GINGIVAL MICROVASCULAR COMPLEX AT THE INDUCTION AND RESOLUTION OF PERIODONTAL INFLAMMATION. AN ANIMAL MODEL STUDY

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Abstract

Introduction. To determine the primary events of gingival microvascular complex damage caused by inflammation (28 days) on an acidotic model of periodontitis in 36 white mongrel rats, and to clarify regulatory factors of the structural recovery after metabolic correction (14 days). **Materials and methods.** Expression and severity of gingival inflammation were visually analyzed with an ANOVA test. The gingival tissue specimens were examined (x 4000 – 6000) after 42 days with a transmission electron microscope JEM-100 CX II (JEOL, Japan). **Results and discussion.** Vacuolation of the endothelial cells' cytoplasm, thickening and loosening of the basal lamina, narrowing of the microvascular lumen, aggregation of the red blood cells and dilation of perivascular space were characteristic features of inflammatory derangement. A significantly lower severity of gingival inflammation ($p < 0.05$), thin condensed basal lamina, tight interendothelial junctions, increased amount of plasma into the lumen of the vessels, no signs of red blood cells aggregation and newly formed thin collagen fibrils in perivascular space were indicative of the progressive resolution of inflammation. **Conclusions.** Reduced vascular permeability after application of an inhibitor of fatty acid oxidation and calcium supplements fostered post-inflammatory recovery of the gingival microvascular complex in rats.

Keywords: periodontitis, endothelial cells, vascular permeability

1.INTRODUCTION

Many studies have confirmed that a properly functioning microcirculation preserves organism adaptability for meeting the metabolic demands [1,2] while, in the pathogenesis of periodontal diseases, impaired gingival microvascular and endothelial functions were substantially [3,4] impaired. Moreover, disruption of the vascular network through protracted inflammation was regarded by Zoellner H. *et al*. as characteristic for gingivitis and chronic periodontitis [5]. During the onset and progression of any periodontal disease, structural damage of capillaries and venules, resulting in microcirculatory disorders, is manifested by cardinal signs of inflammation – pain, redness and swelling (oedema) of the gingiva [6-10]. After the initial periodontal inflammation, the following stages result in advanced lesions, which affect the alveolar bone [11-13].

As generally known, the endothelium plays an important role in vascular regeneration and vascular tone regulation, its disfunction often preceding the clinical manifestation of the disease [14-16]. Recently, there has been significant discussion on the results of periodontal microcirculation *in vivo* assessment [17], expression profile of the macrophage migration-inhibitory factor [18], *in vitro* regeneration of periodontal tissue microvasculature [19], and place of angiogenesis in progression of the periodontal disease [20]. The role of high endothelial postcapillary venules and selected adhesion molecules in periodontal diseases [21] and periodontal microvascular endothelial cells (ECs) for maintaining capillary permeability [22] has been elucidated, as well. At the same time, despite the increasingly recognized role of perivascular spaces in neurogenetics [23] and neuroimmunology [24], many aspects of the homeostatic functions of periodontal vasculature remain unclear [25].

Accordingly, the aim of the present study was to determine the primary events of gingival microvascular complex derangement upon inflammation, on an acidotic model of periodontitis in rats, and to clarify the regulating

factors of its structural recovery after metabolic correction.

2. MATERIALS AND METHODS

The experiment was conducted on 36 white mongrel male rats (2-4 months of age, weighing 210±30g), randomly divided into Ist (control) and two study groups – the IInd and the IIIrd (12 animals each).

The animals were treated under standard vivarium conditions in accordance with the provisions of the European Convention for the Protection of Vertebrate Animals Used for Experimental and other Scientific Purposes (1986), International Standards (Directive 2010/63/EU) and Ukrainian legislation (2012) for the Care and Use of Laboratory Animals. The experimental protocols were approved by the University's Bioethical Committee on Animal Care and Use in Experimental Study.

