Analysis of gingival microvessels ultrastructure in the animal model study

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Abstract
Objective: The aim of this study was to investigate ultrastructural changes that occur in gingival microvessels on acidic model of periodontitis in rats. Materials and Methods: Gingival tissue samples were obtained from 80 mongrel male rats, from which 16 represented the negative control group, 16 the positive control group and the rest of 48 the three study groups with acid-induced periodontitis. Specimens were fixed in 1.5% osmium tetroxide solution and examined (×4000–×6000) in transmission electron microscope JEM-100 CX II (JEOL Ltd., Japan) with an embedded AMT Digital Camera. Results: In study groups, substantially reduced vascular permeability, preserved mitochondrial cristae and enhanced microvesiculation in endothelial cells, condensed basement membranes were observed at the closing stage of experiment that contributed to the significantly lower severity of gingival inflammation on visual examination than in positive controls. Conclusions: Our data suggest that the key role in the recovery of endothelial and perivascular integrity is attributed to the stabilization of vascular cells membranes in rats gingiva as the result of protein and lipid metabolism regulation.

Keywords: periodontitis, animal model, microvessels, endothelial cells.

Introduction
Several clinical investigations and morphological experiments have shown peculiarities in structural and functional relationships of microcircular bed with surrounding tissues [1–3]. Moreover, structural difference between arterial and venous microvessels was described in detail and supplemented by identification of vesicular elements in the cytoplasm of endothelial cells (EC) and disclosure of their involvement in the transcapillary interchange [4, 5].

In the periodontal microcirculatory system, the best adapted for tissues metabolism maintaining is diffusion part, represented by capillaries and postcapillary venules [3]. According to scientific evidence, each of the parts – arterial (precapillary), diffusion and venous (from collector venules) has its specific role in the odonto-periodontal functioning [6]. Many researchers have found distinctive signs of alteration in the endothelial layer and basement membrane, which clearly indicate the implication of capillaries and venules structural damage and resulting microcirculatory disorders in the pathogenesis of periodontitis [7–9]. Numerous techniques associated with experimental periodontitis have been reported, however, the underlying mechanisms leading to endothelial dysfunction, as well as morphological peculiarities of possible impairment and reconstruction of periodontal tissues at the cellular level, are still in question [10–12]. Thus, the aim of this study was to investigate ultra-morphological changes that occur in microvessels gingival upon inflammation on acidic model in rats, to identify mechanisms of vascular derangement and structural recovery after metabolic regulation.

Materials and Methods
Eighty white mongrel male rats (2–4 months of age, weighing 210±30 g) were randomly divided into five groups of 16 animals each: I⁰ (negative) control, IInd (positive) control and I⁰, IInd and IIIrd study groups.

All animals were handled in accordance with the provisions of the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes (1986), International Guidelines (2011) and the Guidelines on protection, care, and handling of laboratory animals prescribed by the Ukrainian legislation (2006, 2012). All experiments were approved by the University Animal Care and Use Bioethical Committee.

The animals were housed under standard vivarium conditions [13]. In the initial – induction of periodontitis – stage animals in IInd control, I⁰, IInd and IIIrd study groups were injected per os 0.04% ammonium chloride solution (NIOCHIM, Kharkiv, Ukraine) at the dosage of 400 mg/kg separately from the main feed daily. In positive controls and rats in study groups after 28 days of the experiment, gingival inflammation was thoroughly expressed and its severity was quantified (as well as at the endpoint of the experiment) according to the Visual Examination Score (0–3). ANOVA test was used to analyze the differences between group means of Visual Examination Scores. Results were considered statistically significant when a probability (p-value) was less than 0.05 and were presented as mean and standard deviation of the mean.

