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Pathomorphological and biochemical changes in the oral mucosa in simulated atrophy

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Abstract

Oral mucosa atrophy is a widespread phenomenon both in prosthetics and in the maxillofacial pathology, having apoptosis as a fundamental mechanism. Modeling of atrophy processes will allow to study its pathogenesis and optimize therapeutic measures. The aim of the study was to investigate the development of oral mucosa atrophy in an experimental model. The study was performed on 50 Wistar male rats, undergone 37% orthophosphoric acid oral mucosa applications. After 30 seconds' exposure the defect of the integumentary epithelium was found. 40-second and 45-second exposure additionally provoked desquamation of the stratum corneum. A 50-second application enhanced infiltration, 90- seconds -aroused significant epithelial defects, lymphocytic infiltration of the lamina propria, and fibers' rupture. The expression of the anti-apoptotic marker bcl-2 remained consistently low in all layers of the integumentary epithelium, regardless of the exposure's time. The pro-apoptotic cell surface death receptor was positive (2+) in the basal and spiny layers of the epithelium, and in the intrinsic plate of the mucosa. The proposed model is valid, based on the apoptotic mechanisms and can be used in the study of prevention and treatment of atrophic processes in oral cavity, including caused by complete removable plate prostheses.

Keywords: Apoptosis, Atrophy, Bcl-2 family, Biological modelling, FAS, Oral mucosa, Prosthetic.

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1. Introduction

The cells of the oral mucosa are constantly exposed to various influences, responding to them by changing the processes of cellular adaptation. Adaptation of cells may manifest by variety of processes, such as hyperbiotic events - hypertrophy, hyperplasia, metaplasia, and hypobiotic processes, including cellular atrophy. Atrophy refers to the decrease in the size of cells due to a loss of cellular material. It occurs in the oral epithelium through a process of terminal differentiation, where basal keratinocytes continuously divide, mature, and differentiate into flat squames. These squames then detach from the epithelial surface as keratin debris [1]. It is known that cell death, or apoptosis, can trigger the process of atrophy. Apoptosis is recognized as a programmed cell death, having specific, natural mechanisms purposed to maintain tissue homeostasis and eliminate damaged, senile and abnormally divided cells. The process of self-destruction is initiated by various exogenous and endogenous factors, such as mechanical, physical and chemical damage, as well as hypoxia. In addition, apoptosis occurs during aging, the natural development of organs and tissues, as well as in a number of other physiological processes, such as postpartum uterine involution and mammary gland involution after the end of breastfeeding.

Apoptosis remains the most studied mechanism of atrophy, having two main pathways: the external pathway, or the death receptor (DR) pathway, and the internal, or mitochondrial pathway. The crossing of both pathways is the system of specific aspartyl-cysteine proteases (caspases), which been activated cleave cellular substrates, leading to disruption of the variety of cellular structures and processes, finally reaching cell contraction or formation of apoptotic bodies. The key connection between two pathways of apoptosis is based on caspase-8, which been involved in the external pathway, is able, at the same time, to cleave Bcl-2 interacting domain (BID), a protein of the Bcl-2 family, involved in the internal pathway. Sequently, caspase-8, activating Bcl-2 after a pro-apoptotic stimulation through DR, ultimately, enhancing the apoptotic signal. The key participants in the controlled induction of cell death are DR. Cell surface death receptor (FAS or CD95) is a prototype of DR activated by its sister ligand CD95L, which causes programmed cell death. As a result, changes in the CD95/CD95L pathway are involved in a number of pathological conditions, ranging from autoimmune diseases to inflammation, cancer and atrophy [2]. Alteration in molecular mechanisms involved in apoptotic signaling contributes to a vast range of oral diseases. An understanding of the regulation of apoptosis has led to the development of many therapeutic approaches and better management of oral diseases. Thus, apoptosis was established to be involved in atrophic epithelium in oral submucosal fibrosis [3]. In addition, various types of prosthetics also have a wide range of effects leading to apoptosis-associated disorders of cell maturation, proliferation and regeneration [4]. An imbalance between cell growth and apoptosis can affect the success of oral mucosa repair processes in case of cell damage. The loss of cell connection with nerve fibers deprives them of trophic support, which leads to death. Mucosal regeneration after injury may occur in parallel with a decrease in the number of apoptotic epitheliocytes and an increase in the index of apoptosis in the deep layers of the mucous membrane at later stages after de-afferentation [5]. Atrophy of the oral mucosa is also found in various pathological processes - changes in endocrine and metabolic conditions, such as vitamin B12 and folic acid deficiency [6] as well as during the processes of cellular proliferation disorders in the framework of the consideration of precancerous and oncological processes proper are highlighted by some authors as one of the most important when considering disorders of cellular adaptation. The "common denominator" of various tumor processes is an uncontrolled increase in the number of cells due to apoptosis' disorders [7]. Reduced apoptosis index and altered values of p53, MDM2, bcl-2, bcl-xL, and bax were also found in salivary gland tumors, lymphomas, and maxillofacial sarcomas [8]. Thus, atrophic processes in the oral cavity are a widespread phenomenon.

