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Original article

Possibilities of correcting iatrogenic mucositis with cyanides in experiment

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Abstract: *Background* — Radiation and chemotherapy of cancer cause complications that drastically reduce the quality of life. This requires the search for effective therapeutic agents for the mucositis treatment. We investigated the effects of amygdalin as a trophic external agent for post-radiation and post-chemotherapeutic oral mucositides.
The study objectives were to conduct an immunomorphological analysis using an experimental model of iatrogenic mucositis, and to evaluate the effectiveness of amygdalin as cyanide with probable protective chemical properties in mucositis correction.
Material and Methods — Our studies were performed on 40 male white rats. Radiation therapy was simulated by irradiating animals with a cranial dose of 6 Gy. Then an intraperitoneal injection of cisplatin was performed. On day 7, a biopsy was taken (model control), and animals were treated for 14 days with a mixture of 0.02% nitrofurazone, Desensitin® gel, Suprasorb® and amygdalin, after which a biopsy was taken again (therapy control).
Results — Immunomorphological studies revealed dystrophic structural changes due to the progress of tissue hypoxia and the launch of Fas-dependent apoptosis in tissues. Using treatment with amygdalin by activating hypoxia-inducible factor (HIF) stimulates the macrophage population to remodel the stroma of the submucosal layer. In addition to activating the cellular components of local immunity, a therapeutic anti-apoptotic effect has been established.
Conclusion — The method of mucositis correction by amygdalin is effective, which is confirmed by increased proliferation and decreased apoptosis due to revascularization and hypoxia reduction.

Keywords: oral mucosa, treatment of iatrogenic pathology, chemotherapy, radiation therapy, morphology.

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Introduction

The relevance of iatrogenesis pathogenetic treatment in oncotherapy is extremely high. Currently, oncological pathology dominates in the structure of serious noninfectious illnesses [1, 2]. In 2019, in Russia, 9287 new cases of oral cancer were registered, the average age of sick subjects was 61 years, while the standardized incidence of the oral cancer per 100 thousand people was 6.65 cases among men, and 1.99 among women. Malignant neoplasms of oral mucosa are represented in 97% of cases by squamous cell carcinoma, less often adenocarcinoma (of smaller salivary glands) and sarcomas [3]. The pandemic, crisis, recession, and restrictive regimen indirectly create a negative background for early detection of cancer patients. With the accentuated support of oncological programs, in a socially problematic period, often for irrational reasons, cancer patients do not seek help in the early stages of the disease [3]. In the context of the developing treatment approaches with staged personification, conventional chemotherapy and radiation therapy remain important. At the same time, the tasks of studying therapeutic pathomorphosis and morphological assessment of complications [1, 4], and pathogenetic substantiation of their correction remain relevant.

Due to the side effects of chemotherapy and radiation exposure in the treatment of oral cancer, complications are possible, leading to oral mucositis [5, 6]. The incidence of oral complications during orofacial radiation therapy is 100% [7]. Both chemotherapy and radiation therapy of the primary focus (or metastatic disease) cause complications that drastically reduce the quality of life; their treatment could be a burden on the staff of the palliative care facility, on the patient and the family. This causes the need of searching affordable, convenient means of treating mucositis, and of identifying pathogenetic approaches to assessing their effectiveness [2-4]. Correction of iatrogenic mucositides has been the subject of many studies. The scientific novelty of our study is that we explored the effects of the pharmaceutical drug amygdalin [9] as an external trophic agent in postradiation and postchemotherapeutic oral mucositides. The choice of this preparation is explained by the fact that, based on contradicting opinions on amygdalin use for cancer treatment [10, 11], its biological properties for usage in combination with other compounds, in order to level the consequences of radiation therapy, are of great interest. There have been attempts to use cyanide for targeted drug delivery [12], but the properties of the

compound itself, which at first glance is toxic, may serve the purpose of repairing cells damaged by radiation.

The use of immunohistochemical methods is extremely convenient for assessing the state of cells after exposure to endogenous and exogenous factors, since it shows the location of phenotypic protein markers – signaling molecules in the cell and tissue. We have identified key indicators in assessing the proliferative and reparative status of the tissue. Determining the expression of markers p53, Bcl-2, and CD95 (Fas) made it possible to estimate the number of cells with oncogenic damage and ready for apoptosis, and to indirectly approximate the extent of damage. The Ki-67 marker is expressed in cells in a phase other than G0, thus indicating in our study the activity of regenerative processes. The number of cells, positive for the cluster of differentiation CD68, could imply the activity of the immune system; while the expression of the hypoxia-inducible factor (HIF) indicated to us a deficiency of oxygen in cells, which was necessary to assess the activity of the experimental pharmaceutical drug.

