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CENTER ZA STOMATOLOŠKE RAZISKAVE CENTER FOR DENTAL RESEARCH

Leading article/Uvodnik

TEN YEARS (1987-1997) OF CENTER FOR DENTAL RESEARCH, LJUBLJANA, SLOVENIA

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The pioneers of Slovenian stomatology headed by prof. dr. Jože Rant established Dental Clinic and education of stomatologists at Faculty of Medicine in first years after the 2nd World War. Prof. dr. Jože Rant and his colleagues and particularly the next generation of professors of stomatology headed by prof. dr. Čedomir Ravnik realized that besides clinical expertise, basic and clinical research is needed for good dental education at Department of Stomatology of Faculty of Medicine in Ljubljana.

In the late sixties first collaboration of stomatologists from Dental Clinic and physicists from Institute Jožef Stefan discovered paramagnetic centre CO_3^{3-} in irradiated tooth enamel (1). This centre was found to be a useful parameter to measure the degree of crystallite arrangement in caries resistant and susceptible permanent and deciduous tooth enamel (2-4) and in root cementum (5).

Electron spin resonance was found to be also appropriate method to study the caries process by diffusion of non-charged molecules (6) and ions (7) through enamel structure. The susceptibility to caries was further studied by evaluation of presence of different trace elements (8, 9) in tooth enamel.

These results in the seventies and early eighties encouraged prof. dr. Uroš Skalerič to promote the establishment of the Center for Dental Research in Slovenia.

After several years of efforts prof. dr. Mitja Bartenjev, director of Dental Clinic and prof. dr. Tomaž Kalin, director of Institute Jožef Stefan signed the act (September 25, 1987) of founding the Center for Dental Research as a joint project of both institutions.

Since foundation of the Center the collaboration between stomatologists from Dental Clinic and scientists from Institute Jožef Stefan became more intense including new fields of interdisciplinary research. The structure of tooth enamel was further characterized by nuclear magnetic resonance (10), which was found to be a useful method for imaging of dental and periodontal tissues (11-13) and for structure analysis of dental materials (14).

From the mid-eighties, besides biophysical dental research, the biochemistry collaborative studies were started. This cooperation lead to the discovery of increased levels of cathepsins B, L and D in inflamed gingiva and crevicular fluid (15, 16). The presence of cysteine proteinase inhibitors (17) and disbalanced proteolitic activity (18) in periodontal inflammation was further evaluated by the decreased concentration of a_2-M in crevicular fluid (19) and cystatin C in inflamed gingiva (20). The role of periodontal inflammation in systemic immunologic response was demonstrated by elevated concentration of proteinase inhibitor a_2-M in the sera of patients with advanced periodontal disease (21).

More recently, the contribution of growth factors and oxygen free radicals in the etiopathogenesis of periodontal disease was studied in collaboration with the Laboratory of Immunology at NIDR, NIH, Bethesda, MD, USA and Veterinary Faculty in Ljubljana (22-24). These studies are aimed to the use of growth factors or inhibitors of oxygen reactive intermediates for treatment of chronic inflammation (25) and periodontitis (26).

Cooperation with Fotona Co. from Ljubljana lead to the new product - a Twin-light laser - where Nsd-YAG and Er-YAG lasers are used for treatment of tooth decay (27) and periodontally involved root surfaces (28).

Collaborative studies with microbiologists, immunologists and physicans revealed the prevalence of oral pathology in HIV-infected population in Slovenia and (29) and pathogenic effects of HIV and HSV viruses on gingival fibroblasts (30). Saliva was shown as a useful body fluid for detection of HIV antibodies in seropositive patients (31).

The role of systemic health in patients in periodontal disease was studied (32). These results demonstrated that not only periodontal disease possess the risk for systemic diseases like cardiovascular disease, stroke, diabetes and preterm low birth weight, but that the different systemic diseases may also influence the development the chronic inflammation in periodontium.

Finally, the interdisciplinary research at Institute Jožef Stefan and Center for Dental Research resulted in five Ph.D. thesis and in ten M.Sc. thesis in dental field. The results of the studies were presented at several international congresses and conferences and contributed to the international affirmation of Slovenian biomedical and oral sciences.

At the tenth anniversary of Center for Dental Research the founder and collaborators are looking forward for further development and promotion of oral and dental research in Slovenia and abroad.

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UROŠ SKALERIČ

was born on April 9, 1945 in Ljubljana. He graduated at Faculty of Medicine - Division of Stomatology in 1968. He received a degree "Public Health" at School of National health "Andrija Štampar" at Zagreb in 1972, M.Sc.degree in 1975 and Ph.D. degree in 1979, both at Faculty of Medicine in Ljubljana. He passed the speciality board exam in dental diseases, oral medicine and periodontology in 1978. He was elected a Chairman of Department of Oral Medicine and Periodontology of University Dental Clinic at Ljubljana in 1983. He was elected Assistant in 1977, Docent (Assistant Professor) in 1985, Associate Professor in 1990, Full Professor in 1993 and Chairman of the Chair of Oral Medicine and Periodontology in 1994. He was elected a Vice-Dean of Faculty of Medicine of Ljubljana in 1995 and Director of Dental Clinic in 1997. He was elected a Research Associate in 1973 and Scientific Councilor in 1994 at Department of Solid State Physics of Institute Jožef Stefan in Ljubljana.

In 1987 he founded and is since Director of the Center of Dental Research, a joint institution of Dental Clinic and Institute Jožef Stefan. In the academic year 1980-81 he was a Senior Fulbright Scholar and Visiting Associate Professor at Department of Periodontology, School of Dentistry, Emory University, Atlanta, Georgia, USA. In the years 1987-90 he was a Senior Fulbright Scholar and Research Associate in Department of Microbiology and Immunology, National Institute of Dental Research, NIH, Bethesda, Maryland, USA.