Currently, there is a rise of interest in studying the pathogenesis of various processes in the oral cavity using alive animal models, cell and tissue cultures. Models of inflammatory processes [9] injury [10, 11] congenital abnormalities [12] the effects of sealing materials [13] widely are used nowadays. Except the animal modelling, monolayer cell cultures and 3D cultures are stated to provide an in vitro model resembling the in vivo situation. Nowadays, four tissue-engineered oral mucosa models are commercially available: The SkinEthic™ Human Oral Epithelium (HOE) and the SkinEthic Human Gingival Epithelium (HGE) constructs from EPISKIN (Lyon, France), and the EpiOral™ and EpiGingival™ tissues from MatTek Corporation (Ashland, MA) [14]. Nonetheless, the commercial 3D models lack the complexity present in *in vivo* systems, as well as immune cells and a vasculature [15]. Also, *in vitro* microphysiological platform and biofabricated full-thickness gingival equivalents (gingiva-on-chip) within a vertically stacked microfluidic device is developed [16]. At the same time, there has been a significant increase in interest in *in vivo* studies due to their effectiveness in drug absorption investigations as well as their low cost for acquiring buccal mucosal samples [17]. The analysis of the last ten years of research has not yielded enough experimental data on apoptotic processes related to oral atrophy. Therefore, the aim of this study is to investigate the development of oral mucosal atrophy using an experimental model.

2. Materials and Methods

2.1. Experimental Animals

2.1.1. Object Of Study was the Modeling of Oral Mucosa Atrophy

The study was performed on 50 white male rats Wistar of the weight of 180-200 g and the age of 10-12 weeks. The animals were kept in standard conditions in accordance with the rules approved by GOST R 53434-2009 on the design, equipment and maintenance of experimental biological clinics (vivarium). *Method of study* was the simulation of mucosal atrophy by the application of 37% orthophosphoric acid to the upper jaw alveolar mucosa by dental applicator. The acid was then washed off with distilled water. The choice of this substance is due to its chemical properties and frequent use in dentistry. Applied 37% orthophosphoric acid has enough grade of acidity, and in contact with human tissues causes necrosis or chemical burns [18].

2.2. Experimental Design

The animals were divided into 7 groups, (n=5 per group).

Group 1: was used as a control and was not exposed.

Group 2: 37% orthophosphoric acid was applied to the mucosa for 30 seconds and then washed out.

Group 3: 37% orthophosphoric acid was applied to the mucosa for 40 seconds and then washed out.

Group 4: 37% orthophosphoric acid was applied to the mucosa for 45 seconds and then washed out.

Group 5: 37% orthophosphoric acid was applied to the mucosa for 50 seconds and then washed out.

Group 6: 37% orthophosphoric acid was applied to the mucosa for 60 seconds and then washed out.

Group 7: 37% orthophosphoric acid was applied to the mucosa for 90 seconds and then washed out.

