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The Effect of Hyaluronic Acid on the Periodontium in Spontaneous Periodontitis in Rats

El Efecto del Ácido Hialurónico sobre el Periodonto en Periodontitis Espontánea en Ratas

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SUMMARY: The aim of the article was to study changes in periodontal tissues in rats with spontaneous periodontitis (SP) and to evaluate the effect of hyaluronic acid (HA) on the state of the periodontium. Wistar rats with signs of SP were divided into 6 groups: 1) intact group; 2) intact animals with HA "HD-1,0 MDa"; 3) SP group; 4) SP with HA "S-2,4 MDa"; 5) SP with HA "ST-2,4 MDa"; 6) SP with HA "HD-1,0 MDa". The study of the periodontium rats with SP noted the main structural changes (collagen reduction, resorption of alveolar bone, dilatation and stasis of the vessels of the periodontium, gingival papilla and tooth pulp), which were assessed as moderate. Morphological evidence of inflammation was infiltration of neutrophils into the connective tissue of the gums, without the formation of abscesses. Local administration of HA did not cause additional structural damage in periodontal tissues of rats with SP, but also did not affect changes in the microvascular system of periodontium and tooth pulp, periodontal ligaments, only a tendency to inhibit alveolar bone resorption in rats was noted. One can consider the tendency to improve the condition of periodontal tissues in the group of rats injected with high molecular HA and HA with mannitol (2.4 MDa).

KEY WORDS: Spontaneous periodontitis; Rat; Periodontal ligament; Hyaluronic acid: Pulp; Inflammation; Blood vessels.

INTRODUCTION

Periodontitis is an inflammatory-dystrophic disease characterized by progressive destruction of the supporting structures of the teeth, including the periodontal ligament (PDL), alveolar bone and gum tissues (Rusyanti *et al*., 2019). The presence of inflammation, periodontal rearrangement and impaired tooth fixation are interdependent structural and functional factors of dentol-alveolar complex damage, considering that the condition of the periodontium is one of the crucial factors in the stable attachment of the tooth root (Könönen *et al*., 2019).

Changing the biomechanical properties of root dentin is considered as a possible way to influence the restoration and fixation of the periodontium (Lin *et al*., 2020). By changing the surface layer of dentin, which is a thin layer of less mineralized collagen, the dentinal tubules open and thus the fixation of the collagen of the periodontal ligament to the dentin surface changes. Due to the development of structural disorders in the soft tissues of the periodontium in periodontitis, the qualitative attachment of the periodontal ligament to the alveolar part of the bone and the fixation of the tooth root (Herber *et al*., 2012) deteriorate at the same time. Different therapies have been tested to activate the regenerative effect on the damaged periodontium: the use of BMP (Bone morphogenetic proteins), hydrogel (Kato *et al*., 2015), stem cells (Inoue *et al*., 2010), collagen type I (Rosa *et al*., 2013), platelet-rich fibrin (PRF) (Chen *et al*., 2015), platelet-rich plasma (PRP) (Kopchak, 2017), polylactide (Polylactic acid), polymer poly-D, L-lactide and glycolide (Huang *et al*., 2010), ceramic powder (Miura *et al*., 2003), hyaluronic acid (Bansal *et al*., 2010). Various advantages and disadvantages of cell therapy are evaluated, but the simplest and the most convenient to perform is the introduction of components that can be used by periodontal tissues in the restoration of the extracellular matrix (extracellular matrix). Hyaluronic acid (Zhai *et al*., 2020)

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is a natural biological component of connective tissue, which is proposed in restorative medicine (surgery, traumatology, dermatology, orthodontics, etc.).