Initially, acidotic induction of the periodontal inflammation stage was performed along 28 days, the animals in the IInd and IIIrd groups separately from the main feed being injected daily per/os with 0.04% ammonium chloride solution (Biochim, Kharkiv, Ukraine) at a dosage of 400 mg/kg. There followed resolution of the inflammation stage, with a duration of 14 days, animals in the IInd group alike in the Ist (control) group receiving per/os (400 mg/kg) a normal saline solution (InDar, Kyiv, Ukraine), while animals in the IIIrd group – intramuscular injections (0.25 mg/kg) of 5% solution of meldonium dihydrate (OlainFarm, Olaine, Latvia) combined with calcium glycerophosphate (Chim-Pharm, Lubny, Ukraine) per/os (135 mg/kg) daily. All animals survived to the end of the experiment. After 28 and 42 days of experiment, expression of gingival inflammation and its severity were quantified according with the Visual Examination Score (0-3). The ANOVA test was used to analyze the differences between group means of Visual Examination Scores, the results being presented as mean and standard deviation of the mean and considered statistically significant, when probability (p-value) was below 0.05.

42 days after the experiment, the animals were euthanased by decapitation under ether inhalation anesthesia. Jaws were dissected and sectioned. After fixation of the gingival samples in an 1.5% solution of osmium tetraoxide (0.2 M buffer, pH – 7.2) at 0°C for 2 h and washing in buffer solution, they were dehydrated in ethanol, treated by propylene oxide and embedded in epoxy resin Epon–812. After polymerization, the resin blocks were labelled and sectioned with ultramicrotome Sorvall MT 6000. 300-500 nm thick specimens were displayed (x 4000 – 6000) on a transmission electron microscope JEM-100 CX II (JEOL, Japan) with an embedded AMT Digital Camera.

3. RESULTS AND DISCUSSION

In the Ist group, moderate hyperemia and rarely occurring minor gum bleeding at the end of the experiment (0.74±0.08) were comparable ($p > 0.05$) to the observation results on the initial stage (0.82±0.08). The transmission electron micrograph of the gingival vascular bed and perivascular space displayed the layer of ECs of typical ultrastructure in the walls of the capillaries, facing the vascular lumen, and a 20-100 nm thick electron-dense *basal lamina*, lining the external surface of the ECs membrane. The proximal postcapillary venules presented similar anatomical features, exhibiting the endothelial inner layer and a thin basal lamina as the outer layer. Interendothelial junctions in the gingival microvessels were narrow, of high electronic density, slits clearly visible. Smooth muscle cells of spindle-shaped form with the cytoplasm of moderate electronic density were also found in gingival microvessels' walls. Venular lumen was filled by plasma with a few red blood cells (RBCs), as well as with small cytoplasmic fragments (Fig. 1). The perivascular connective matrix contained activated fibroblasts, recognized by their abundant rough endoplasmic reticulum, having two or more nucleoli within a branched cytoplasm, and extracellular fibrillar and nonfibrillar components (Fig. 2).

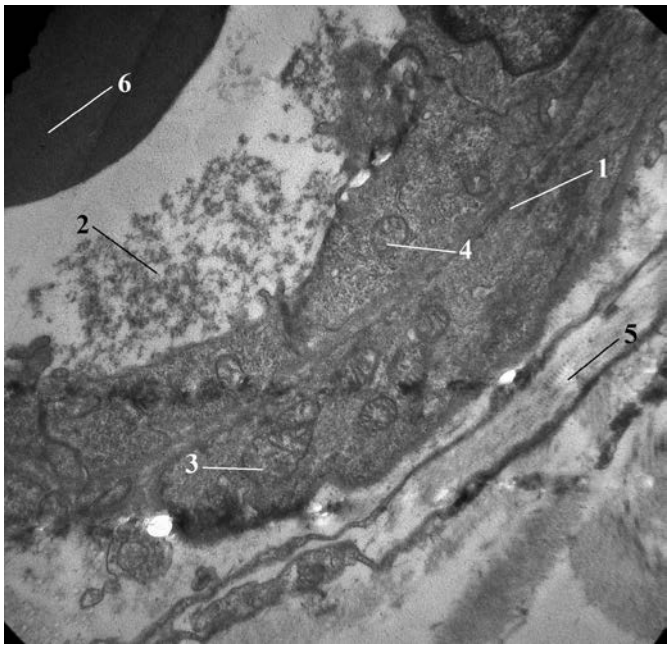

Fig. 1. Venular intra- and perivascular space: 1 – basal lamina, 2 – plasma with cytoplasmic fragments, 3, 4 – EC' mitochondria, 5 – collagen fibrils in perivascular space, 6 – RBC in venular lumen (x 6000)