2.3. Tissue Sampling

The experiment was completed at the 6th day after exposure. Under the influence of local anesthesia, 1 cm of the upper jaw alveolar mucosa samples between the canine and the first premolar were taken by a disposable surgical scalpel (15 S). The samples were placed in cassettes and fixed in 10% formalin solution. Fixed tissue samples were dehydrated and impregnated with formalin in a histoprocessor of the MTP carousel type (Slee, Germany), poured into paraffin blocks and semi-thin sections of 4 microns' thickness were made on a rotary microtome RM2255 (Leica, Germany). The sections were stained with hematoxylin-eosin according to the standard protocol. Immunohistochemical staining was performed in an automatic Bond-Max immunohistostainer according to protocols recommended by the antibody manufacturer.

2.4. Measurement of Apoptotic Markers

The Bond Polymer Refine Detection System (Leica, UK) was used to visualize the markers of antibodies. Antibodies to the anti-apoptotic protein bcl-2 (clone BCL-2/100/D5, Leica, UK, dilution 1:100) and to the pro-apoptotic receptor CD95 (clone 13/Fas, BD Biosciences, USA, dilution 1:100) were used as primary antibodies. BCL-2 expression was evaluated in the cytoplasm of epithelial cells, lymphocytes and vascular endothelium, the number of positively colored cells in the field of view was calculated at 40x. Markers with cytoplasmic localization (bcl-2, CD95) were evaluated using a semi-quantitative method. The following scale was used (Table 1):

Table 1.
Evaluation criteria for IHC markers with cytoplasmic localization.

Staining	Nonexistent	Mild	Moderate	Intense
<25	0	0	0	+
25-50	0	+	++	++
50-75	0	+	++	+++
>75	0	+	+++	+++

2.5. Method of Statistical Analysis

Descriptive statistics were performed using STATISTICA version 10.0 with median calculation and comparison according to the Mann-Whitney criterion. The significance level was set at $p<0.05$.

3. Results

3.1. Histological and Morphological Changes in the Oral Mucosa During Atrophy Modeling

In the control group of animals, the gingival mucosa was observed as a multilayer flat keratinizing epithelium containing a basal, spiny, granular and horny layers (Figure 1A). Lamina propria was revealed as a loose fibrous connective tissue, with blood vessels and small salivary glands laying.

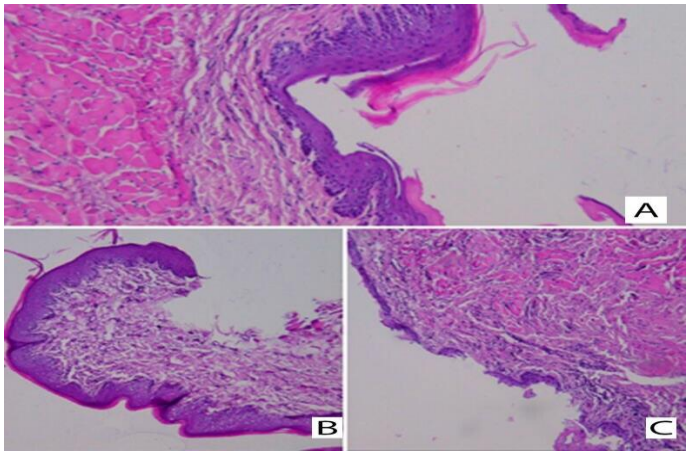

Figure 1.
Fragment of the rat gingival mucosa, stained with hematoxylin and eosin (magnification 10x) A – control group, B – the first experimental group exposure of 30 seconds, C – the second experimental group exposure of 40 seconds, D – the third experimental group exposure of 45 seconds.

In the first experimental group, there was a partial absence of the integumentary epithelium, partial necrosis of lamina propria and moderate lymphocytic infiltration (Figure 1B).

In the second and third experimental groups, the above-mentioned defects were accompanied by desquamation of the stratum corneum in the areas adjacent to the wound (Figure 1 C, D).

In the fourth experimental group, an even more pronounced response from the local immune system was observed, as manifested by a larger area of infiltration into the lamina propria (Figure 2A).

In the fifth experimental group, a remnant of granulation tissue was preserved, along with abundant lymphocytic infiltration and desquamation of the stratum corneum in the adjacent areas of the lamina propria (Figure 2B).