His early research focused on biophysics of hard dental tissues, including studies of crystallite arrangement and permeability of tooth enamel and cementum by electron spin resonance. Later his interdisciplinary research with biophysicists, biochemists, mathematicians, biologists and psychologists , focused on prevalence and natural progression of periodontal disease among Slovenian population, role of cysteine proteinases and their inhibitors, growth factors, cytokines, oxygen reactive intermediates and stress in pathogenesis of periodontal disease and chronic inflammation, role of HIV and other viruses in periodontal inflammation, MR imaging periodontal tissues and treatment of periodontitis in experimental animals by free radical and enzyme inhibitors.

He presented the results of his collaborative studies in 15 invited lectures and 35 presentations at International Congresses, Conferences and Universities. His bibliography numerate 270 items, including 115 articles, 30 of them published in International Journals and Proceedings. In 1997 he was appointed the Visiting Professor on Eastman Dental Institute for Oral Health Care Sciences, University of London.

He is President of Slovenian Dental Health Association and President of Slovenian Society for Oral Medicine and Periodontology. He is member of the following international professional and research organizations: IADR, ORCA, FDI, AAP, IAP, EAP and ICO and EAOM. He was president of the Continental European Division of the International Association for Dental Research in the years 1990-92 and 1994-95. He is vice-president of the International Academy of Periodontology. He is representative of Slovenia in EU COST 8 "Odontogenesis" Project and in the Council of European Chief Dental Officiers. In 1997 he was nominated the Ambassador of Science of Republic of Slovenia.

Research article/Raziskovalni prispevek

AN EPR APPROACH TO DENTAL TISSUE RESEARCH

RAZISKAVE ZOBNIH TKIV Z ELEKTRONSKO PARAMAGNETNO RESONANCO

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Key words: *microcrystal alignment; molecular diffusion; spin labels; electron paramagnetic resonances*

Abstract – *A short review describing the possibilities of electron paramagnetic resonances for structural and functional characterization of dental tissues is discussed. In the first part the structural characterization related to the microcrystal alignment in the enamel and cementum is reviewed, in the second part the methods to characterize the molecular diffusion in dental tissue are discussed. Finally, the methods of kinetic imaging as well as 1D-EPRI imaging are described.*

Introduction

The dental tissues are well described from the histological point of view. Though, the pathology of the complicated hard tissue structure draw attention of several other approaches, searching for a microscopic view , by which the functional relations involved in physiological processes could be explained. There are ample evidences, that there should be communication, between the tissues composing the tooth, via the molecular and ionic transport. Composition and structure of teeth have been studied by chemical methods and × rays diffraction was used for the evaluation of mineralization. Electron paramagnetic resonance (EPR) method was used much later to elucidate the basic phenomena in structure and permeability of mineralized dental tissue.

The morphology of tooth tissue is well documented by classical microscopic examination (1). Additional insight was gained by X-ray scattering, where the hydroxyapatite microcrystals have been studied (2), as well as the inorganic amorphous components have been identified (3).

Chemical analysis combined with other techniques produced a relatively sound presentation of the enamel, dentine as well as root cementum constituents (4, 5). Several questions related to tissue transport and the possible transport pathways remained. In our studies by electron paramagnetic resonance the crystallinity was studied by the radiation damage (6). CO_3^{3-} radicals have been identified as products of gamma irradiation, located within the hydroxyapatite microcrystals. It is the electronic structure of these radicals which is anisotropic and by EPR spectra provide the information of their orientation in the mineralized tissues.

The basic idea that the microcrystals contain also carbonate ions, has been well documented (7). The primary orientation of the CO_3^{3-} radicals is defined by the microcrystal matrix. Since the microcrystals are partly oriented in the enamel and cementum the EPR spectra could provide the information on such microcrystalline alignments.

This approach demonstrated the increase of microcrystal alignment from the dentine enamel junction towards the enamel surface (8–10).

Additionally, from the local radical concentration measurement we found that the carbonate concentration is also depth dependent. It increases from the enamel-dentine junction towards the enamel surface (11).

Similar measurements in cementum showed that the alignment of microcrystal is different from that measured in the enamel. A model was proposed where an axially symmetric arrangement of the microcrystals is found with the symmetry axes perpendicular to the cementum surface normal (12).

Another important question is how molecules bind to and transport across the tooth matrices. In these measurements we came up with the following values of the diffusion coefficients for the enamel, cementum and dentine, evaluated by the penetration rate of spin probe EPR spectra in human permanent enamel 4×10^{-7} cm^2/s, human deciduous enamel 8×10^{-7}cm^2/s, and horse enamel 5×10^{-7}cm^2/s (13). Evaluation by other techniques showed the following values: tritiated water molecules diffusion across human dental enamel 1×10^{-10} cm^2/s (14), fluoride ions and C^{14} -chlorhexidine across human enamel $(1–9)\times10^{-10}$ cm^2/s in the diaphragma cell (15), Na^{24} ions in human dental enamel 2×10^{-10} cm^2/s (16), and water molecules in human dentine 2×10^{-6}while albumin 4×10^{-8} cm^2/s (17). For steady state flow of spin probe molecules across human cementum we found $(3.1\pm2.7)\times10^{-10}$ cm^2/s, on the other hand the intrusion measurements furnished two rates: the fast $(2.2\pm2.7)\times10^{-6}$ and the slow $(4.7\pm3.9)\times10^{-9}$ cm^2/s (18). Mn^{2+} ions penetration from both sides into the enamel sample showed two different diffusion coefficient values for the penetration from the enamel surface 7.8×10^{-8} cm^2/s and for the penetration from the dentine enamel junction side 9.3×10^{-8} cm^2/s. After longer penetration time both sides produce the diffusion constant 2×10^{-9} cm^2/s. Concentration profiles in the tissues as well as the flow across the enamel have been measured (19, 20), using the nitroxide spin probes.