In the next, closing (regulation) stage, duration 14 days, animals in the IInd control group alike rats in the I control group received throughout the experiment a normal saline solution (Indar, Kyiv, Ukraine) per os (400 mg/kg), while animals in the I⁰ study group – intra-
muscular injections (0.25 mg/kg) of 5% solution of meldonium dihydrate (OlainFarm, Olaine, Latvia), II study group — *per os* (135 mg/kg) calcium glycerophosphate (Chim-Pharm, Lubansk, Ukraine), III study group — 5% solution of meldonium dihydrate and calcium glycerophosphate daily. All of the animals survived to the end of the experiment. After 42 days, the animals were euthanized by decapitation under ether inhalation anesthesia. Jaws were dissected and sectioned. After fixation of gingival samples in 1.5% solution of osmium tetroxide (0.2 M buffer, pH 7.2) at 0°C for two hours, and washing in buffer solution, they were dehydrated in ethanol, treated by propylene oxide and embedded in epoxy resin Epon™ 812. After polymerization, resin blocks were labeled and sectioned with ultramicrotome Sorvall MT 6000. Specimens of 300–500 nm thickness were displayed for viewing (>4000×6000) in transmission electron microscope JEM-100 CX II (JEOL Ltd., Japan) with an embedded AMT Digital Camera.

Results

Minor bleeding, moderate hyperemia of gums were only rare observations at the closing stage of experiment in the I control group — 0.93±0.12, similarly to the initial stage — 1.08±0.14 (*p*>0.05). The findings were supplemented by electron microscopy of sectioned tissue samples of the blood vascular bed and perivascular space. Inter-endothelial junctions in the gingival microvessels presented narrow, of high electronic density, slits faintly revealed. Cytoplasmic matrix of EC homogeneous, containing mitochondria with preserved cristae, granular endoplasmic reticulum and inconsiderable number of micropinocytotic vesicles, some of them fused with lysosomes. Venular lumen was filled by plasma with a few erythrocytes, while basement membrane was visible as a thin layer of moderate electronic density (Figure 1). Smooth muscle cells (SMC) in the vessel walls were of typical ultrastructure, with multiple mitochondria in the cytoplasm. The connective tissue in the perivascular space contained processes of fibroblasts surrounded by unstrained collagen fibrils in the form of separate bundles.

In the II control group, after application of normal saline solution bleeding, swelling and hyperemia of gums (1.80±0.12), compared to the initial stage (2.08±0.18), were insignificantly reduced (*p*>0.05). Thickening and loosening of the basal membrane, accompanied by vacuolic dystrophy that impaired turgor pressure within the EC, led to the critical constriction of the capillary lumen (Figure 2).

Irregular nuclei of EC, found in quantities with condensed chromatin at the periphery, vacuolated cytoplasm, isolated, partially destroyed or missing mitochondrial cristae were indicative for the destructive changes in the capillary wall. Endoplasmic reticulum was seen as a dilated interconnected membranous network with numerous vesicles, swollen and vacuolized cisterns, detached ribosomes. A layer of osmiophytic cellular debris was deposited alongside the loosened and thickened basement membrane. Cytoskeletal abnormalities, caused by swelling, hydroptic dystrophy, typical for cellular dysproteinosis, and increased cytoplasmic eosinophilia were regarded as morphological appearance of reversible EC injury.

Aggregation of erythrocytes that under unfavorable conditions can result in irreversible “erythrocyte sludge” was observed characteristically to the microcirculatory derangement (Figure 3). Increased endothelial permeability was manifested by enhanced pinocytosis and swollen perivascular space, containing a solitary collagenic fibrils and considerable number of polysomes.

Bleeding, swelling and gum hyperemia in the I control group at the closing stage, weakened obviously even to the naked eye, comparing with the initial stage (1.29±0.10 vs. 1.95±0.15, *p*<0.05). Cytoplasmic matrix of homogeneous translucency, marked by the substantially increased number of microvesicles and exosomes that are important as mediators of intercellular communication, as well as non-swollen preserved mitochondrial cristae indicated restored functional ability of EC. A variety of intercellular connections between pericytes and EC through a relatively thin condensed basement membrane of moderate electronic density that have a crucial role in the formation and functionality of the selective permeability space, were seen. Vascular SMC with the typical cellular substructures were detected. Peripheral processes of fibroblasts were surrounded by thin collagen fibrils. Despite the contact of erythrocytes with the luminal surface of the venular endothelium, no signs of aggregation of red blood cells were observed (Figure 4).