Table 1. Antibodies for IHC reaction, method of expression assessment, and purpose of administration

Antibody, clone, dilution	*Manufacturer*	*Purpose of administration*	*Method for assessing staining in a light field*
Ki-67 (RTU)	Leica Biosystem, Germany	Mitotic proliferative activity of the epithelium	At ×400 magnification, the absolute number of immunopositive cells was calculated in 10 fields of view, and the average values were computed
FAS-R (marker CD 95)	Leica Biosystem, Germany	Caspase pathway of apoptosis activation	
Bcl-2 (apoptosis regulator NCL-L-BCL-2)	Leica Biosystem, Germany	Apoptosis inhibitor protein, anti-apoptotic factor	
p53 – (NCL-L-p53-DO7) dilution 1:800	Novocastra, UK	Tumor suppressor gene protein	
CD 68 (NCL-L-CD68), dilution1:100	Novocastra, UK	Inflammation intensity, macrophage marker	
HIF-1alpha, AC-0108RUO HIF-1alpha EP118 0,1 dilution 1:200	Epitomics, USA	HIF-1alpha – hypoxia-inducible factor; potentiates regeneration	Semiquantitatively 0 – absent, or there is weak / moderate amount (less than 25% of cells); 1+ – low-intensity staining of over 25% of cells, or strong staining of less than 25% of cells; 2+ – 25-75% of cells with medium intensity staining, or 25-50% of cells with high intensity staining; 3+ – over 75% of cells are medium-intensive, or over 50% of cells are intensely stained