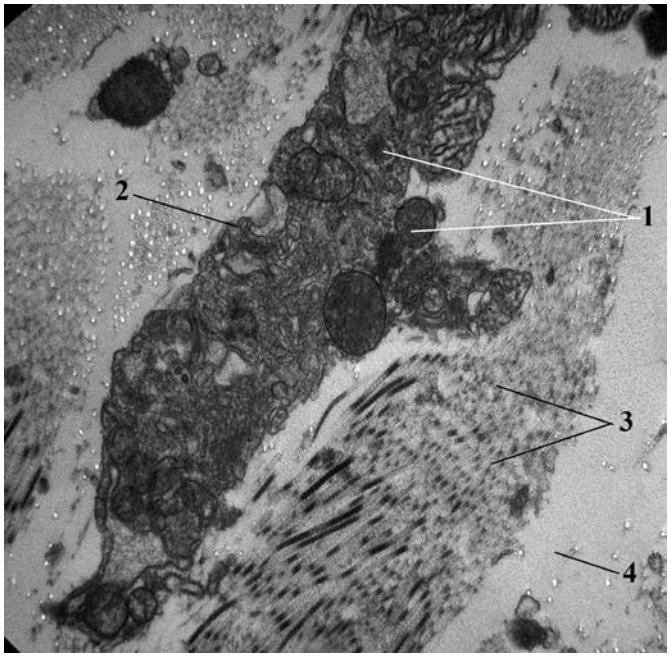

Fig. 2. Activated fibroblast in perivascular space: 1 – nucleoli and 2 – endoplasmic reticulum of the fibroblast, 3 – extacellular matrix and 4 – collagen fibrils in the interstitium (x 6000)

In the IInd group, gingival bleeding, swelling and hyperemia after application of normal saline solution were insignificantly reduced (1.86±0.12 *vs* 2.02±0.14) compared to the initial stage ($p > 0.05$). In both study groups, upon development of periodontal inflammation due to "metabolic acidosis" in the induction stage, in the walls of capillaries, hydropic distrophy of ECs with increased number of microcytopinocytic vesicles was observed. As a result, increase in ECs' volume caused narrowing of the capillary lumen, which acquired a slit-like shape, that may cause insufficient tissue perfusion with subsequent development of hypoxia. The basal lamina was thickened, fluffy in focal points, with a pericyte projecting finger-like extensions in its duplication. Characteristic features of microcirculatory derangement – virtually no boundary layer of plasma by the inner capillary wall, aggregation of RBCs in the lumen of the capillary close to the endothelial lining, resembling "erythrocyte sludge", and cell fragments were also observed (Fig. 3). Alteration in the venular wall was evidenced by vacuolation of ECs' cytoplasm, microclasmatosis with cellular debris alongside the loosened and thickened basal lamina, accompanied by extensive disruption of the plasma membrane. Faintly revealed ECs junctions and enhanced pinocytosis denoted increase of interendothelial permeability. Perivascular space, containing solitary collagenic fibrils and a considerable number of polysomes, was dilated due to interstitial edema (Fig. 4).