The spin probe molecules are molecules containing the paramagnetic nitroxide group. Such molecules can diffuse and are relatively stable in the tooth matrices as well as in aqueous phases. These EPR spectra reveal a series of information on the molecular concentration, mobility as well as the polarity of the matrix in which the spin probe are dissolved.

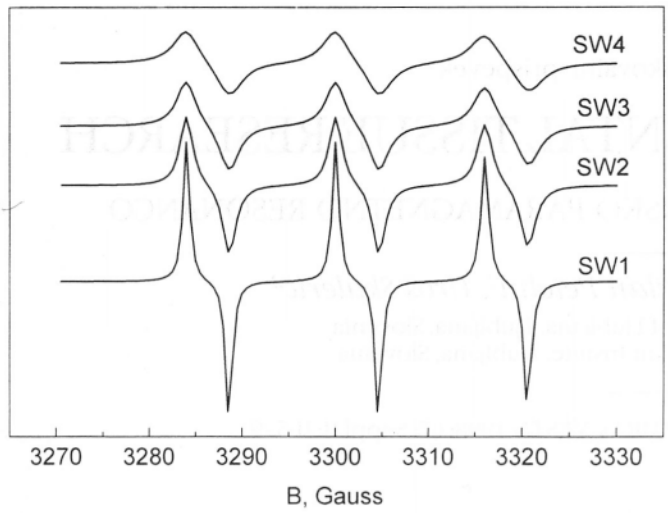

Fig. 1. *Calculated 1D-EPRI spectra for a sample of 0.1cm thickness and a uniform constant concentration across the sample. The possible spectra for the magnetic field gradient G = 46.0 Gauss/cm reconstructed with different individual line widths. Taken from the upper spectrum downwards (SW4 to SW1) the linewidts DHpp are 2.0, 1.5, 1.0, and 0.5 Gauss.*

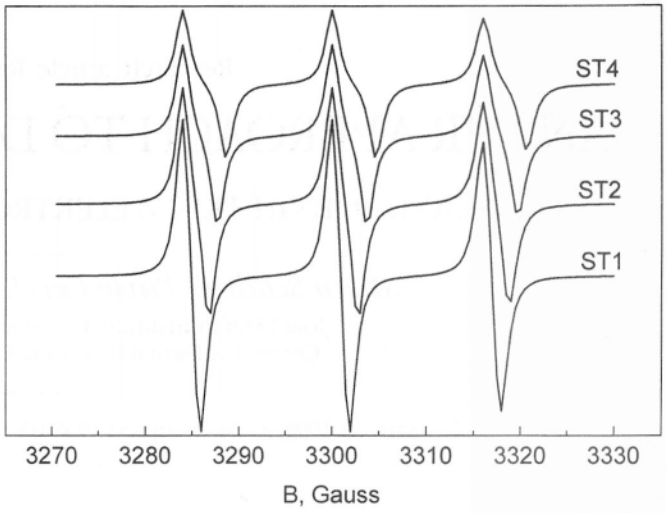

Fig. 2. *Calculated 1D-EPRI spectra, for samples from ST4 to ST1 being 0.1, 0.08, 0.06, and 0.04 cm thick, with DHpp = 1.0 Gauss, and G = 46.0 Gauss/cm./ The concentration profile is uniform like in Fig. 1.*

First of all, we tried to estimate the tissue content of the relatively exchangeable mobile aqueous phase by simple air-drying of the sample (21). There was a significant difference resulting in a smaller weight loss in deciduous as in permanent ones.

The organization of microcrystals in prismatic structures in enamel was studied with respect to the size of microcrystals and possible correlation with the caries resistance of teeth. We found that the size of microcrystals was not correlated with the caries resistance and that only microcrystal alignment in the enamel is significantly correlated with the caries resistance (22). Initially we believed that larger crystals would be less vulnerable, due to the lower rate of solubility, but this parameter is probably not decisive in the processes leading to decay.

Due to the structure and composition of the enamel it was tempting to compare the hard tissue with a biological membrane. Namely, the lipid component in the aligned hard matrix of microcrystals as well as lipids and proteins, which might be in part fastened to the inorganic surfaces in a relatively free aqueous domains, should provide a matrix to perform at least some functions of molecular transport as well as ion permeability resembling those in the biological membranes.

We would like to discuss the basic idea that the hard tissue could exhibit some regulative functions like a biological membrane. Enamel is delineated by two compartments, the internal is blood supplied pulp and the outer is oral cavity. It also provides the sensitivity to mechanical stresses, which presumably could be a piezoelectrically induced electrical potential.On the other hand the sensitivity to pH alterations probably relays on the build up electrochemical potentials imposed by the slight diffusion rate differences between the penetrating ions.The spontaneous transport of molecules could represent a way to sustain the tissue elasticity. The presence of the fluid should be essential. However, it is typical that a biological membrane is a highly anisotropic system with several coexisting regions-domains (23) and gradients in the lateral direction and across the membrane. On the other hand, the development of hard tissue relays on the cells and subsequent degradation of their components. In part some organic structures are still left in spite of the specific deposition of the inorganic microcrystals.

Though, the tooth matrix integrity can be sustained by the viscoelastic damping elements represented by the organic gel like structure which are best kept via the adequte retention of water, in order to be capable to dissipate the mechanical energy induced by the imposed mechanical stresses Though, such a structure might be easily destroyed by the ionic fluxes or the possible chemical reactions, as well as solubility gradients and fluxes. Static deformations in an living systems have to be degraded in order to keep the system responsive to new stresses.

Tooth is a composite structure made up of a surface layer of enamel or cementum and the inner layer the dentine. Special attention should be ascribed to the interface junction between the inner and outer layers.We recently described a barrier which is the rate limiting segment for crossing of molecules in the tooth roots.