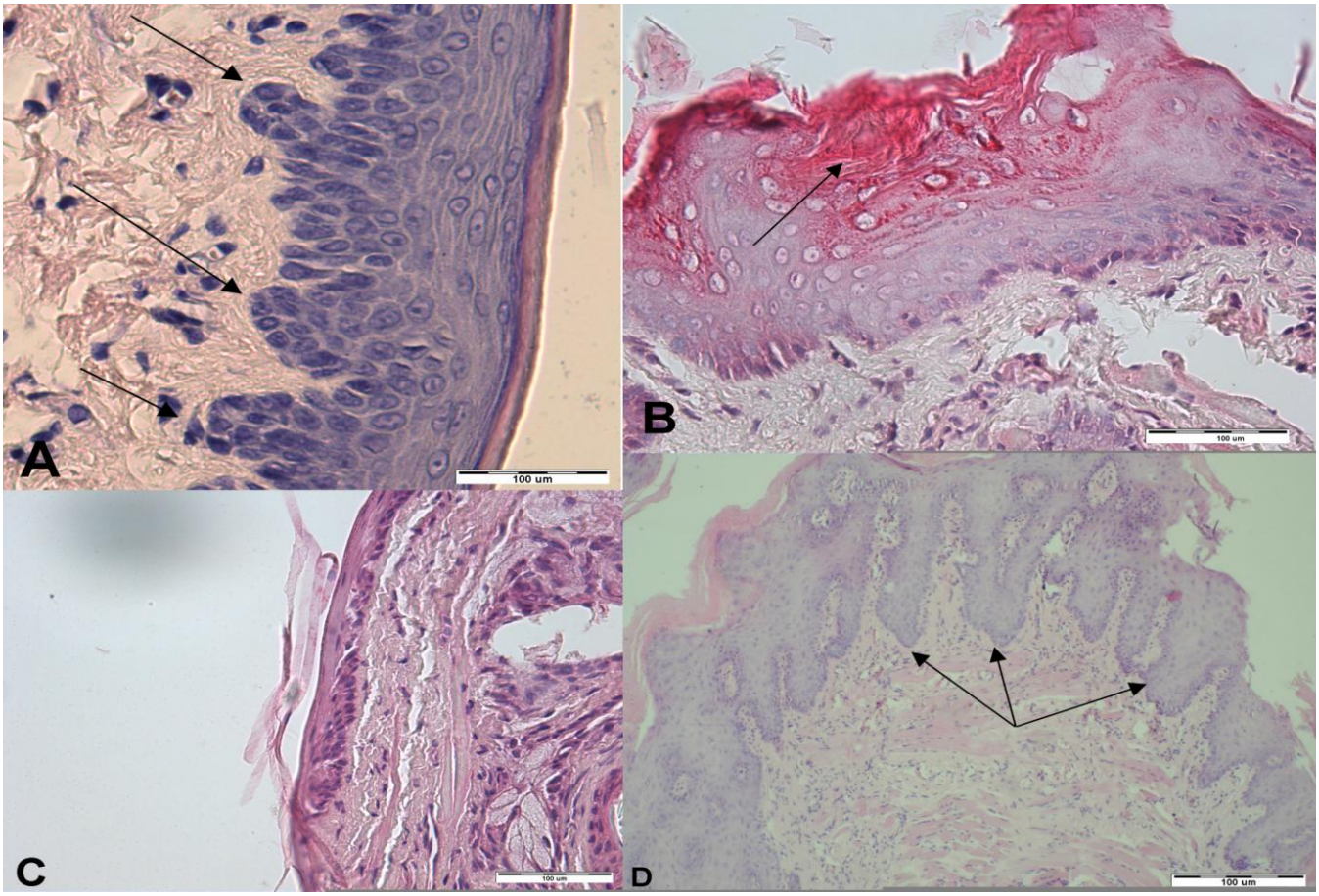

Figure 1. Fragments of the buccal mucosa in a male white rat. Staining with hematoxylin and eosin. A – Control, norm. Magnification ×400. Stratified epithelium, papillae (arrows). B, C – Conventional therapy. Magnification ×400. Tissue defect (arrow), flattening of the basement membrane. Tissue separation, thinning, lack of papillae. D – Treatment with amygdalin. Magnification ×100. Normal structure, acanthosis (arrows).

Table 2. Quantitative indicators of CD68+ cells in experimental and control groups (the average number of positively stained cells in the field of view)

Group	*CD68⁺ M±m*
Control (n=10)	1.89±0.01
Radiation (n=10)	2.13±0.01
Radiation followed by therapy (n=20)	6.27±0.05# (p=0.023)

– intergroup differences among experimental groups, p<0.05. Hereinafter, M – the sample mean for the group; m – a standardized error of the mean; n – the sample size.

Table 3. Quantitative indicators of Ki-67+ cells in experimental and control groups (the average number of positively stained cells in the field of view)

Group	*Ki-67⁺ M±m*
Control (n=10)	3.12±0.01
Radiation (n=10)	11.01±0.11*(p=0.003)
Radiation with subsequent therapy (n=20)	48.31±1.03*# (*p=0.003. #p=0.002)

* p<0.05 – difference with control group; # p<0.05 – the difference among experimental groups.

Table 4. Intensity of HIF-1alpha expression in experimental and control groups (average indicators of expression intensity, points)

Croup	*HIF-1alpha M±m*
Control (n=10)	0.87±0.01
Radiation (n=10)	1.01±0.03
Radiation with subsequent therapy (n=20)	2.12±0.03* (p=0.068)

* p<0.05 – difference with control group.

Table 5. Quantitative indicators of cell apoptosis activity in experimental and control groups (the average number of positively stained cells in the field of view)

Group	*p53 M±m*	*Fas M±m*	*bcl2 M±m*
Control (n=10)	0	0.11±0.01	0.16±0.01
Radiation (n=10)	0	17.32±1.35* (p=0.009)	3.14±0.03* (p=0.005)
Radiation with subsequent therapy (n=20)	0	2.98±0.05*# (*p=0.006 #p=0.037)	6.12±0.07*# (*p=0.064 #p=0.006)

* p<0.05 – difference with control group; # p<0.05 – the difference among experimental groups.

The goal of our study was to conduct an immunomorphological, using an experimental model of iatrogenic mucositis, and to evaluate the effectiveness of its correction with amygdalin.

Material and Methods

Study design

We created iatrogenic mucositis model on rats under the conditions of combination therapy of malignant lesions on head and neck. We then assessed the immunomorphology of oral mucosa in conditions of mucositis. Finally, we tested the pathogenetically substantiated correction method, using amygdalin, and verified its morphological and immunohistochemical (IHC) effectiveness.

While working with animals, the principles and norms of ethical treatment were used, and all manipulations were carried out under reversible ether anesthesia. The experimental rats were not euthanized: they completed their ontogeneses in a natural way, being kept in a vivarium.