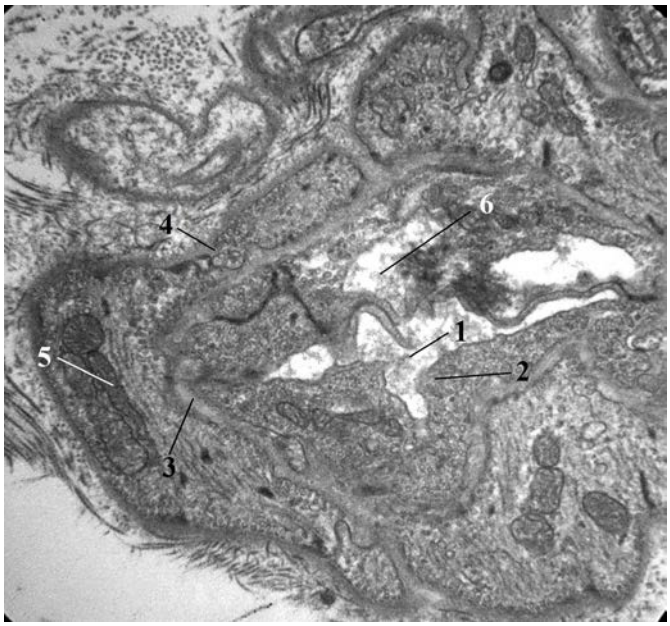

Fig. 3. Intra- and pericapillary tissues: 1 – cytoplasmic fragments in slit-like lumen, 2 – EC, 3, 4 – basal lamina, 5 – pericyte, 6 – vacuolated EC' cytoplasm (x 4000)

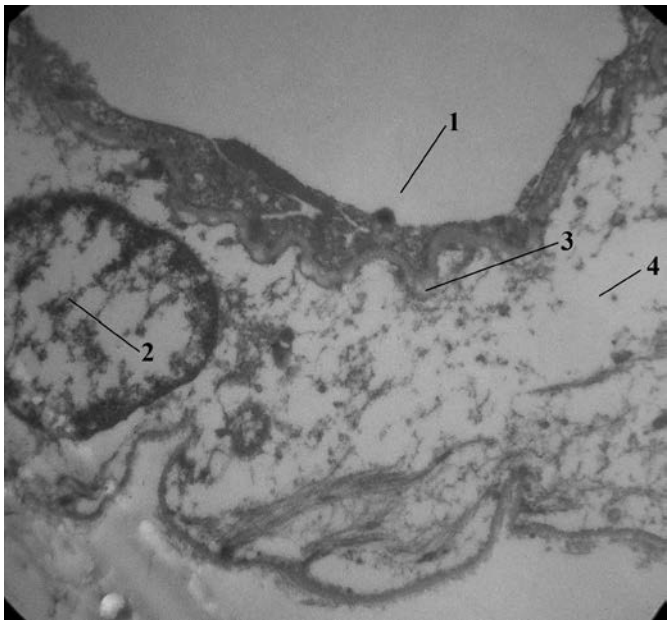

Fig. 4. Venular fragment: 1 – lumen with plasma, 2 – vacuolated mitochondria of the EC, 3 – basal lamina, 4 – cytoplasmic matrix of the EC (x 6000)

During the resolution stage, only mild swelling and substantially reduced hyperemia of gums were noticed, comparatively with the results recorded in the induction stage in the IIIrd group (1.28±0.10 vs 1.92±0.14, $p < 0.05$), and no signs of gingival bleeding. The ultrastructural features of the hemo-microcirculatory complex evidenced a progressive reduction of gingival inflammation. In the walls of capillaries, ECs were predominantly of typical structure and size, with plasma membrane preserved, and cytoplasmic matrix of homogeneous electron density. Only a single EC displayed vacuolated cytoplasm and swollen mitochondria. In the inner layer of capillaries, EC smaller junctions produced intercellular clefts, and larger ones – intercellular gaps. In the capillary lumen, an increased amount of plasma and a single RBC together with small cytoplasmic fragments and vesicles, enclosed by the cytoplasmic membrane, as well as residual signs of interstitial edema indicated the activation of ECs during resolution of gingival inflammation (Fig. 5).

In the walls of the venules, relatively thin condensed basal lamina of moderate electronic density and slightly swollen mitochondria with a reduced number of cristae were observed. In the venular lumen, an increased amount of plasma and single red blood cells RBCs of a "domelike" shape in contact with the luminal surface of the venular endothelium were evidenced, yet with no signs of RBCs aggregation. The endothelial cell-to-cell junctions with restored boundary integrity and newly formed thin collagen fibrils of low electronic density in perivascular spaces were detected, revealing the progressive recovery of the gingival microvascular complex (Fig. 6).