Hyaluronic acid (HA) is one of the main components of the extracellular matrix of connective tissue. HA is found in all periodontal tissues, more in the connective tissue of the gums and periodontal ligament, compared with cementum and alveolar bone. In chronic inflammatory and inflammatory-dystrophic diseases of the gums, HA undergoes degradation due to the release of hyaluronidases by bacteria. Therefore, to stimulate the regeneration of damaged periodontal tissues, the introduction of exogenous HA was proposed (Pilloni *et al*., 2019). Some studies have shown successful attempts to use hyaluronic acid in the treatment of gingivitis and periodontitis (Lobato *et al*., 2019). When interacting with collagen and proteoglycans, HA provides structural stability to the matrix, and through its hydrophilic properties binds to water molecules and proteins, forming a viscous physical state that facilitates its introduction into tissues compared with fluid. It is thought that the hygroscopic and elastic properties of HA may slow the penetration of infectious agents into periodontal tissues (Bansal *et al*.). This leaves permeability, diffusion and the possibility of migration and proliferation for cells in the interstitial space. According to scientists, the influence of HA is determined by its molecular weight. Thus, low-molecular HA activates angiogenesis, and high-molecular, on the contrary, blocks it (Deed *et al*., 1997). But these studies are debatable and need clarification.

Considering these facts and the biological properties of hyaluronates, the aim of the study was to examine the effect of hyaluronic acid of three different shapes and molecular sizes on the state of the periodontium in a model of spontaneous periodontitis (SP) in rats.

The aim of the article is to investigate changes in periodontal tissues in rats with spontaneous periodontitis and to evaluate the effect of hyaluronic acid of different molecular sizes (1.0MDa and 2.4MDa) on the state of the periodontium.

MATERIAL AND METHOD

The incisors of the lower jaw of Wistar rats with signs of SP were studied. Visual manifestations of gingivitis around the incisors were the selection criteria for the animals under study. The average weight of rats was 220 g, age - 6 months. After selection of animals included in the study, they were divided into 6 groups (5 animals each) depending on the method of treatment, which differed in the form of HA. Rats of the 1 and 3 groups were not administered HA.

Rats of the groups 2, 4, 5 and 6 were injected with HA (0.05 ml) in the alveolar process once a week, 3 times. Three forms of HA were used: "HyaDENT BG" 1.0 MDa ("BioScience GmbH, Germany)" ("HD-1.0 MDa"), "SERTOBEC" 2.4 MDa (SC Rompharm Company SRL, Romania) ("S-2.4"), form with manitol"SERTOBEC Tendon"2.4 MDA (SC Rompharm Company SRL, Romania) ("ST-2.4"). The distribution of experimental animals by groups depending on the diagnosis and method of treatment are shown in Table I.

In 4 and 8 weeks of observation histological examinations were performed, in 8 weeks – electronic-microscopic ones. Histological samples were fixed in 10 % neutral formalin, dehydrated in isopropanol and embedded in paraffin (Leica Surgipath Paraplast Regular). Paraffin sections were prepared on a Thermo Microm HM 360 microtome. The sections were dewaxed and stained with hematoxylin and eosin (H&E) according to the picro-Mallory method. Micropreparations were examined on an Olympus BX51 microscope, quantitative measurements using Carl Zeiss software (AxioVision SE64 Rel.4.9.1). The assessment of structural changes was performed by the semi-quantitative method, as proposed by Aguirre *et al*. (2017). Evaluation criteria: gingivitis, PDL changes, epithelial changes, alveolar bone resorption. For each criterion, the sample score was differentially assigned (absence (0), slight (1), mild (2), moderate (3), severe (4)) and presented as the mean value of the points (M ± SD).

Table I. Distribution of experimental animals by groups depending on the diagnosis and method of treatment.

Nº	Diagnosis	Treatment
Group 1	Intact animals	-
Group 2	Intact animals + HA "HD-1,0"	hyaluronic acid "HyaDENT BG" 1,0 MDa (BioScience GmbH, Germany)
Group 3	SP	-
Group 4	SP + HA "S-2,4"	hyaluronic acid "SERTOBEC" 2,4 MDa (S.C. Rompharm Company S.R.L., Romania)
Group 5	SP + HA "ST-2,4"	hyaluronic acid with mannitol "SERTOBEC Tendon" 2,4 MDa (S.C. Rompharm Company S.R.L., Romania)
Group 6	SP + HA "HD-1,0"	hyaluronic acid "HyaDENT BG" 1,0 MDa (BioScience GmbH, Germany)

For scanning transmission electron microscopy (TEM), tissue samples were fixed in a 2.5 % solution of glutaraldehyde on phosphate buffer with fixation in 1 % buffered saline (OsO_4). Dehydration was performed in ethanol and acetone, impregnated and poured into epon-araldite epoxy resin (Epon 812, Araldite 502). Ultrathin sections were made on a Reihart ultratome, contrasted with 2 % uranyl acetate solution and lead citrate. Ultrathin sections were examined and photographed under an electron microscope Tescan Mira 3 LMU (Czech Republic).