Explanation of research methods

The biopsy of the buccal mucosa, 0.5×0.5×0.2 cm each, after 18-hour formalin fixation, was impregnated with paraffin, and 4-µm thick cross-sections were made. The IHC reaction was performed on a Bond™-maX device on adhesive glasses with a Bond Polymer Refine Detection System (Leica Biosystem, Germany). A panel of 6 antibodies was used (*Table* 1). The following protocol was used: 4-µm thick cross -sections were dewaxed, subjected to high -temperature unmasking at a temperature of 96°C in a pH=6.4 buffer; endogenous peroxidase was suppressed with 4% hydrogen peroxide at room temperature for 5 minutes; and then incubated with the primary antibody for 30 minutes at room temperature. Evaluation of the expression of markers was carried out on images obtained from a scanner of tissue preparation specimens. Each glass was evaluated in a minimum of 10 fields of view (*Table* 1). The measurement results were presented as M±m, that is a mean value of the number of positively stained cells (or their percentage in the case of HIF-1alpha) ± the standardized error of the mean.

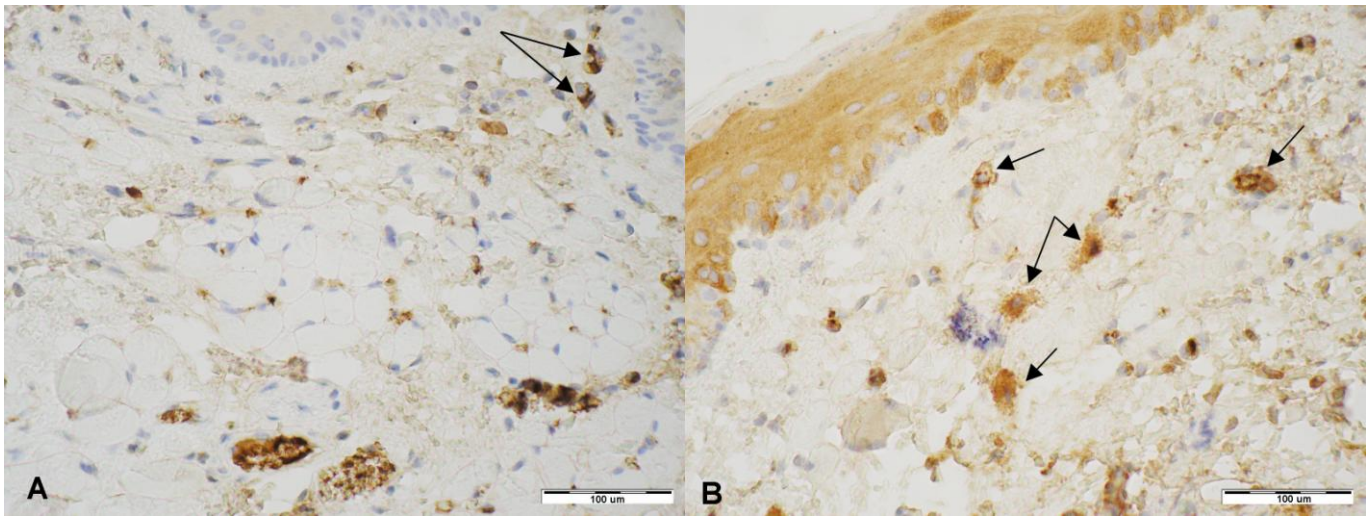

Figure 2. Fragments of the buccal mucosa in a male white rat. IHC with CD68. Magnification ×200. Macrophages with positive cytoplasmic expression in the submucosa (arrows). IHC: A – Conventional therapy. B – Amygdalin therapy.