As there is a constant difference in the concentration of substances between the saliva and the pulp compartment, a constant flow of substances can be expected. Such flow might be important in sustaining some other active components necessary to keep up the ionic concentration or the flow of organic substances.

For example a constant flow from the interior of the tooth to the oral cavity would establish a potential, which could be efficient in preventing pH variation in the saliva to be transferred to the pulp, while on the other hand, it might be efficient in sustaining other flows or chemical reactions, by being coupled with them.

In this review we would like to discuss the results that characterize the molecular flow across and into the tooth mineralized tissue.There are two interesting effects: the first is the rate of diffusion, and the second are the effective transport pathways.

In enamel the presence of lipids is known and we found that the fluidity, detected by the inserted fatty acid spin probe molecules, increases with the temperature rise. This part has a special importance as the fatty acid molecules in the already existent organic components/ matrix may be the lipid components. However, we have also shown that such molecules enter the enamel and can be again washed out. That could be an efficient way to exchange the possibly chemically altered, peroxidated molecules. Lipid peroxidation per se is quite pos-

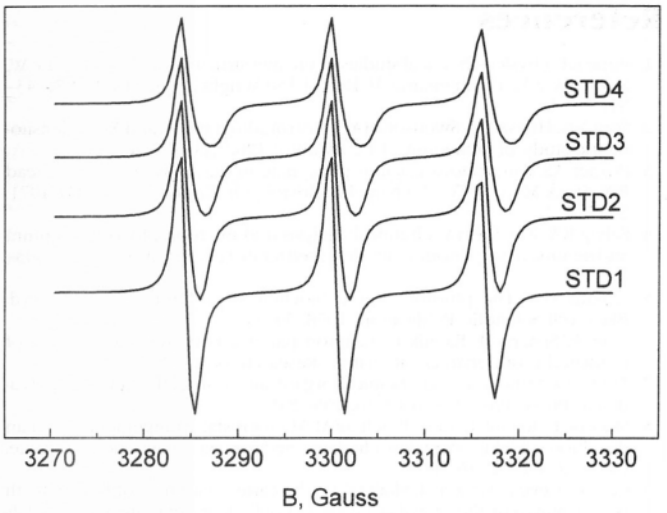

Fig. 3. *The same calculation and conditions like in Fig. 2, where in samples from STD4 to STD1 of different thickness 0.1, 0.08, 0.06, and 0.04 cm (except that here the concentration profile is not uniform but decreases linearly from 1 to zero across the sample.*

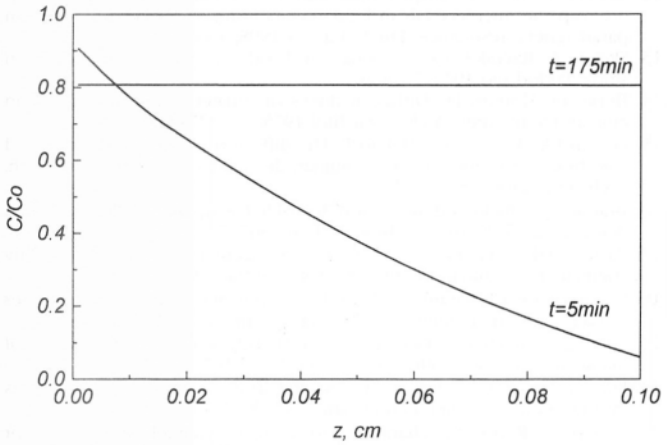

Fig. 4. *The calculated concentration profiles for a 0.1 cm thick sample established after five or 175 minutes, when the diffusion coefficient is 1×10^{-6} cm²/s.*

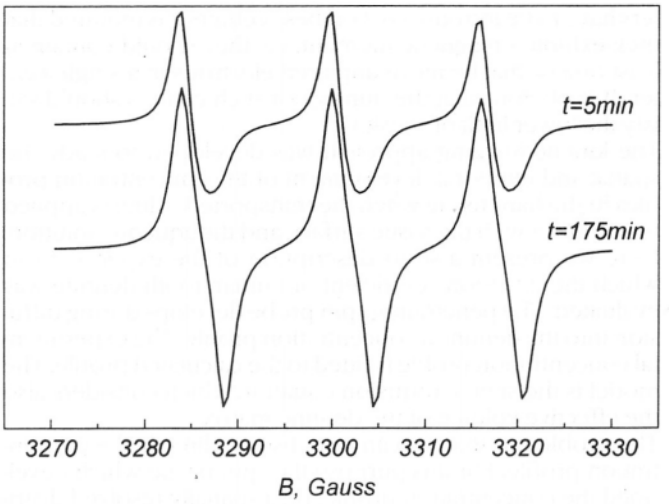

Fig. 5. *The corresponding calculated 1D-EPRI spectra with DHpp = 1.0 Gauss, and G = 46.0 Gauss/cm are shown.*

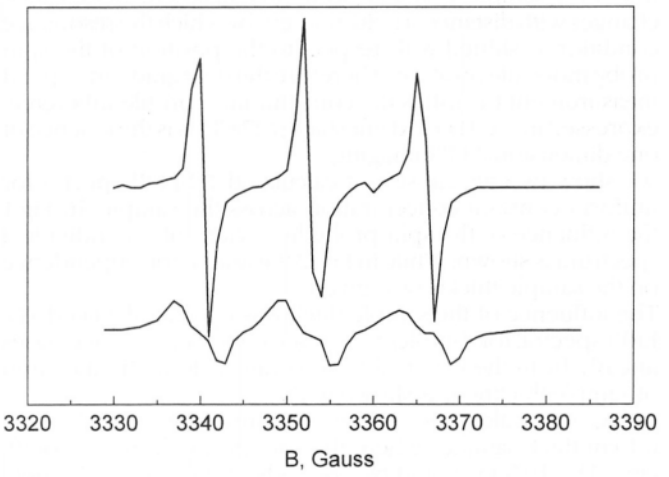

Fig. 6. *The experimental spectra in absence (narrow high amplitude lines) and in presence of the field gradient 46.0 Gauss/cm (broad line spectrum) of a 0.1 cm thick dentine sample are shown. They have been measured after 175 minutes using a one sided contact of the sample with the 1×10^{-2} M aqueous solution of the spin probe 2,2,6,6 tetramethyl-4-acetamido-piperidine-1-oxyl.*

sible, therefore, the lipids should be replenished, as damaged gel phase is less effective in maintaining the tooth matrix integrity.

