in group 3.

cellular elements similar to groups 1 and 2. The absolute number of p53-positive cells was lower ($p<0.05$) compared to the absolute number of Ki-67-positive cells (Fig. 2). The absolute number of Ki-67- and p53-positive cells increased ($p<0.05$) from days 10 to 22, which was due to the active pace of regenerative processes, and from days 22 to 45 the indicators decreased ($p<0.05$) due to the reduction in the rate of regeneration and organotypic wound healing.

Analyzing the dynamics of apoptotic and proliferative processes in groups 2 and 3, it was noted that in group 2 the activity of these processes increased from 14 to 52 days of the experiment. In group 3, apoptotic and proliferative processes increased from 10 to 22 days of the experiment, and from 22 to 45 days their intensity decreased. In group 3 compared to group 2, apoptotic and proliferative processes were more active and balanced.

DISCUSSION

Exposure of cells to ionizing radiation results in a number of complex responses, including the activation of apoptosis and proliferative processes inhibition which could be one of the reasons of abnormal or delayed healing of radiation-induced skin ulcer [11]. Apoptosis is a major route of radiation-induced cell death [12]. Apoptosis can occur through two main pathways: the extrinsic pathway (death receptor-mediated) and the intrinsic pathway (mitochondria-mediated) [13].

In this study, it was detected the expression of monoclonal antibodies to the proliferation marker Ki-67 and apoptosis marker p53 by the cells of fibroblastic differon, immune cells and vascular endotheliocytes in granulation tissue from the regenerate filling the cavity of Staphylococcus aureus-infected radiation skin ulcer. The authors revealed the predominance of apoptosis processes over proliferation processes at all experiment periods; increased apoptotic activity and unchanged activity of proliferative processes in the direction from 30 to 52 days of the experiment. Inhibition of the proliferative potential of cells against the background of activation of their apoptotic activity takes place, according to many scientists, in the morphogenesis of erosive and ulcerative processes of various genesis and localization [13, 14].

Under the conditions of photodynamic therapy in granulation and connective tissues from the regenerate filling the cavity of Staphylococcus aureus-infected radiation skin ulcer, firstly, proliferation processes dominated over apoptosis processes, and secondly, apoptotic and proliferative activity increased in the direction from 14 to 52 days. On the 52nd day of the experiment, these processes led to complete healing of Staphylococcus aureus-infected radiation skin ulcer with the formation of a pathological skin scar, which we noted in a previously published article [7]. It is well known fact that balanced activation of the processes of apoptosis and proliferation is one of the important conditions for ulcer healing [15]. In the literature, it was noted that photodynamic therapy killed bacterial cells and balancedly stimulated apoptosis and proliferation [16].

In cases of simultaneous use of photodynamic therapy and platelet-rich plasma, compared with photodynamic therapy alone, the processes of apoptosis and proliferation were more balanced, active, with a shift in the proliferative-apoptotic ratio towards proliferation processes and met the needs of the regenerative process. It is interesting that from the 10th to the 22nd day of the experiment these processes

increased, which indicated active healing processes, that, during survey microscopy on the 22nd day, were manifested by the complete filling of the wound cavity with granulation and connective tissues with the presence of an epithelial layer on the surface of the regenerate [7]. From the 22nd to the 45th day of the experiment, a decrease in the rate of regeneration was recorded, as evidenced by a decrease in the intensity of apoptotic and proliferative processes. The intensity of the latter was sufficient, which led to the healing of Staphylococcus aureus-infected radiation skin ulcer on the 45th day with complete restoration of the original structure of the skin [7].

The positive effects of the complex treatment, which we previously called photometabolic therapy, were due not only to the photodynamic therapy, but also to the use of platelet-rich plasma [7]. Platelet-rich plasma (PRP) is an autologous serum prepared from whole blood by centrifugation, containing high concentrations of platelets, growth factors, and cytokines, which can promote cell regeneration and tissue remodeling [17]. Many scientists have found that platelet-rich plasma influenced the processes of apoptosis and proliferation at various stages of wound healing, inhibiting apoptosis and stimulating proliferation [18].

CONCLUSIONS

Photodynamic therapy in combination with the use of platelet-rich plasma balancedly activates apoptotic and proliferative processes with a predominance of the latter in granulation and connective tissues filling the lumen of Staphylococcus aureus-infected radiation skin ulcer, which on the 45th day of the experiment leads to wound healing with complete restoration of the original structure of the skin.

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CONFLICT OF INTEREST
The Authors declare no conflict of interest

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