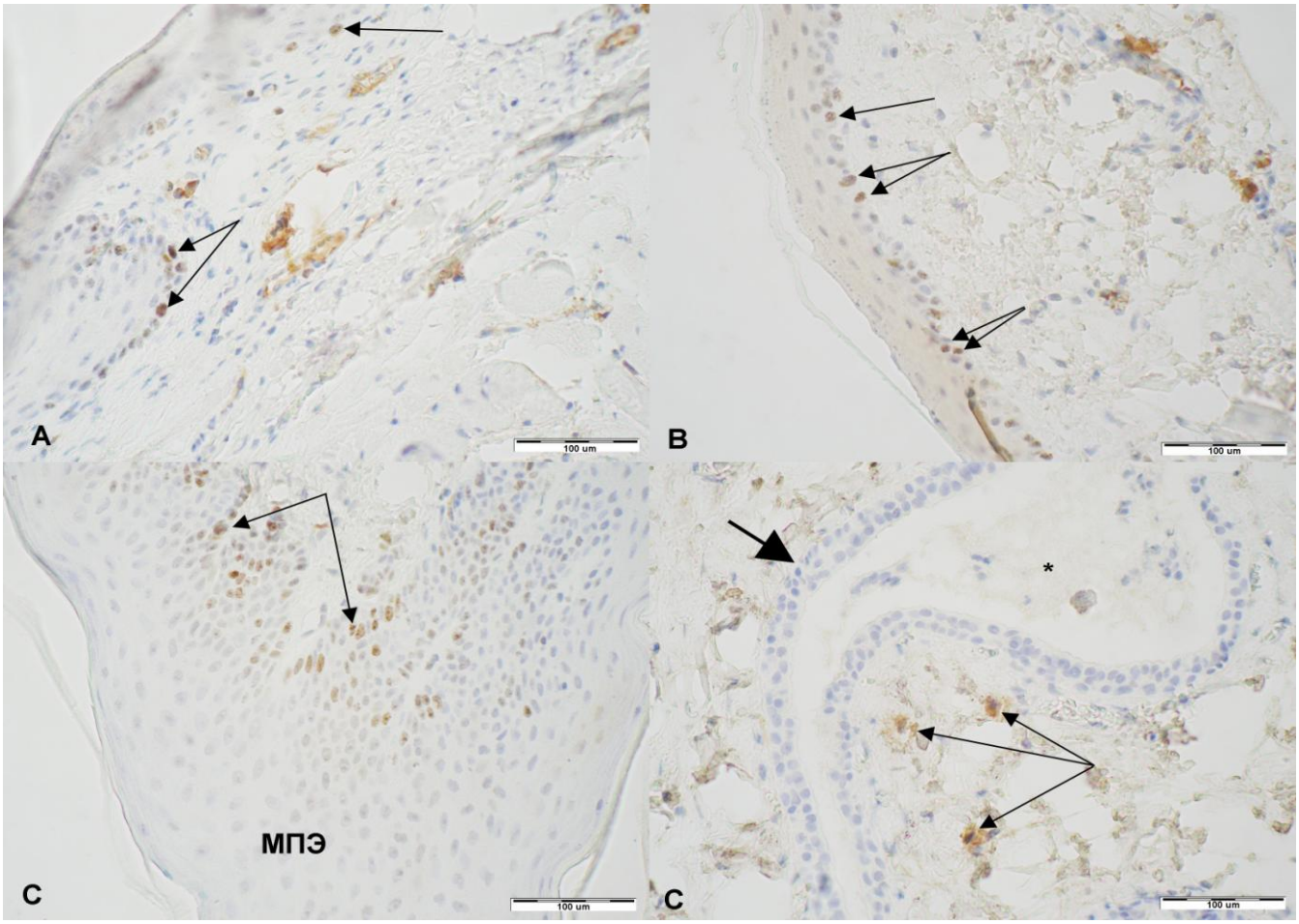

Figure 3. Fragments of the buccal mucosa in a male white rat. IHC Ki-67 expression. Magnification ×200. Nuclear-positive cells (thin arrows). A – Control group. B – Conventional therapy. C – Amygdalin therapy. Epithelium with basal cell hyperplasia and acanthosis. D – Amygdalin therapy. Salivary gland duct (*). Duct epithelium with negative reaction (thick arrow). A positive nuclear reaction in stromal cells.

Statistical data processing

The normal distribution of the trait within the group was assessed using the Shapiro-Wilk method. The identification of differences between the groups in the level of HIF-1alpha expression was performed by the Mann-Whitney method, while in other markers – by the Student's method. Calculations were performed using Microsoft Excel software. Differences were considered significant at 5% probability of an error.

The research was carried out with the financial support of the development program at the Federal Autonomous Institution of Higher Education V.I. Vernadsky Federal University of Crimea. All studies were conducted in a licensed laboratory, using equipment and reagents with clinical approvals, and the HIF-1alpha antibody, properly certified for research purposes (Research Use Only certificate).

Results

Histological examination of the buccal mucosa in experimental animals (*Figure* 1) made it possible to establish the following:

(1) In the control, the connective tissue papillae in the lamina propria of the mucosa penetrated into the epithelial layer by 1/4-1/6 of its thickness, which increased the area of the basement membrane and contributed to trophism;

(2) In the course of the radiation therapy and chemotherapy, against the background of conventional treatment, ulcerative and necrotic damage to the buccal mucosa occurred, epithelium layers were partially flattened and desquamated; the terminal sections of the smaller salivary glands were overfilled; and the connective tissue papillae in the lamina propria were sharply smoothed;

(3) During therapy with amygdalin, the integumentary epithelium did not differ from the control group; the stratification of layers was preserved; moderately expressed parakeratosis and hyperkeratosis were present. Inflammatory infiltration and dystrophic processes were not visualized on the day 14. It is worth noting the formation of multiple acanthotic epithelial extensions one-half of the thickness deep into the submucosal layer in animals with amygdalin application, which indicates the activation of regeneration.