The protocol of this study was approved by the Ethics Committee of the Bogomolets National Medical University (Protocol Number: 01-2019). In experimental studies on animals adhered to the "European Convention for the protection of vertebrate animals used for experimental and other scientific purposes" (Strasbourg, 1986), "General ethical principles of animal experiments", approved by the First National Congress of Bioethics (Kyiv, 2001), Directive 2010/63/EU of European Parlament and Council on the protection of animals used for scientific purposes, Law of Ukraine Nº 3447-IV dated February 21, 2006, order Ministry of Education and Science, Youth and Sports of Ukraine Nº 249 dated March 1, 2012. All surgery was performed under general ketamine anesthesia and every effort was made to minimize suffering.

Statistical data processing was performed in Origin Lab version 8.0 by one-way analysis of variance (one way ANOVA) followed by Tukey test (post-hoc test). Differences between groups were considered statistically significant at $p<0,05$.

RESULTS

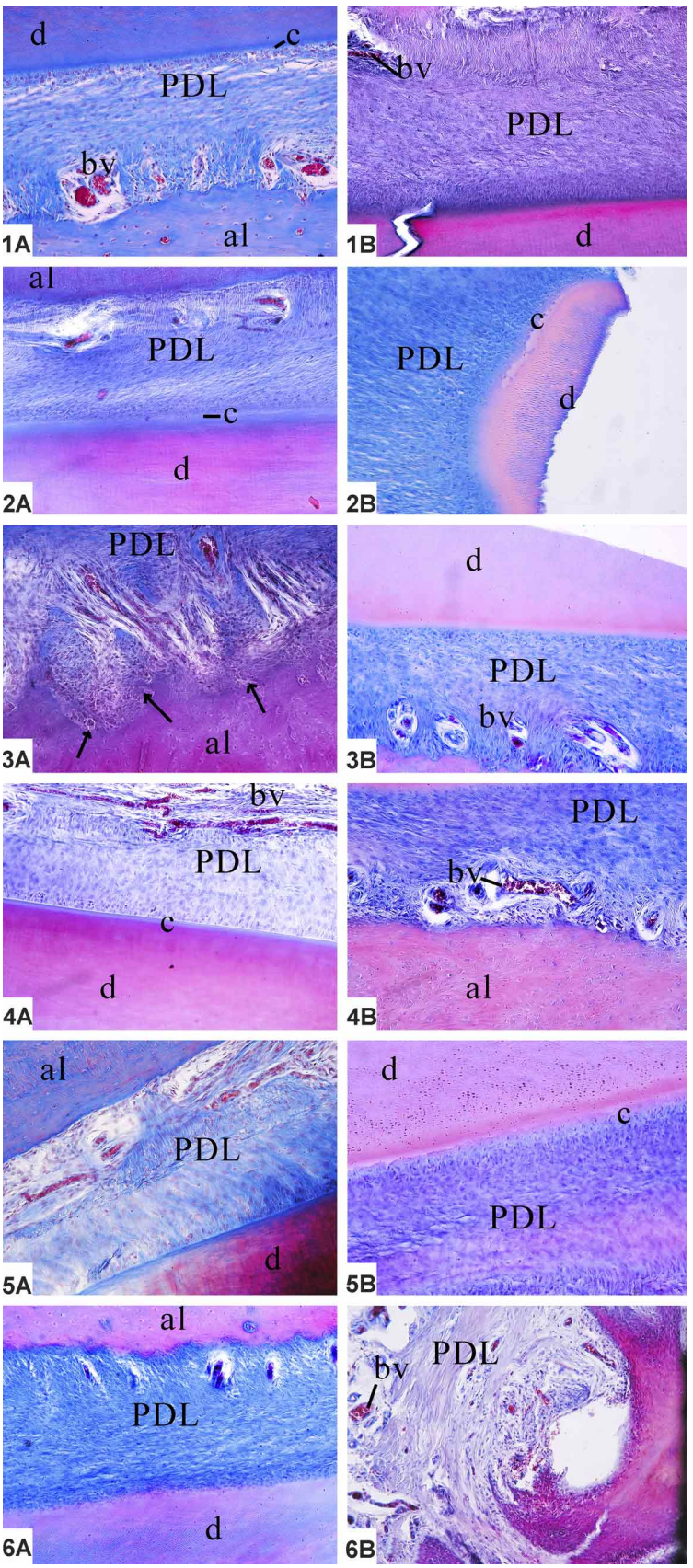
The experiment investigated the morphofunctional formations of the periodontium of the lower incisors of rats with spontaneous periodontitis (SP). In groups 1 and 2, structural periodontal disorders were not detected, although in the 1st sample of group 2 infiltration of neutrophils was noted in the connective tissue of the marginal part of the gums (apparently a consequence of the introduction of HA to intact rats). In an intact periodontium, collagen fibers asclusters connect the tooth root cementum with the alveolar bone (Sharpey's fibers), penetrating it to form periodontal ligaments (PDL). Sharpey’s fibers around the root of the tooth are directed in three projections, radial (perpendicular to the cementum) and obliquely oriented fibers dominate in the ligament. We investigated PDL in the projection of the longitudinal section of the tooth. This made it possible to detect Sharpey’s fibers at different levels of the tooth root, and repeatedly recorded them in separate clusters in the radial direction from the cementum to the alveolar bone tissue (Figs.1a,b and 2a).