The sixth experimental group showed a significant defect in the integumentary epithelium, accompanied by abundant lymphocytic infiltration of the underlying region of the lamina propria and disorganization of its fibers (Figure 2 C).

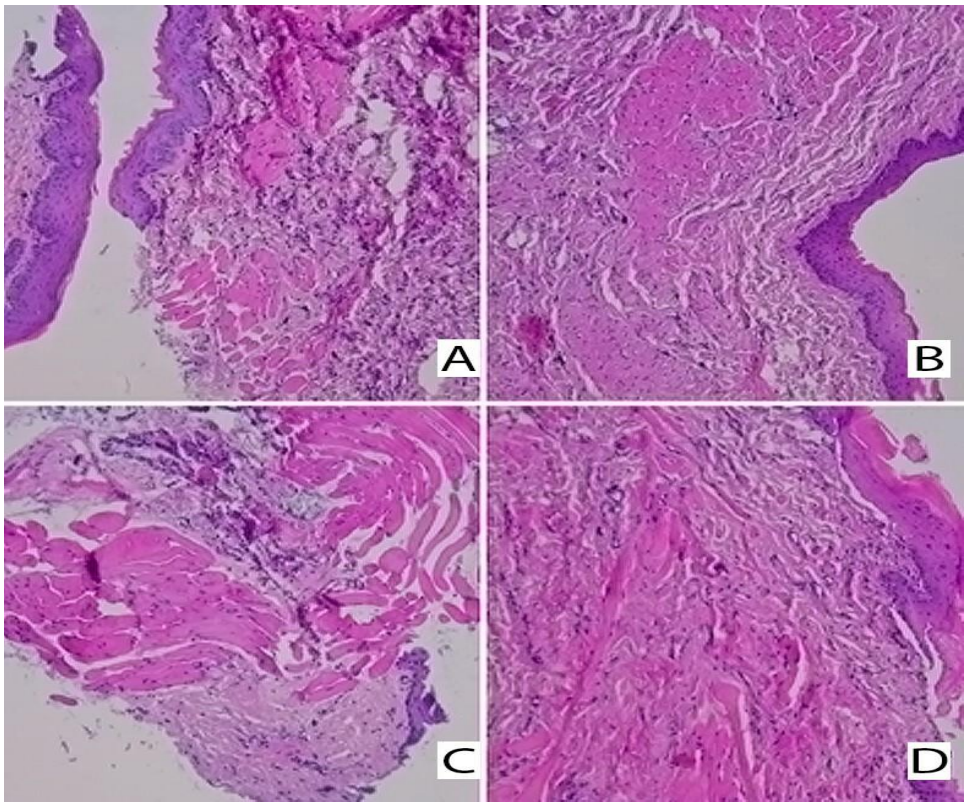

Figure 2.
Fragment of the rat gingival mucosa, stained with hematoxylin and eosin (10x magnification), A – the fourth experimental group of exposure for 50 seconds, B – the fifth experimental group of exposure for 60 seconds, C – the sixth experimental group of exposure for 90 seconds.

3.2. Expression of Apoptotic Markers in Simulated Atrophy

The expression of the anti-apoptotic marker bcl-2 remained consistently low in all layers of the integumentary epithelium, regardless of exposure time. Positive expression was observed only in lymphoid cells in lamina propria and vascular endothelium. In the control group, the number of positive cells averaged 7 cells in the field of view with at 40x magnification, and for the experimental groups this indicator was slightly lower (2-4 cells in the field of view), however, there was no statistical significance in the differences between the groups or depending on the duration of exposure.

The pro-apoptotic FAS receptor (CD95) was found to be moderately positive in the basal and spinous layers of the epithelial cells (2+) in the control group, as well as in the lamina propria of the rats' mucosa. In the experimental groups, expression of FAS was slightly higher in the epithelial cells, without a clear correlation with the duration of exposure (3+).