In the case of higher local concentration an increased spontaneous flow of this substance could be expected. The effective adjustment of the matrix might be an important regulative phenomenon.

From that point of view it was interesting to see the incorporation of the amphiphiles like fatty acids into the enamel matrix. The first results showed that the hydrocarbon chains in the tooth enamel melt above 37° C (24).

In a series of experiments penetration as well as the elution of the aqueous phase soluble spin probe 2,2,6,6, tetramethyl-4-acetamide piperidine-1-oxyl again showed the diffusion coefficients in the range from 4×10^{-7} to 9×10^{-7} cm²/ s (25).

It is interesting to note, that for human tooth enamel is typical in fact that the surface layer renders a substantial resistance for molecular penetration. In a study where the flow across the enamel was measured it could be shown that a slight abrasion of the enamel surface layer provides the steady state conditions for several hours. However, in enamel samples with non-treated surface the flow comes to a halt. A typical concentration saturation occurs (26). It is not easy to fit together the described observation. Though it is observed that the surface layer of enamel is loaded with the carbonate ion, the microcrystal alignment is best close to the enamel surface. So it is not surprizing that in presence of organic material in this region is prone for irreversible alteration, due to solubility and possible chemical alterations, which inhibit the molecular flow across the sample. This experiment speaks in favour of the idea that in conditions in vivo the material flows are involved in and sustained by processes taking place in the enamel surface layers.

Ilustration of the 1D-EPRI method

Electron paramagnetic resonance, a spectroscopic method, detects the absorption of the microwaves in the frequency region (most often 9 GHz) by the paramagnetic sample. The sample might be paramagnetic per se or paramagnetic cen-

ters have to be introduced. For these centers it is required that they exhibit a magnetic moment, i.e. they should contain at least one or maybe more unpaired electrons in a single center. It is obvious that the number of such centers should satisfy the lower limit of sensitivity.

The kinetic imaging approach was developed to study the spatial and temporal development of the concentration profiles in the hard tissue when the transported solute is applied by a contact with the tissue surface and the aqueous solution. Here we present a short description of the experiment in which the diffusion coefficient of human tooth dentine was evaluated. The penetrating spin probe develops during diffusion into the dentine a concentration profile. The experimental concentration profile is fitted to the calculated profile. The model is the simple diffusion equation which considers also the effective volume of the dentine matrix.

The problem is that we can not observe directly the concentration profile. For this purpose the spin probe which developed the concentration profile, i.e. a spatially resolved distribution of the diffusing molecules in the sample.

If the EPR spectrum is taken all the observed molecules appear in the spectrum irrespective to their position in the sample more or less as a single spectrum typical for the matrix of the sample. Using an additional magnetic field, which changes with distance, a field gradient by which the resonance condition is shifted with respect to the position of the spin probe molecule position. Therefore the field gradient applied measurement furnishes the concentration profile inherently expressed in the 1D-EPRI lineshape (27). This is the essence of one dimensional EPR imaging.

We show two typical sets of calculated 1D-EPRI spectra for uniform constant concentration across the sample. In Fig.1 the influence of the spin probe line width of the individual spectrum is shown. While in Fig. 2 the line shape dependence on the sample thickness is given.

The influence of the sample thickness on the calculated 1D-ERPI spectra, for samples where the concentration decreases linearly from the maximal to zero value, along the direction of sample thickness, is shown in Fig. 3.

In Fig. 4 the calculated spin probe concentration profiles for 0.1 cm thick samples where the spin probe diffusion coefficient $D = 10^{-6}cm^2/s$, and two periods of the spin probe penetration. The corresponding calculated 1D-EPRI spectra for these concentration profiles are given in Fig. 5.

The experimental spectra for a human dentine sample measured after 175 minutes of spin probe penetration are shown in Fig.6. The broad 1D-EPRI spectrum together with the high amplitude EPR spectrum. The later is taken in absence of the magnetic field gradient. Here it should be stressed that the spectrum resembles the calculated one with the notion that in the calculation a partition coefficient 0.1 was used. That means that only 10% of the sample's volume is actively involved in the molecular transport.

Conclusions

We believe that the tooth's tissue by the virtue of its composition and structural complexity offers a plethora of functions which have to be operative to sustain this extraordinary efficient organ with respect to its strength and sensitivity.

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Ključne besede: *urejenost mikrokristalov; difuzija molekul; spinski označevalec; EPR*