Between Sharpey's fibers, the entire interstitial space (from the root of the tooth to the alveolar bone) is filled with connective tissue with blood vessels. A thin layer of cementum (basophilic line between dentin and ligament on the H&E and Picro-Mallory stained sagittal section) was recorded on the tooth root contour, along its entire length the layer is regular, and on the root apex the layer grows and contains cementocytes (cellullar cementum).

The contour of the alveolar bone, to which the PDL joins, is regular and only focally has channels through which vessels penetrate (Volkmann's canals). The vascular architecture of PDL is interesting. It contains a relatively dense network of small blood vessels, which are oriented longitudinally and radially relative to the root of the tooth. Several longitudinal vessels form a network of small collaterals that penetrate into the alveolar bone. That is, the vessels in PDL are integrated into the alveolar bone, being the only system of vascularization of the periodontium of blood vessels. We also found that at the level of the apex of the root of the tooth, the vessels have a higher density and vascularize the pulp of the tooth. Given this, it is obvious that changes in the microcirculation of periodontal vessels can affect the trophism of the pulp.

The pulp cavity in group 1 and 2 contains intact pulp: connective tissue with blood vessels and nerves. On the margin of the pulp and dentin a regular layer of odontoblasts was recorded. In the dentin, tubules formed by dentinoblasts were clearly traced. These tooth structures of group 1 were control for comparison groups with SP. In group 2, at the 8th week of the experiment, a slight reorganization of the collagen fiber bundles at the level of the root apex was detected, without damage to the nerve trunks, which is probably due to a mechanical factor (consequence of HA “HD-1.0”).

In all groups of rats with SP (groups 3-6) a weak infiltration of neutrophils and fewer eosinophils and basophils into the connective tissue of the gums, rarely into the marginal periodontium was found, which is morphological evidence of a local inflammatory reaction. The detected changes are limited to these regions and over the other root length, apical periodontium were not registered. Structural elements of PDL connective tissue – Sharpey’s fibers, fibroblasts, blood vessels - as in groups 1 and 2 were also found. Total reduction of collagen was not detected, but a focal loss of Sharpey’s fiber density was observed (Figs. 1: 3a, 3b), although there was more free