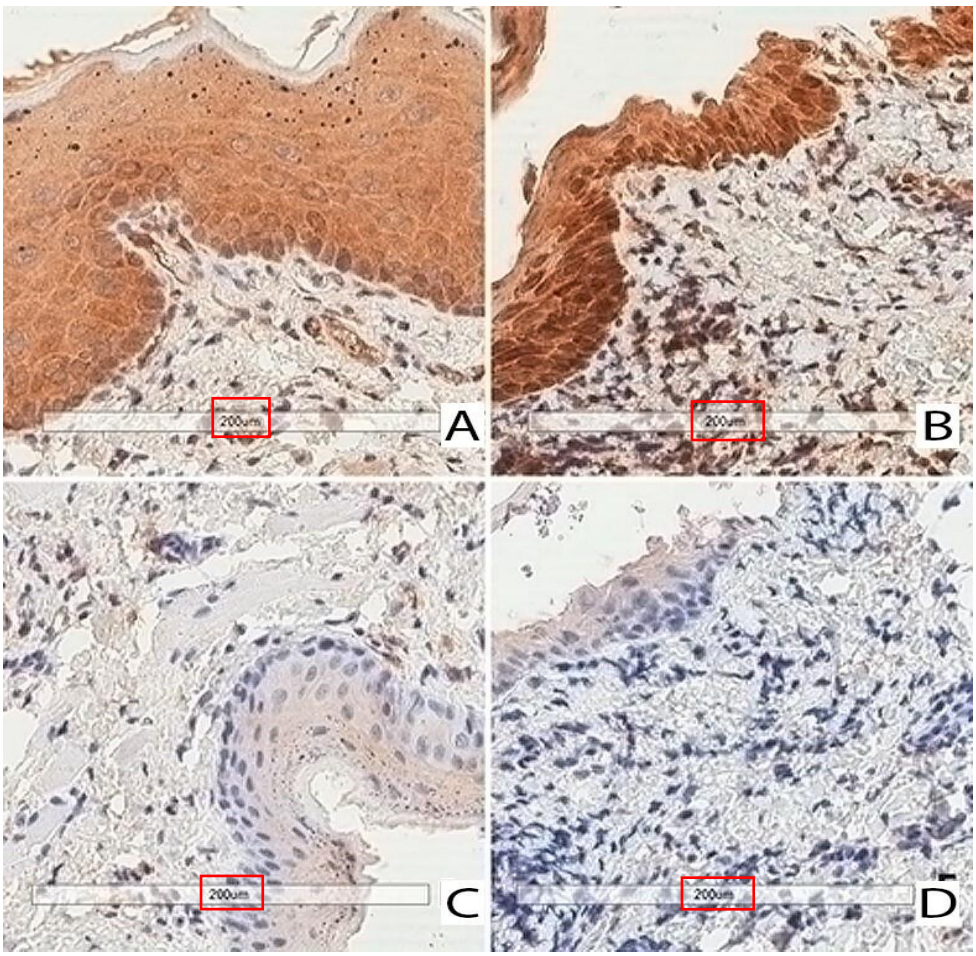

Figure 3.
Sections of rat gingival tissue. A paraffin slide at 200× magnification. A, B – immunohistochemical (IHC) staining for apoptosis marker CD95, visualized using the Bond Polymer Refinement Detection System.

A. Gingiva of the control group of rats.

B. Gingival tissue of rats after 90 seconds of orthophosphoric acid exposure. Increased staining (3+) is observed in the epithelial cells.

C, D – IHC staining for anti-apoptotic protein Bcl-2 marker, visualized with the Bond Polymer Refinement System.

C. Gingiva of the control rats. D. Gingival tissues from rats after 90 second exposure to orthophosphoric acid, with a decrease in positive staining cells in the gingival stroma.

Therefore, the rise of pro-apoptotic CD95 receptor expression the and the drop of anti-apoptotic Bcl-2 protein suggest the activation of apoptotic processes during simulated atrophy.