Izvleček – *Metoda elektronske paramagnetne resonance je omogočila vrsto meritev, ki osvetljujejo strukturo in funkcijo zobnih tkiv. Želeli smo dokazati, da so zobna tkiva debele membrane z nekaterimi lastnostmi, ki so vsaj delno podobne biološkim membranam. Seveda so tkiva mineralizirana in njihove mehanske lastnosti odražajo organizacijo anorganskih mikrokristalov. Heterogena zgradba tkiva omogoča transport vodotopnih molekul in ionov. Voda, ki vzdržuje primerno gelsko strukturo med kristali je pomembna tudi za poraz-*

deljevanje napetosti in razsipanje energije, ki nastaja v tkivu zaradi žvečnih pritiskov. Po drugi strani pa transport molekul in ionov vzdržuje tokove, ki pogojujejo nekatere procese kot so antioksidacija in regeneracijske zamenjave peroksidiranih acilnih verig v lipidih. Podrobneje smo opisali primer merjenja difuzije molekul v dentinu človeškega zoba in tako ilustrirali uporabo 1D-EPRI spektroskopije za ugotavljanje koncentracijskih profilov, ki se vzpostavijo v nestacionarnih pogojih vdiranja molekul. To je del mehanizma, ki pogojuje tokove med zobno pulpo in ustno votlino oziroma obzobnimi tkivi. Fluidnosti lipidnih sestavin in oviran prenos molekul in ionov skozi vrhnjo plast sklenine, ki je bila opisana kot plast z najboljšo urejenostjo mikrokristalov, odražajo potrebo po ohranjanju sestavin, v medprizmatskem oziroma medkristalnem prostoru v zobne sklenine. Problem transporta skozi zobni cement je opisan z modelom, s katerim smo pojasnili prenos molekul preko troplastne strukture v področju zobnih korenin, kjer je poleg cementa in dentina, med njima tudi tanka plast z najmanjšo permeabilnostjo.

Review article/Pregledni prispevek

THE APPLICATION OF MACROMOLECULAR CONTRAST MEDIA TO DEFINE THE ABNORMAL MICROVASCULAR PERMEABILITY IN THE TEMPOROMANDIBULAR JOINT BY MRI

UPORABA MAKROMOLEKULARNIH KONTRASTNIH SREDSTEV ZA DOLOČANJE NENORMALNE PREPUSTNOSTI MALIH ŽIL V ČELJUSTNEM SKLEPU PRI SLIKANJU Z MAGNETNO RESONANCO

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Key words: *diagnostics; inflammation; macromolecules; temporomandibular joint*

Abstract - *The application of magnetic resonance (MR) imaging in medicine represents a novel approach in the detection of fine tissue structure and organ function in vivo. An improved enhancement of MR images is achieved by using paramagnetic contrast media. These substances enable faster relaxation of water protons in a magnetic field, what is presented as a positive contrast enhancement at the sites where the medium is concentrated. Historically, small molecule contrast media were developed first. They were successfully used in different clinical research fields. Their property of rapid diffusion to the interstitial space rendered their use limited in further clinical exploitation. The development of macromolecular contract media (MMCM) enable these molecules to dwell inside the vascular compartment. Contrast-enhanced MR imaging is a useful tool for the detection of early inflammatory changes of joint tissues (like temporomandibular joint) and for the follow up therapeutic strategies of affected joints.*

Introduction

The application of magnetic resonance imaging (MRI) in medicine represents a relatively novel approach in the detection of fine tissue structure and organ function in living organisms. The introduction of enhancement techniques of MR imaging has made possible to measure some parameters such as changes in microvascular permeability that plays an important role in pathologic conditions (1-3). Its understanding would facilitate better diagnosis and increase success of systemic drug therapy (4, 5).

The introduction of a paramagnetic substance to the biological system accelerates enhancement of MR image and allows increased contrast by the selection of suitable imaging parameters. When this paramagnetic substance is placed in a magnetic field it affects also its molecular neighbours, not only itself. A magnetic dipole of a paramagnetic agent can interact with nearby nuclear dipoles. Within an external magnetic field this interaction causes the nuclear dipole to align more quickly. When a proton is in question as a nuclear dipole, this effect is called proton relaxation enhancement (6). Relaxation times of the protons close to the paramagnet will be shortened, and this behaviour can be measured in a magnetic spectrometer as a change in longitudinal and transverse relaxation times, T_1 and T_2 respectively. Enhancement of proton relaxation rates by a paramagnetic substance is termed relaxivity (r). A higher relaxivity indicates a greater proton relaxation enhancement, and generally a stronger contrast-enhancing effect. Relaxivity depends on the magnetic field strength. Using gadolinium Gd(III) ions as a paramagnet, a positive tissue enhancement on the MRI image can be achieved, since Gd(III) exerts a particularly strong T_1 relaxivity effect in pulse sequence MR imaging settings.

Small molecule contrast media

Contrast enhancing agents in conjunction with MRI provide an opportunity to extract a detailed information on the microvascular integrity, in addition to superb anatomical data offered by unenhanced images.

A strategy was developed to eliminate incompatible disadvantages of inorganic Gd(III) salt used as a MR contrast in the biological systems. Metal ion toxicity can be avoided by incorporating Gd(III) within the core of polybasic organic ion to get a chelate, which improves Gd's in vivo solubility, defines its distribution in the extracellular fluid space, permits renal elimination by glomerular filtration, and enables safe intravenous administration of the Gd ions.

In the last decade several Gd complexes were developed including gadopentetate dimeglumine (Gd-DTPA), gadoterate

meglumine (Gd-DOTA), gadoteridol (Gd-HPDO3A), and gadodiamide (Gd-DTPA-BMA).
Gd-DTPA has a limited value and inherent disadvantages for estimation of the blood volume and perfusion, since it is a small molecule, and rapid equilibration takes place between the vascular and interstitial space after the intravenous administration. That encompasses enhancement of proton relaxation in both spaces (7, 8). Moreover, a rapid renal elimination of Gd-DTPA produces a rapid decrease in tissue Gd concentration; biological half-life in men is 90 minutes; in rats less than 20 minutes (9).