interstitial space. This is vividly illustrated by TEM (Fig. 2: 3), given the higher resolution and technical capabilities of the method. TEM allowed to detect in detail the ultrastructural PDL in the marginal zone of PDL. Two main features of PDL changes in SP have been identified: infiltration of macrophages and neutrophils, which actively phagocytose various detritus, and migration of basophils (mast cells) between clusters of collagen fibers with their active degranulation. A significant number of free electron-dense granules of mast cells were found in the interstitial space. In some samples it was found that macrophages phagocytosed these granules. The leukocyte density in PDL was high in areas with extracellular matrix (ECM) edema (recorded as the appearance of a free electron-transparent space between collagen fibers and cells). In general, periodontal changes were variable, mainly including a decrease in the density of collagen fibers, blood vessel stasis, local edema. In rare cases, resorption of alveolar bone tissue has occurred. At the morphological level, this consisted in a change in the contour of the alveolar bone, the appearance of resorption fissure with macrophages (Fig. 1: 3a). Changes in the cement contour were detected in such samples (focal loss of the cement line, in the sample at 4th weeks of the experiment). Interestingly, at the same time in other samples an increase in the contour of the cement line by 10-40 μm ($P < 0,05$) (basophilic line between dentin and PDL on H&E and Picro-Mallory stained sagittal section) was recorded. There was no significant difference between PDL changes at 4th and 8th weeks, which we could quantify.

In other groups with SP - groups 4, 5 and 6, the state of PDL was similar to that described in group 3.

Fig. 1. Periodontal changes of mandibular incisors of intact rats and rats with spontaneous periodontitis. Note: 1 - control group (group 1); 2 - intact group + HA "HD-1.0"; 3 - SP group; 4 - SP + HA "S-2.4"; 5 - SP + HA "ST-2,4"; 6 - SP + HA "HD-1.0"; a - period of 4 weeks; b - period of 8 weeks; al - alveolar bone; PDL - periodontal ligament; d - dentin; bv - blood vessel; c - cementum; → bone resorption. Picro-Mallory staining, × 200.