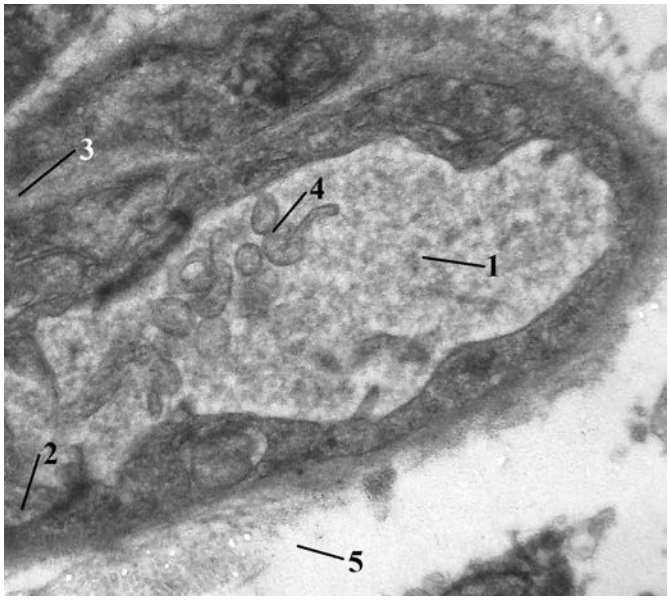

Fig. 5. Capillary fragment: 1 – lumen with plasma, 2 – EC, 3 – basal lamina, 4 – cytoplasmic fragments 5 – loose connective tissue in perivascular space (x 4000)

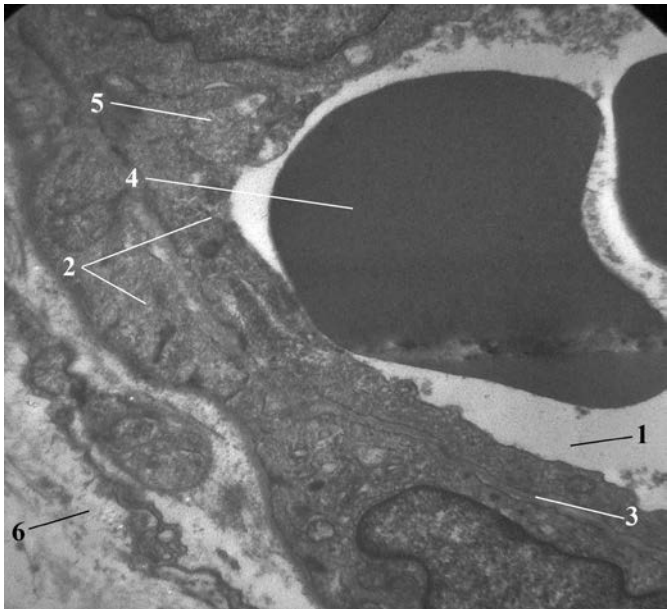

Fig. 6. Venular fragment: 1 – lumen with plasma, 2 – ECs, 3 – basal lamina, 4 – RBC, 5 – EC' mitochondria, 6 – loose connective tissue in perivascular space (x 6000)

It was clearly demonstrated that a large area of the terminal vascular network in conjunction with a low velocity of the peripheral blood flow creates appropriate conditions for the exchange of nutrients and oxygen between the blood and teeth-supporting tissues and, consequently – adaptability to either acute or chronic changes in its environment [2,11]. Therefore, an appropriate vascularization and consistent microcirculatory beds were prerequisites for an adequate periodontal tissues perfusion in the animals of the Ist group. The ultrastructural study of the periodontal vascular bed and perivascular space revealed distinct interendothelial cell gaps, and a luminal boundary layer, created by capillary plasma, separating the inner surface of the microvessel from the RBCs stream. In such conditions, extravasation of macromolecules was expected to maintain a balanced blood and interstitial pressure. ECs, with their distinct functions and morphologies, formed a vascular barrier between the blood and the surrounding tissues in the rats of the Ist group all along the experiment. According to L. Claesson-Welsh, the intercellular connections between ECs and pericytes, whose variety may be found in the venular wall, and also the terminal arterioles and capillaries have a crucial role in the formation and functionality of the selective permeability space [25]. As reported by M. Kouhsoltani *et al*. [26] and H. S. Sheethal *et al*. [27], molecular regulators of vascular permeability also include growth factors and inflammatory cytokines (not approached in the present study).