At the endpoint of the experiment, after the regulation stage in the II study group bleeding was not visible, only mild swelling and reduced gum hyperemia, if compared to the observations at the initial stage (1.57±0.11 vs. 2.11±0.16, *p*<0.05) with inter-dental papillae detached from the teeth, were noticed. Swollen pericytes, with a decreased number of cristae, indicating a partial regain of their regular activity, projected finger-like extensions to wrap around the EC in the walls of capillaries and venules. Inter-endothelial cell slits widened because of residual swelling and excessive collagen synthesis by fibroblasts for the tissues replacement in the pericapillary spaces. Newly formed elements of low electronic density were predominantly represented in the structure of collagen fibrils that are involved in regulation of the flow of tissue fluid in the space. Together with the increased amount of plasma into the lumen of the vessels, separate red blood cells become common in observations (Figure 5).

When comparing results in the III study group after the closing stage with those at the initial stage (1.20±0.08 vs. 1.98±0.14, *p*<0.05), bleeding was substantially reduced, only mild swelling and hyperemia of inter-dental papillae that were closely adjoining to the teeth, was seen. In the capillary lumen with irregular contours of the luminal surfaces, domelike shape of the red blood cells indicated their adequate membrane elasticity and, thus — ability to pass uninterruptedly through microvessels (Figure 6).

Cytoplasm of EC contained sufficient amount of vesicles and a few lysosomes. In most mitochondria, numerous cristae were visible in the clear matrix enhancing production of adenosine triphosphate (ATP) due to expansion of the inner membrane surface area. SMC with reduced number of mitochondria cristae were noticed in the vessel walls. In the pericapillary spaces, demarcated by basal lamina of both capillary and parenchymal cells, no signs of edema were observed.
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Figure 1 – Fragment of the venular lumen with erythrocyte and plasma layer. Well-structured endothelial cells, basement membrane and collagen fibers in the vessel wall (×6000).

Figure 2 – Capillary lumen surrounded by deformed nucleus of the endothelial cell with vacuolated cytoplasm. Basement membrane loosened and thickened (×4000).

Figure 3 – Aggregation of red blood cells on the luminal surface of venular endothelium. Endothelial cell with vacuolated mitochondria, perivascular space widened (×4000).

Figure 4 – Diminished plasma layer by the luminal surface of the venular endothelium, erythrocyte in short length of contact with the endothelium (×6000).

Figure 5 – Pericytes at the external surface area of the capillary wall. The capillary lumen is filled with plasma, contains red blood cell. Excessive amount of collagen fibrils in the pericapillary space (×4000).

Figure 6 – Fragments of the capillary lumen with erythrocytes and equable layer of plasma. Endothelial cells with partially preserved mitochondrial cristae. Basement membrane of typical structure (×6000).
**Discussion**

Many authors asserted that the most prevailing mechanism of endothelial dysfunction is an increase in reactive oxygen species which can influence nitric oxide production from the endothelium and activity via several mechanisms that is thought to be one of the key events in the development of periodontitis [9, 14, 15]. Hypoxia, free radicals (superoxide, hydrogen peroxide, hydroxyl ion), chemical agents are among the causes of severe or prolonged injury of the cell that is characterized by the lack of energy production and membrane damage. According to Deanfield et al. [4], Pietropaoli et al. [8], endothelial dysfunction is associated with progressive loss of phospholipids due to activation of endogenous phospholipases, reduced anticoagulant properties as well as increased adhesive molecules expression, chemokines and other cytokine release. Other studies have shown that alteration in endothelial function precedes the emergence of morphological changes in EC, which interacts with blood components and the abluminal tissues, as well as in basement membrane, which is in close cooperation with SMC, pericytes and extracellular matrix [2, 6, 10].