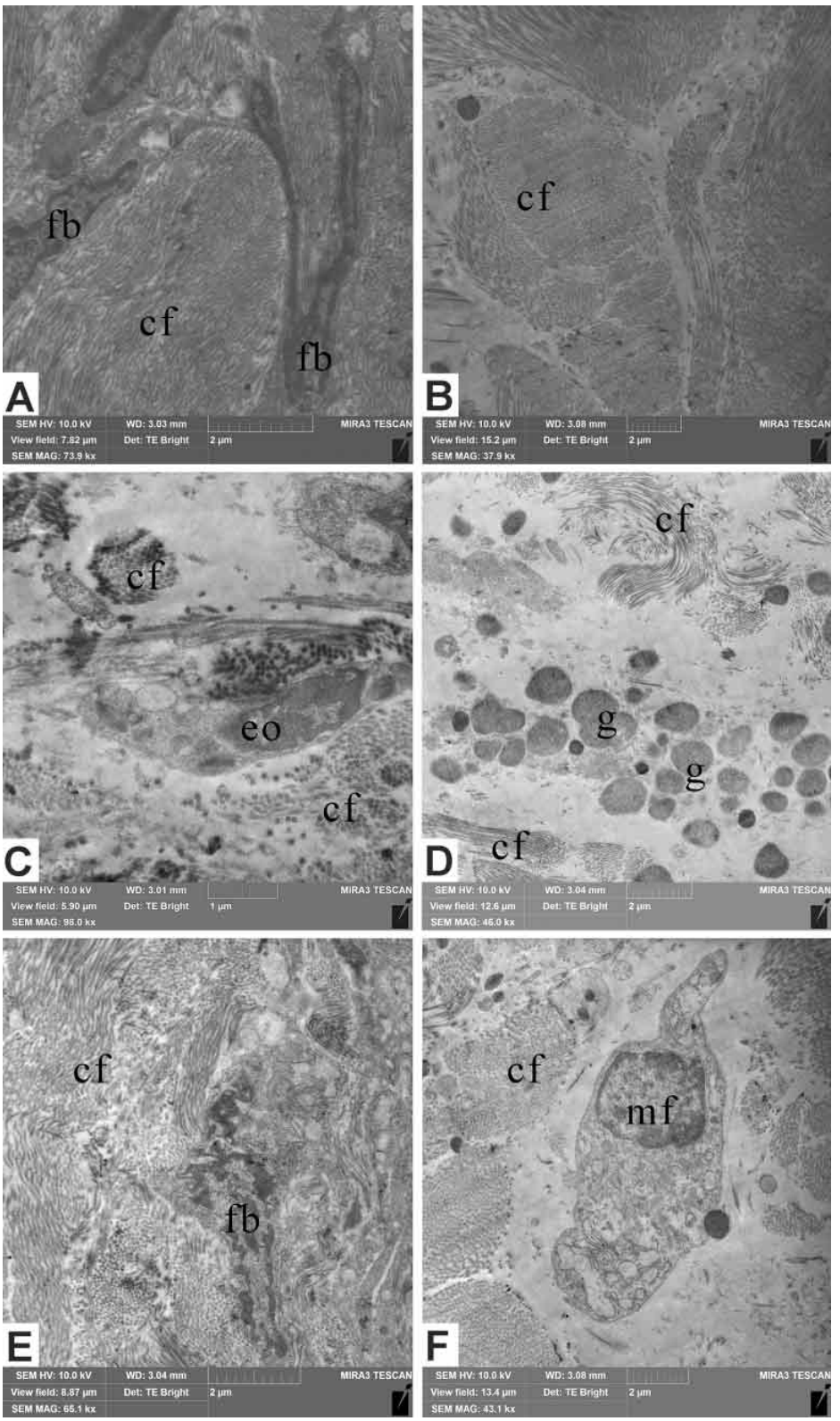


Fig. 2. TEM of PDL in rats with SP. Note: A – intact control; B – HA "HD-1,0"; C– SP; D – SP + HA "S-2,4"; E – SP + HA "ST-2,4"; F – SP + HA "HD-1,0"; cf – collagen fibbers; mf – macrophages; fb – fibroblast; eo – eosinophils; g – mast cells granules.

Group 4 also showed an increase in the interstitial space between collagen fibers and stasis of blood vessels in PDL (Fig. 1: 4a, 4b). No sharp differences were found between the 4th and 8th weeks. TEM showed a reduction in collagen fibers and an abundant density of free mast cell granules, as already noted in groups 3 (Fig. 2: 4). Although at 8th week a change in the contour of the cement and its multifocal reduction (up to 9-11 µm against 17-20 µm at 4th week, $P < 0,05$) was revealed. That is, changes in cement have progressed. We did not detect foci of free HA, but macrophages contained a significant number of phagosomes with a homogeneous content (electron-dense), which may indicate phagocytosis and elimination of HA by migrating macrophages.

In group 5, a higher density of both collagen fibers and fibroblasts was observed, but macrophages and neutrophils were equally present as in groups 3, 4 and 6. It should be noted that the ECM in marginal PDL contained mainly collagen fibers, which are oriented by clustered in different projections (Fig. 1: 5a, 5b). Multidirectional collagen fibers are successfully visualized by TEM (Fig. 2: 5).

At the 8th week, multifocal reduction of cement was also detected (up to 10-13 µm against 18-25 µm at 4th week, $P < 0.05$). Structural signs of alveolar bone resorption were also not detected, except for the 1st sample at 4th week (focal displacement of the alveolar bone contour, osteomucoid resorption).

In group 6 at 4th week there was a fairly well-preserved PDL (dense bundles of collagen fibers, fibroblasts), but at 8th week there were already destructive changes and focal infiltration of leukocytes, closer to the apical periodontium (Fig. 1: 6a, 6b). Cellular reorganization of apical PDL involved the migration of fibroblasts and nonresident cells, and among them macrophages, neutrophils and mast cells dominated, i.e. those leukocytes that implement nonspecific immune protection and support inflammatory responses. Fig. 2: 6 illustrates phagocytic macrophage and altered ECM in PDL.

In some samples HA was detected. In group 5 spherical inclusions at the optical level at 4th weeks of the experiment (diameter from 4.2 μm to 7.3 μm) were revealed, and in group 6 at 8th week - at the ultrastructural level (diameter from 0.23 mm to 1.17 μm) . We concluded that HA "ST-2,4" and HA "HD-1,0" were gradually eliminated from marginal periodontal tissues within a time period, but as the TEM results showed, fine inclusions still remain in the interstitial space of the connective tissue of the damaged periodontium. In other observations where hyaluronic acid preparations (group 2 and 4) were administered, no inclusions at the optical and ultrastructural levels were recorded.

Unfortunately, no significant differences in PDL between comparison groups with SP were found in the experiment. Although due to the lack of resorption of alveolar bone in groups 4-6, statistical analysis showed intergroup differences. This is reflected in the evaluation results by the scale of Aguirre *et al*. (Fig. 3). The trend of improvement in groups 4 and 5 can be considered, as the changes in the comparison groups were moderate or weak, and the quantification is difficult.