Macromolecular contrast media (MMCM)

As opposed to relatively small molecules, such as Gd-DTPA dimeglumine (MW = 547 Dalton), which rapidly equilibrate between the plasma and the interstitial space, MMCM are designed primarily to enhance the blood pool and sites of abnormal endothelial permeability. They have sufficient molecular weight, generally more than 20.000 Dalton, that assures prolonged intravascular retention. Enhancement of normal tissues using MMCM is nearly identical at 5 minutes or 50 minutes after administration (10-12). MMCM will tend to have higher proton relaxivity than small molecular Gd complexes. They are the result of binding of many Gd-DTPA ligands to a bulky macromolecular carrier, to yield albumin-$(Gd\text{-}DTPA)_{30}$, dextran-$(Gd\text{-}DTPA)_{15}$, and polyslysine-$(Gd\text{-}DTPA)_{60}$ (1, 2, 11-20) and most recently cascade-24-polymer ((21, 22)).
The major research goal is to identify a superior MMCM to carry forward into clinical trials, based on dose effectiveness, desired biodistribution, metabolism, complete elimination, and safety. Safety is an essential element in selecting compounds with promise for advancement to clinical trials. One of the major drawbacks is immunological intolerance. A significant antibody response could be induced in rats to albumin-$(Gd\text{-}DTPA)_{30}$ (23). Dextran-$(Gd\text{-}DTPA)_{15}$ and cascade polymer showed little or no immunologic activity, respectively, but relaxation characteristics, uniformity of the product and chelate stability for both agents are not optimal. LD50 values of polylysine-$(Gd\text{-}DTPA)_{60}$ in mice are higher than LD50 of Gd-DTPA dimeglumine.

Applicability of MRI to joint tissue inflammation

The synovial tissue inflammation is characterised in its early phase by leakage of plasma proteins from microvessels. In response to inflammatory mediators, released by injured tissues, endothelial cells of the microvessels contract, forming intercellular gaps which allow macromolecular solutes dissolved in the intravascular fluid to leak into the interstitial space.
The disease model of the snivel tissue inflammation has been developed in the rabbit temporomandibular joint (25, 26) with abundant synovial and villous hyperplasia and pannus formation early in the development of the disease. Using a recently developed automated computer-based technique for data analysis, acquired by contrast-enhanced MRI by albumin-$(Gd\text{-}DTPA)_{30}$ as a blood pool contrast agent, blood volume and permeability surface area maps were generated in 30 minutes after the application of the contrast medium (27). In these maps the pathologically increased permeability, restricted to the synovial membrane, was estimated to be about 8 times higher comparing to controls (25, 27).
This specially designed MRI method used in the antigen induced arthritis model has a potential for clinical application since it offers a convenient basis for the identification of lesions, for the evaluation of responses to therapy, and for the more complete characterisation of vascular abnormalities (24).

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Ključne besede: *diagnostika; vnetje; makromolekule; temporomandibularni sklep*

Izvleček – *Slikanje z magnetno resonanco (MR) predstavlja v medicini relativno nov pristop k prikazovanju podrobne anatomske strukture tkiv in funkcije organov in vivo. Boljšo resolucijo MR slik dosežemo z uporabo paramagnetnih kontrastnih sredstev. S temi sredstvi dosežemo hitrejšo relaksacijo vodnih protonov v magnetnem polju, kar se na MR sliki prikaže kot ojačen pozitiven kontrast na mestu, kjer se kontrastno sredstvo nabira. V razvoju teh sredstev so najprej razvili nizkomolekularne vrste, ki so sicer našle mesto v raziskovalni klinični uporabi, vendar prehitro difundirajo iz žilnega prostora. Zato je resolucija MR posnetkov nezadovoljiva. Glavni predstavniki med temi sredstvi so gadolinijevi kelati z večvalentnim organskim anionom, med katerimi se najpogosteje uporablja Gd-DTPA. Razvoj nove vrste visokomolekularnih kontrastnih sredstev (MMCM), v katerih je gadolinijev kelat vezan na makromolekularen nosilec, ki je lahko albumin, dekstran ali polilizin, pa je omogočil zadrževanje molekul v žilnem prostoru. Lahko je kelat polimeriziran v kaskadno kontrastno sredstvo, ki ima določene prednosti zaradi popolnega izločanja iz organizma pred albumin-(Gd-DTPA)$_{30}$, ki povzroča nezaželen imunski odziv. MMCM prestopa v intersticij le na mestih, kjer je povečana prepustnost kapilarne membrane zaradi vnetja. S tako prirejeno metodo je možno ugotavljati zgodnje vnetne spremembe na strukturah, ki tvorijo sklep (npr. na temporomandibularnih in drugih sklepih), kot so sinovijska ovojnica, sklepni hrustanec in pod njim ležeča kostnina, možno je tudi zasledovati učinke zdravljenja prizadetega sklepa.*

Case report/Prikaz primerov

POSSIBLE USE OF MRI IN PERIODONTOLOGY

A case report

MOREBITNA PREISKAVA MRI V PERIODONTOLOGIJI

Prikaz primera

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Key words: *periodontal inflammation; MR techniques; signal intensity; contrast agent*

Abstract – Background. *Accurate diagnosis is a prerequisite for a good treatment plan in periodontology. The imaging diagnostic methods which are now used in stomatology show mainly the changes in the hard dental and periodontal tissues. In our research we tested the MR imaging techniques to be used for the diagnosis of inflammation in soft periodontal tissues.*

Methods. *Periodontal tissues of a patient with periodontitis marginalis chronica was evaluated with a whole body MR imager. The MR imaging was performed at the Institute of Radiology of the Clinical Center Ljubljana before and after the periodontal therapy. The MR imaging results were compared with other periodontal parameters.*

Conclusions. *MR imaging is offering a new diagnostic modality of the inflammation diagnosis in soft periodontal tissues.*