Quantitative analysis of CD68 expression showed no significant intergroup differences between the control group and the conventional therapy group. At the same time, against the background of local therapy with amygdalin, intensification of cells of histiocytic origin was noted (*Figure* 2, *Table* 2). The proliferative activity of cellular elements varied in intergroup analysis. The minimum values were recorded in the control group and were determined in single cells of the basal layer of the integumentary epithelium (*Figure* 3A, *Table* 3).

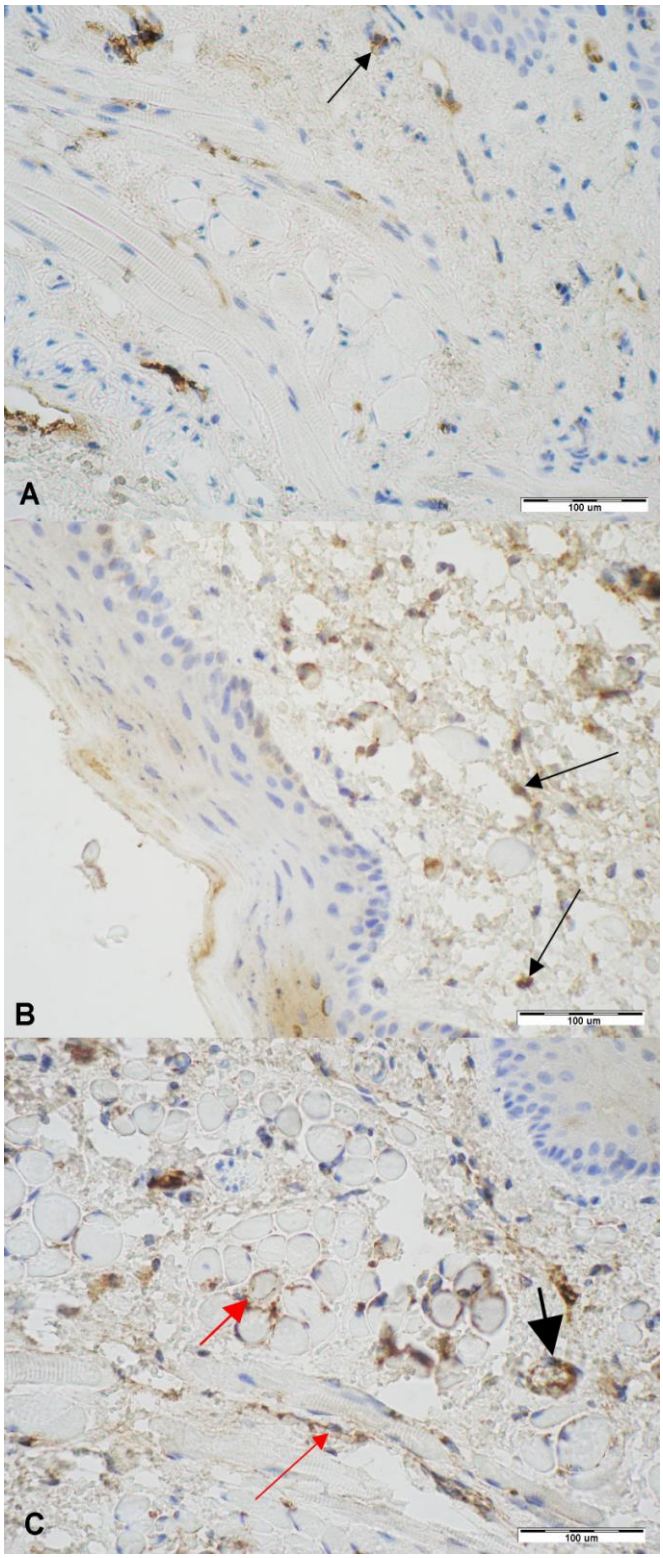

Figure 4. Fragments of the buccal mucosa in a male white rat. IHC. Expression of HIF-1alpha. Magnification ×200. Cells with a positive membrane-cytoplasmic reaction (thin arrows). A – Control group. B – Conventional therapy. C – Amygdalin therapy. Positive expression of capillary vascular endothelium (thick arrows). Myocytes with membrane expression of HIF-1alpha (red arrows).