We have previously determined that the acid-mediated periodontal inflammation in rats is predominantly a vascular reaction with reversible or irreversible destruction of ECs' and smooth muscle cells' organelles and basement membrane, accompanied by changes in the permeability of the capillary and venular walls [28]. Vacuolation of the cytoplasm, that impaired turgor pressure within the ECs in the vascular walls, thickening and loosening of the basal lamina were prominent in the animals of the study groups in the induction stage. Exit of the inflammatory cells, differently regulated in acute and chronic inflammation, and initial sclerosis of vessels' walls and perivascular spaces may have caused an insufficient tissue perfusion with subsequent development of hypoxia. The lumen of the capillaries was narrowed, due to an increase in ECs volume, and obstructed by the presence of cell fragments. Apparently, the blood flow and vascular permeability were also affected by degradation of a surface layer lining the luminal walls of blood vessels (endothelial glycocalyx), resulting in altered barrier function, impaired intracellular and interstitial hydrostatic pressure. Vasodilation of gingival venules in rats, as observed by M. Gyurkovics *et al*., was related in part to an increased production of the vascular endothelial growth factor, which also induces proliferation of ECs and small blood vessels in the gingival *lamina propria* [29,30]. Therefore, an insufficient passage of the fluid has been suggested as one of the possible causes for dilated perivascular spaces, as markers of the inflammatory activity and interstitial oedema in the mentioned groups. Inflammatory dilation of perivascular spaces with accumulation of perivascular macrophages, aiding T-cells, as reported by Cekici *et al*., is often correlated with many other symptoms and conditions, which affect the arterial walls, including sclerosis [31].

F.-M. Chen and Y. Jin [32] suggested that a predictable structural and functional reconstruction of a tooth-supporting apparatus still remains a challenging clinical task. Since changes in the blood flow might lead to insufficient perfusion and deranged metabolic pathways, the vasoactive compounds affecting the vascular tone and perfusion could, in our opinion, indirectly improve tissue metabolism. The results of S. Statsenko *et al*. [10] demonstrated the ability of meldonium to significantly improve the endothelial function, and thus – the state of the microcirculatory bed. Obviously, the main target of the metabolic correction referred to the fatty acids, as an integral part of the phospholipids that form the bulk of the cell membranes. In the present study, clearly visible on electron micrographs, tight endothelial cell-cell junctions, increased plasma into the lumen of microvessels, initial collagen formation by fibroblasts in perivascular space after use of an inhibitor of fatty acid oxidation and calcium supplements were considered as important morphological signs of the post-inflammation gingival recovery in rats. Whereas the periodontal disease resulting

from excess inflammation may be considered a failure of resolution pathways [5,11], we fully support the thesis that an adequate resolution – return of the tissue to homeostasis, defined as absence of inflammation, is an essential goal of interventions in periodontal diseases [31]. Hence, the results of this study elucidate some of the regulatory mechanisms of gingival endothelial function and microvascular permeability in response to the proacidotic stimulation–induction stage and also after the metabolic correction–resolution stage of the experiment.

4. CONCLUSIONS

Widened interendothelial junctions, reduction of plasma and aggregation of erythrocytes in vascular lumens, dilation of perivascular spaces evidenced the acid-mediated periodontal inflammatory reaction of intra- and perivascular structures. Reduced vascular permeability after use of an inhibitor of fatty acid oxidation and calcium supplements fostered post-inflammatory recovery of the gingival microvascular complex in rats.

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