4. Discussion

In modeling of mucosal atrophy in experimental animals, we used 37% orthophosphoric acid because it is routinely used in clinics as dental acid etching solutions, has high acidity and in contact with human tissue causes necrosis or chemical burns. D. Kim and colleagues [19] studied the effects of orthophosphoric acid on epithelial cells in the oral cavity, including the degree of viability and nature of damage. It was found that formation of vacuoles is an early stage in cell damage, along with signs of autophagy, apoptosis, and necrosis. Additionally, cell necrosis causes nuclear pyknosis and karyolysis, as well as cell survival reduction in the application of orthophosphoric acid.

In our experiment, we observed the presence of damage in the epithelial lamina propria and necrosis of the inner mucous layer after 30 seconds of exposure of 37% phosphoric acid. As the duration of exposure increased, further damage of the epithelium occurred as exfoliation of the lamina propria. Ultimately, after 90 seconds of exposure, significant defects in the epithelium, including lymphocytic infiltration, were observed. These findings indicate the presence of atrophic changes.

In recent years, there has been compelling evidence to support the important physiological role of apoptotic cell death in maintaining optimal cellular numbers in multicellular organisms [20]. Bcl-2 is an intracellular protein that inhibits apoptosis induced by various stimuli in different cell types, while FasL is a transmembrane protein belonging to the TNF-α family of receptors. The activation of the Fas receptor through its natural ligand or specific antibodies leads to apoptosis. An imbalance in the regulation of cell death and growth, or in the expression of Bcl-2, Fas, or FasL has been implicated in the pathogenesis of several diseases [21-23].

The study of apoptotic markers in our experiment revealed changes in the expression of Bcl-2 protein. As it was mentioned, the Bcl-2 family comprises both pro-apoptotic and anti-apoptotic proteins. Anti-apoptotic ones, such as Bcl-2

and Bcl-XL, inhibit apoptosis by preventing the release of mitochondrial cytochrome C, whereas pro-apoptotic factors, like Bax and Bad, stimulate apoptosis. A disruption of this balance can lead to both suppression and activation of apoptosis in affected cells. This can be caused by overexpression of either anti-apoptotic or pro-apoptotic proteins, or a combination of both [24]. In healthy proliferating epithelial cells of the oral mucosa, Bcl-2 expression is observed in certain cellular regions, such as basal layers, which prevents cell death during active regeneration. Overexpression of Bcl-2 leads to changes in programmed cell death, with the preservation of cells that would not normally undergo apoptosis. This has been observed in the context of reactive oral lesions with atypical epithelial cells and in oral epithelial dysplasia associated with exposure to carcinogens [25]. Additionally, research of the radiation-induced damage of oral mucosa, and the transplantation of bone marrow-derived mesenchymal stem cells and platelet-rich plasma found the positive effect in promoting healing in an experimental model in albino rats [26]. Furthermore, studies explored the age-related effects of cell death on muscle fiber morphology and number in the tongue muscle of aged experimental animals [27]. The results demonstrated the rise in Bcl-2 expression, which is associated with the inhibition of apoptosis in aging.

The low expression of Bcl-2 observed in our experiment under orthophosphoric acid exposure suggests that apoptosis is the main mechanism responsible for the atrophic effects induced by the chemical, which activates apoptotic activity. Positive expression of Bcl-2, as mentioned above, was observed only in lymphoid cells in lamina propria and vascular endothelium, which could be explained by lymphoid tissue activation for the further removing of apoptotic remnants.

One of the key pathways involved in apoptosis is the receptor-mediated pathway, which includes Fas and its ligand FasL. Fas, also known as Apo-1 or CD95, plays a crucial role in this process. The reduction of Fas expression is linked to low sensitivity of tumor cells to apoptosis, leading to a disruption of the normal cellular regeneration and potentially contributing to the development of cancer. According to De Carvalho-Neto, et al. [28] the expression profile of Fas and FasL can be used as a prognostic marker for squamous cell carcinoma of the oral cavity, correlating with worse outcomes in the mentioned pathology. Deyhimi and Alishahi [29] stated that apoptosis is a potentially preceding phenomenon to acantholysis in pemphigus vulgaris. This occurs mostly through an extrinsic pathway, using pro-apoptotic mediators such as TNFR1 and FasL. In addition, studies gave evidences that lichen planus may also be associated with changes in apoptosis and Fas expression. Neppelberg, et al. [30] along with other researchers, have investigated apoptosis in oral lichen planus and identified potential links between apoptosis and the disease. A pronounced expression of FasR/FasL has been observed, and the expression of these receptors in the epithelial cells was more prominent in the basal layer compared to the suprabasal layer. The increased expression of the pro-apoptotic receptor Fas/CD95 obtained in our study also confirms the presence of atrophic processes, with apoptosis as a leading mechanism.