Fragments of the buccal mucosa after amygdalin therapy exhibited the maximum proliferation of cellular elements. A positive nuclear reaction was observed in almost all basal epithelial cells, especially in the histologically verified foci of basal cell hyperplasia (*Figure* 3C). Subepithelial mitotic activity was detected in cells of fibroblastic and histiocytic populations. Epithelial cells of the salivary glands were characterized by mitotic stability (*Figure* 3D). Micropreparations of cheeks in rats, undergoing conventional therapy, were characterized by intermediate values of proliferation with maximum activity in the basal layer of stratified squamous epithelium, especially in the areas of thinning (*Figure* 3B).

The increase in the number of Ki-67$^+$ stromal cells in the experimental groups with amygdalin, relative to conventional therapy, was comparable with the number of elements expressing HIF-1alpha (*Figure* 4A, B). This fact was an indicator of the intensification of the functional activity in cells of histiocytic origin and indicated the activation of the angiogenesis mechanism in order to normalize oxygen delivery to hypoxic areas. A fundamentally important observation was the intensification of HIF-1alpha expression in animals of the group with amygdalin therapy, against the background of radiation therapy, since it was recorded in the increasing pool of macrophages (as in the group with radiation therapy and conventional therapy), as well as in myocytes, fibroblasts, and vascular endotheliocytes (*Figure* 4C, *Table* 4).

The study of markers of programmed cell death and genetic disorders of the cell cycle enabled us to establish the absence of mutagenic activity in all studied groups, despite the intensification of the processes of cell division and proliferation. IHC reactions with markers Fas and Bcl-2 showed statistically significant activation of cell death processes in all animals of the experimental groups in comparison with the control group. However, in the cases of the group with radiation therapy and conventional therapy, there is a prevalence of cells demonstrating proapoptotic readiness over Bcl-2 positive blockers of apoptosis. Against the background of amygdalin therapy, the opposite trend was identified (*Figure* 5, *Table* 5).

Discussion

Modeling iatrogenic mucositis in rats using the described method is fully effective: it manifests itself in characteristic pathomorphological changes of the buccal mucosa. Immunomorphology of oral mucosa reflects the progression of apoptosis induced by increasing tissue hypoxia. The result of an immunomorphological study showed that radiation therapy of the buccal mucosa in rats formed dystrophic structural changes, caused by progressing tissue hypoxia and by triggering Fas-dependent apoptosis, which leads to ongoing destruction, exudation, formation of erosions and ulcers, and a chronic course. This finding does not contradict the published data [13, 14].

The use of a novel, amygdalin-based topical treatment method via activating HIF stimulates the macrophage population to remodeling of the submucosal stroma by means of launching the processes of angiogenesis. In addition to activating the cellular components of local immunity, a therapeutic antiapoptotic effect has been discovered, coupled with a compensatory activation of the proliferative activity in epithelial and stromal cells without causing mutagenic effect.