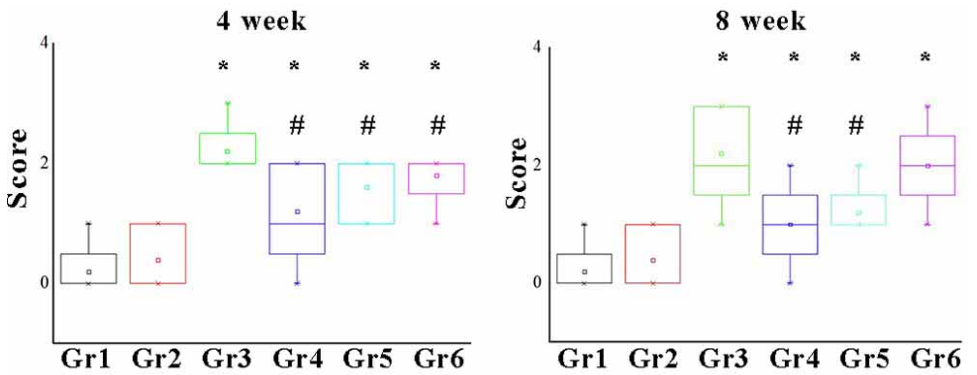


Fig. 3. The value of the level of structural changes of the periodontium (score). Note: * - significant to group 1 (P <0,05); # - significant to group 3 (P <0,05).

DISCUSSION

Thus, the experiment was set to investigate the structural changes of the periodontium in rats with SP and to assess the effect of HA on the disorder. We used histological and morphometric methods, the scale of periodontal evaluation by the methods proposed in the literature. H&E staining technique is routine, but gives good results in the identification of structural formations, including cell nuclei, blood vessels, bone tissue. But the best and contrasting result was achieved by the Picro-Mallory method, namely the identification of collagen fibers in PDL (Sharpey's fibers, Sharpey's fibers), their density, direction relative to the root of the tooth and even focal loss of density in rats with SP. TEM allowed to study the ultrastructural state of the ECM in PDL, namely bundles of collagen fibers, to assess in more detail the changes in PDL.

Histomorphometric assessment of the periodontium is a difficult task, so the publications mainly give the results of semi-quantitative analysis, as shown in various publications (Nakahara *et al*., 2014). Periodontal evaluation is to measure the relative amount (%) of damaged tissue (Salomão *et al*., 2014) or the degree of periodontal damage (+ / ++). Mild periodontal changes may not affect morphometric parameters at all, such as periodontal thickness (de Souza *et al*., 2014), so quantitative analysis may have advantages. The expressed changes are additionally combined with increase in distance between a cement-enamel junction and an alveolar bone and then linear measurements are rational (Irie *et al*., 2008).

However, in our experiments we found only moderate

changes in the periodontium, which were limited to the gums, occasionally the marginal part of the periodontium and occasionally focal changes in the cementum (increase or decrease) were found. Unfortunately, the identification of early changes in cement is limited by the resolution of the optical device used (light microscopy), ultrastructural changes in the ability to prepare samples for transmission electron microscopy (TEM). Although a number of authors have examined the tooth root and alveolar bone by scanning electron microscopy (SEM) (Chen *et al*.), but we believe that the limited quantitative measurements and the probability of the formation of artifacts in the preparation of samples for SEM does not provide fundamental advantages in the study of periodontitis.

In rats, SP was noted in changes in other tooth tissue structures, including pulp vessels and PDL (dilatation and stasis). The condition of the pulp and root canal is important because damage to the pulp impairs trophism and over time can affect the condition of the hard tissues of the tooth (Herber *et al*.). Changes in tooth biomechanics, as well as spontaneous periodontitis in laboratory rats, did not have pronounced inflammation, such as abscess. This is due to the fact that the experiments include intact animals and simulate weak changes without infection, so the regional microenvironment of the defect is rich in cells from healthy tissues, while in clinical conditions inflammation prevents periodontal healing. Therefore, the reproduction of the model of periodontitis in rats is promising, which is as close as possible to the pathophysiological processes of the disease and allows to evaluate various treatment modalities.