The present study allowed us to notice the fact that minimum degree of permeability of gingival vascular walls in negative control group has been provided by compacting inter-endothelial cells contact in capillaries and venules. Our results with increase in capillaries and venules permeability accompanied by aggregation of red blood cells under the influence of ammonium chloride (positive control group) are in agreement with several reports on the evolvement of periodontal inflammatory reaction, manifested by microcirculatory disorders, dysregulated microvesicular and intercellular transport [2, 3, 7]. Ganz and Vita [15] concluded that mild reversible cellular injury results in dysfunction of the cellular membrane, while in a case of irreversible cell injury the changes seen are extensive membrane damage, lysosomal swelling and vacuolization of mitochondria that are actively involved in cellular metabolism. Recent findings suggest that disruption of the outer mitochondrial membrane permits proteins in the inter-membrane space to leak into the cytosol, leading to certain cell death [11, 12].

In periodontal pathology investigations, Garg et al. [17] have substantiated particularly the important role of disrupted oxidation in mitochondria that generate most of the cells supply of chemical energy (ATP), and ATP-ase with its direct effects on the degree of vascular permeability. Studies have shown that mitochondrial inner membrane contains the ATP synthase, while outer membrane can associate with the membrane of endoplasmic reticulum – which is concerned with protein and lipid synthesis as well as cell metabolism secretory pathways – into the structure that is increasingly recognized as influential for overall cellular physiology and homeostasis [5, 7].

Regeneration of microcirculatory network was reported by Owens [1], Scannapieco et al. [3], Deanfield et al. [4] as highly important because even after short-term ischemia profound changes in the structure of the walls of microvessels can be observed. Garg et al. [16], Allt and Lawrenson [18], Dore-Duffy and Cleary [19] postulate, that the conditions, under which in response to injury EC, pericytes – that regulate capillary blood flow, and SMC produce a large number of growth factors and other substances that stimulate reparative processes in the vessels, can be regarded as prognostically favorable.

Weakening of hyperemia and edema of the inter-dental papillae in the study groups at the closing stage of the experiment, statistically significant ($p<0.05$) in comparison with the results after the initial stage ($1.35\pm 0.10$ vs. $2.01\pm 0.16$), justifies the rejection of the null hypothesis within the limits of the present study. Beyond the aim of this investigation, we also have observed typical for the animal model study hyperplasia of squamous epithelium, focal inflammation of lamina propria, formation of new vessels with various wall thicknesses (the most probably caused by increased collagenase action or other metabolic abnormalities) in rats gingival biopsy samples stained with Hematoxylin–Eosin. In the present study, we were concerned primarily on resultant to the impaired metabolic control of systemic acidity in rats’ ultrastructural gingival changes, similar to those in patients with diabetes mellitus. In such a context, we have found tight inter-endothelial cells contact, initial signs of collagen formation in perivascular spaces, enhanced microvesiculation, preserved mitochondrial cristae, condensed vascular basement membranes, increased plasma into the lumen of microvessels in study groups after use of an inhibitor of fatty acid oxidation and calcium supplements. Taken together, the data in this study provides further insight into some of the mechanisms involved in gingival endothelial function and their response to the pro-acidotic stimulation – induction stage, and after metabolic regulation – closing stage of the experiment.

**Conclusions**

The acid-mediated inflammation in rats’ periodontium is predominantly vascular reaction with reversible and/or irreversible destruction of EC and SMC organelles and basement membrane, increasing permeability of the capillary and venular walls, accompanied by exudation of plasma, and aggregation of erythrocytes. Our data suggest that recovery of the gingival endothelial and smooth muscular cellular structures, reduced permeability of the vascular walls and increased plasma into the lumen of microvessels occurred primarily due to the microvascular membranes stabilization after experimental metabolic regulation in rats.

**References**


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