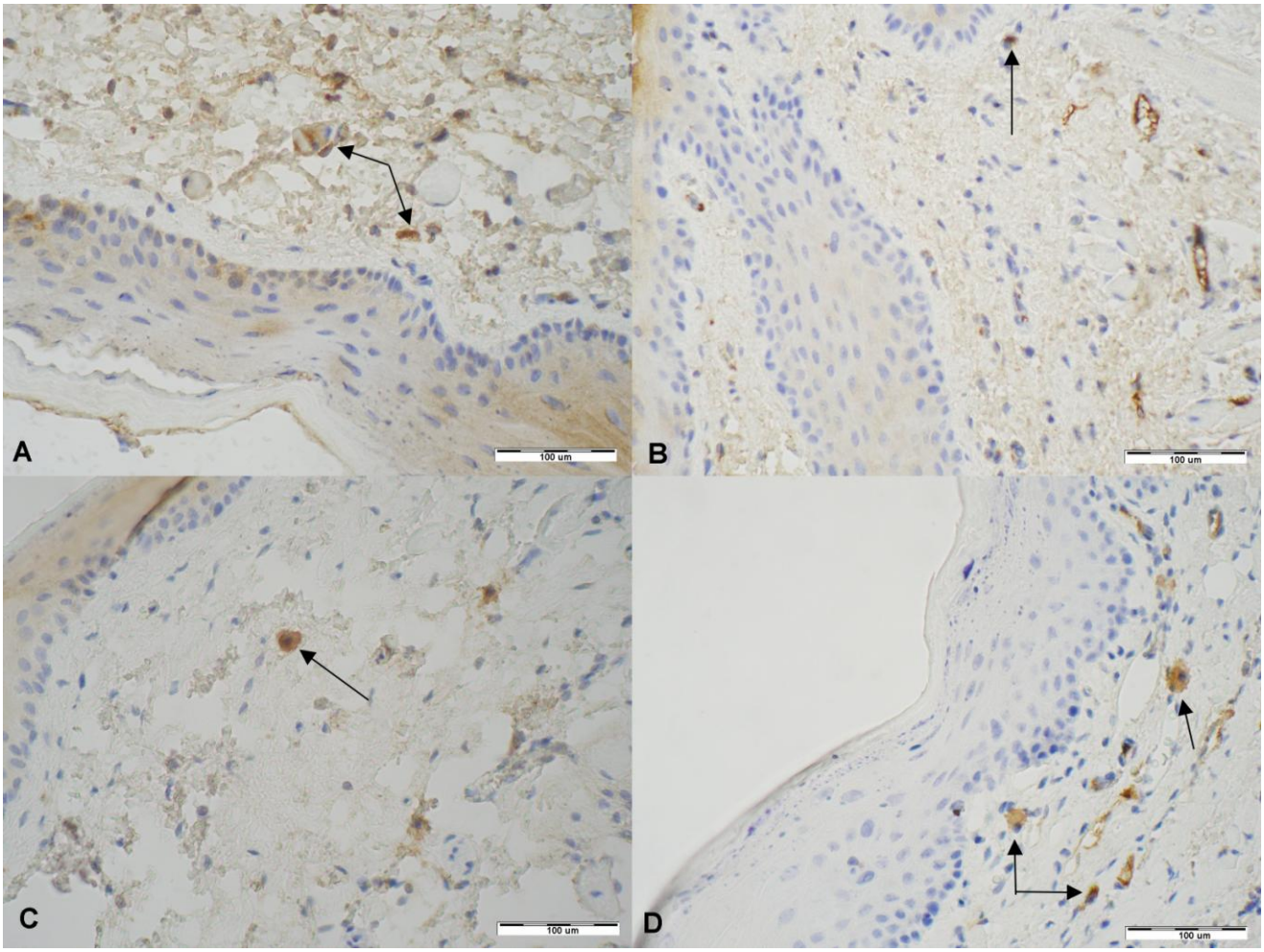

Figure 5. Fragments of the buccal mucosa of a male white rat. IHC. Expression of FAS and Bcl-2 Uv. 200x. Cells with a positive membrane-cytoplasmic reaction (arrows). A – Conventional therapy. Fas. B – Amygdalin therapy. Fas. C – Conventional therapy. Bcl-2. D – Amygdalin therapy. Bcl-2.

As a result of the ionizing radiation effect on live tissues, a number of changes occur inside cells, including physical (energy absorption and ionization of molecules), physicochemical (emergence of active radicals and their interaction with organic molecules), chemical (damage to biologically active molecules), and biological (pathophysiological processes in damaged cells) processes [15]. Oxygen molecules are actively ionized. Among reactive oxygen species (ROS), there are (according to the degree of increase in their activity) superoxide anion radical (O_2*), hydrogen peroxide (H_2O_2), and hydroxyl radical (*OH). Their presence in a healthy body is normal, because they enter the mitochondrial respiratory chain. However, when exposed to exogenous factors of various origins, the amount of ROS can significantly increase and have a destructive effect on biologically active molecules, especially on lipids in membranes of cell organelles [16].

It is well-known that the body contains a number of catalytic enzymes that can inactivate ROS. These include superoxide dismutase (SOD), catalase, and others [17]. At the same time, SOD is a synthesized enzyme, which means that its amount can increase with an increase in ROS. However, as a result of oxygen radiolysis after radiation treatment, antioxidant systems do not have time to cope with the sharply increased amount of ROS, as a result of which the inactive superoxide radical transforms into more active hydrogen peroxide and then into the most active hydroxide ion. This happens especially quickly in the presence of cytochrome oxidase, which contains iron ions. It is acknowledged that cyanide, which is part of amygdalin, blocks cytochrome oxidase by binding to iron cations, and also inhibits catalase [18]. As a result, both the formation of ROS and their transition to the most active phase are slowed down. This permits the cell to adapt the antioxidant system to new conditions and to better preserve the structure of the organelles. The slight oxygen starvation, resulting from these reactions, activates angiogenesis in the damaged tissues.

Conclusion

The method of correcting mucositis with cyanides, using the case-study of amygdalin, is effective, which is confirmed by stroma remodeling, increased proliferation and decreased apoptosis due to revascularization and decreased hypoxia, identified by immunohistochemical methods with quantitative mathematical confirmation.

Conflict of interest

We declare no conflict of interest.

Ethical approval

All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

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