In this study we also studied the effect of HA of different molecular weight on periodontal tissues. As is known from the literature, HA in vitro stimulates early osteogenic differentiation of human periodontal cells (Fujioka-Kobayashi *et al*., 2017). Application of 0.2 % hyaluronan gel (Xu *et al*., 2004) once a week for 6 weeks did not give the desired clinical result, which can be explained by the low content of hyaluronic acid from the optimal one to achieve significant improvement. In our studies, we administered three forms of HA with a molecular weight of 1.0 MDA and 2.4 MDA once a week for 3 weeks, which expressed in terms of a 2 % solution of HA, but this did not affect the changes in PDL, and significant changes in the microcirculatory system of the periodontium and pulp were not revealed either. Only the lack of resorption of alveolar bone tissue resulted in a lower score by the scale of Aguirre *et al*. in groups 4 and 5, so the effect of HA "S-2,4" and HA "ST-2,4"was assessed as a trend. In addition, in groups 5 and 6, residues of introduced HA were detected, and at the ultrastructural level macrophages phagocytosed a significant amount of electron-dense homogeneous material, including granules of mast cells other than cell debris, this indicates the elimination of HA by phagocytes from tissues. Migration and degranulation of mast cells in connective tissue is a universal cellular mechanism of initiation and maintenance of inflammation, being a nonspecific response to existing HA. Recent studies suggest that mast cells have CD44 receptors for HA and, with their participation, adhere to HA, thereby migrating into the ECM of connective tissue [28]. Abundant degranulation of mast cells was a nonspecific response of these cells in all groups with SP. Other concomitant changes in PDL associated with inflammation were blood supply and vascular stasis of PDL. In addition, dilatation of blood vessels was observed in the pulp and gingival papilla. We concluded that the angioarchitectonics of the PDL vessels and the pulp are morphofunctionally related, so this explains the detected vascular blood supply in the pulp.

CONCLUSION

The study examined the structural changes of the periodontium in rats with SP and noted the main disorders (collagen reduction, resorption of alveolar bone, dilatation and stasis of the vessels of the periodontium, gingival papilla and tooth pulp), which were assessed as moderate. Morphological evidence of inflammation was infiltration of neutrophils into the connective tissue of the gums, without the formation of abscesses. Local administration of HA did not cause additional structural and functional disorders in periodontal tissues of rats with SP, but also did not affect changes in the microvascular system of periodontium and tooth pulp, PDL, only a tendency to inhibit alveolar bone resorption in rats was noted. One can consider the tendency to improve the condition of periodontal tissues in the group of rats injected with high molecular HA and HA with mannitol (2.4 MDa).

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RESUMEN: El objetivo del artículo fue estudiar los cambios en los tejidos periodontales en ratas con periodontitis espontánea (PE) y evaluar el efecto del ácido hialurónico (HA) sobre el estado del periodonto. Las ratas Wistar con signos de PE se dividieron en 6 grupos: 1) grupo intacto; 2) animales intactos con HA "HD-1,0 MDa"; 3) grupo PE; 4) PE con HA "S-2,4 MDa"; 5) PE con HA "ST-2,4 MDa"; 6) PE con HA "HD-1,0 MDa". En las ratas con PS se observaron los principales cambios estructurales (re-

ducción de colágeno, reabsorción del hueso alveolar, dilatación y estasis de los vasos del periodonto, papila gingival y pulpa dentaria), que fueron evaluados como moderados. La evidencia morfológica de inflamación fue la infiltración de neutrófilos en el tejido conectivo de las encías, sin la formación de abscesos. La administración local de HA no causó daño estructural adicional en los tejidos periodontales de las ratas con PE, pero tampoco se produjo cambios en el sistema microvascular del periodonto y en la pulpa dental y ligamentos periodontales.Se observó una tendencia a inhibir la resorción del hueso alveolar. Se puede considerar la tendencia a mejorar el estado de los tejidos periodontales en el grupo de ratas inyectadas con HA de alto peso molecular y HA con manitol (2,4 MDa).

PALABRAS CLAVE: Periodontitis espontánea; Rata; Ligamento periodontal; Ácido hialurónico; Pulpa; Inflamación; Vasos sanguíneos.

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