It should be noted that in the scientific literature of recent years there has been a lack of research on the role of apoptosis in atrophic processes in the oral cavity during the installation of removable dentures and the possibilities of influencing this process. Meanwhile, removable dentures remain the leading cause of oral mucosal atrophy, because a number of social, demographic and anthropogenic factors lead to the fact that removable prosthetics is still an alternative option for many patients [31-33]. Atrophic processes that occur under the influence of complete removable dentures worsen the prognosis of wearing a prosthesis and the quality of life of patients. It has been established that the nature and degree of these changes depend on the quality of the prosthesis manufacture, the duration of their use, oral hygiene, and the reactivity of the patient's body Trunin, et al. [34] and Berlov and Rybal'chenko [35]. Domian, et al. [36] analyzing the results of the activity of p53, NF-kB and caspase-3 in oral mucosa smears and saliva samples from patients with dentures in the oral cavity, found raised expression of caspase-3, NF-kB and p53 in the epithelial cells of the oral cavity of patients undergone the prosthetic restorations, compared with subjects from the control group without removable dentures. The strongest immunological reactivity and highest expression of caspase-3, NF-kB and p53 were registered in patients having a complete prosthesis for less than two years. Thus, the results of research on the role of apoptosis in oral atrophy mechanisms remain largely conflicting. Therefore, further investigation of the role of apoptosis in the processes of atrophy of the oral mucosa, including those caused by complete removable dentures remains relevant.

5. Conclusions

Programmed cell death is an essential process for maintaining the homeostasis of organs and tissues. It prevents the accumulation of structural, functional, and genetic damage, as well as the occurrence of maladaptive processes. The head and neck region is exposed to various environmental influences that can affect programmed cell death, including the disruption of programmed cell death or apoptosis. Disruptions in apoptosis in the maxillofacial area and oral cavity can lead to various cellular adaptation disorders, such as impaired reparative abilities, dystrophic, paraneoplastic, and neoplastic processes as well as atrophy. One of the factors that can cause atrophic processes in the oral mucosa is wearing of removable dental prostheses. Currently, due to the ageing of the population and the senile processes occurring in the oral cavities of elderly groups of people, as well as the lack of dental care and oral hygiene in many regions, the number of patients requiring removable dentures is significantly increasing. The use of removable dentures can cause a number of inconveniences and complications, such as allergic reactions, dental irregularities, difficulty chewing, pain syndrome, inflammation, necrosis, and the initiation of superficial and deep atrophy of oral cavity tissues. The study of these processes in humans brings well-known ethical, physiological, and personal challenges, that is why biological modeling at the tissue, cellular, and whole organism's levels in animals has a great relevance. When analyzing the current state of biological modeling in experimental medicine, a shortage of research on modeling oral cavity atrophy in animals was identified. Therefore, in order to more effectively study atrophy and apoptosis in the oral cavity, we have proposed an animal model based on orthophosphoric acid. The choice of this acid was based on its widespread use in dentistry, known coagulating action on tissues and the lack of systemic effects on the entire organism. The orthophosphoric acid model has resulted in

the development of injuries, inflammation, and both superficial and deep atrophic changes as in oral epithelium, as in *lamina propria*. These changes were confirmed both morphologically and biochemically, through the exfoliation of epithelial cells and the *lamina propria*, significant defects in epithelial tissue, including lymphocytic infiltration, and alterations in the concentration of apoptosis markers (the rise of pro-apoptotic receptor Fas/CD95 and the low expression of anti-apoptotic Bcl-2). Therefore, this model is a reliable tool that can be used in the investigation of strategies to prevent and manage atrophic conditions, including those that may result from the use of complete removable dentures.

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