Introduction

Magnetic resonance imaging (MRI) has become an accepted mode of examination in medicine. It allows a generation of images of the interior of the human body. It is capable of direct imaging in any plane, producing no harmful biologic effects in the diagnostic range currently utilized and has better soft tissue contrast than the best x-ray computer tomography. MRI offers a three dimensional presentation of the tissues observed (1). Image slices in any orientation can be generated without recourse to ionizing radiation. It provides the information of the tissues in three dimensions. MR imaging depends on at least seven parameters which can be selectively enhanced in order to obtain the greatest difference between normal and inflamed tissues. In stomatology MRI is used mainly for the diagnosis of pathology of temporomandibular joint, floor of the mouth, tongue, salivary glands and paranasal sinuses (2, 3). As it has been shown in other fields of medicine and in imaging of orofacial region, MRI proved to be a better modality than X-ray techniques for assessing the pathology of soft tissues (4). The imaging diagnostic methods currently used in periodontology show changes in the hard periodontal tissues, especially the resorptive changes of the alveolar bone. As periodontal inflammation is first limited to gingiva and periodontium, more precise methods are needed to evaluate the extent of inflammation in soft periodontal tissues. In our previous studies we applied the magnetic resonance imaging (MRI) as a diagnostic method in animals with experimental periodontal disease (5, 6). In this report we are presenting the first application of MRI to the patient with periodontal inflammation.

Patients and methods

A 42 yr old male patient with periodontitis was chosen for the study. The patient was in a good physical condition with no contraindications for the MR imaging procedure (1). An informal consent was given by the patient. Periodontal parameters including P1, bleeding on probing at 4 sites of all the teeth, and plaque index were taken.

After the clinical examination and X-ray analysis (plain radiography), MR images were made. The patient was imaged in the whole body MR imager (Siemens Magnetom-SP 63), with the magnetic field strength of 1.5 T, working at the frequency of 63 MHz. A head surface coil was used. Transversal 3 mm thick slices that offered the best resolution on the level of gingival tissue were performed. They were chosen from the sagital MR images of the head. Spin echo T2 weighted (TR = 2200 ms, TE = 80 ms) and T1 weighted (TR = 470, TE = 15ms) pre- and post-contrast images were performed. As the contrast agent gadolinium-DTPA (0.2 ml/kg) was used. Fat suppression T1 weighted techniques (TR = 550 ms, TE = 15 ms) were used to suppress the signal from fat, which is similar to the signal of the inflamed tissues (2).

The clinical examinations and MR imaging were taken before and after the therapy. X-ray images were taken only before the therapy.

In studying the pretreatment MR images it was evaluated that the best diagnostic sensitivity was accomplished by T1 pre and post contrast images. Thus after the therapy of the patient, only T1 pre and postcontrast images were taken.

The periodontal therapy includes root planning and scaling with curettage under local anesthesia. The therapy was performed on each tooth and was repeated after a period of three and six months. The patient was motivated and instructed for good oral hygiene including appropriate tooth cleaning and interdental flossing.

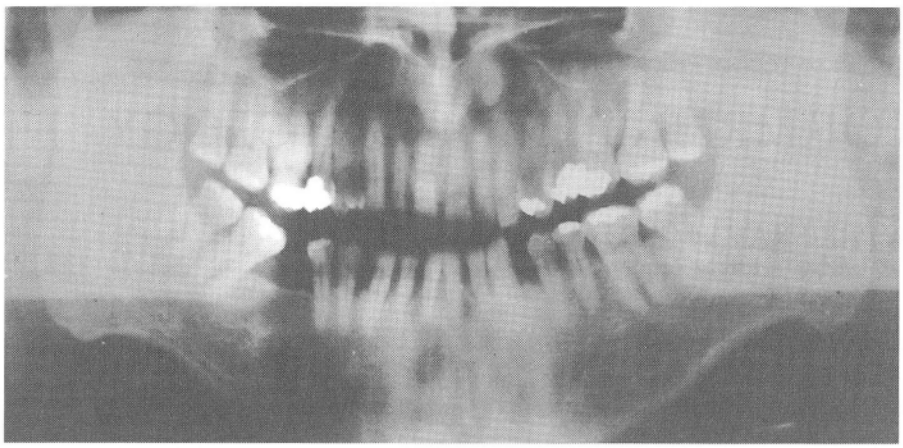

Fig. 1. *X-ray plain film (orthopan) changes in the alveolar bone could be observed.*

Sl. 1. *Rtg posnetek (ortopan) prikaže spremembe v trdih zobnih in obzobnih tkivih.*

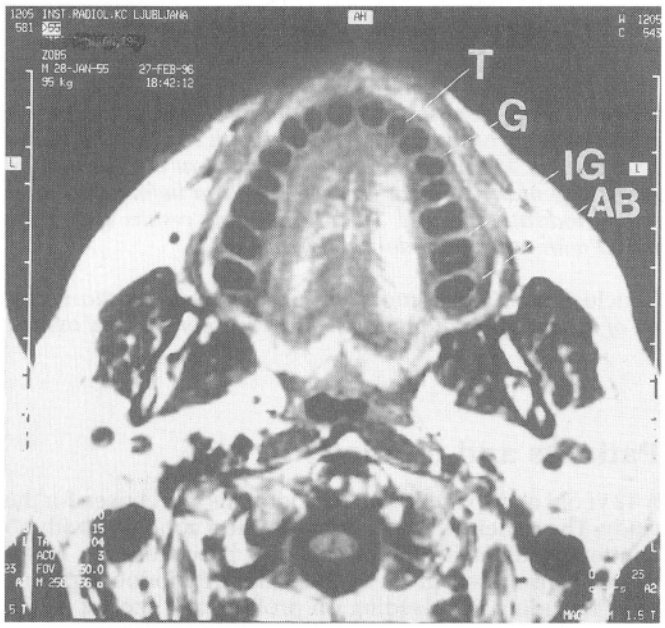

Fig. 2. *MR image of a 3 mm thick transversal slice through the maxilla. We can see the teeth (T), gingiva (G), inflamed gingival tissue (IG), alveolar bone (AB).*

Sl. 2. *MR Slika 3 mm debele transverzalne rezine skozi zgornjo čeljust. Vidimo zobe (T), dlesen (G), vneto dlesen (IG), alveolarno kost (AB).*

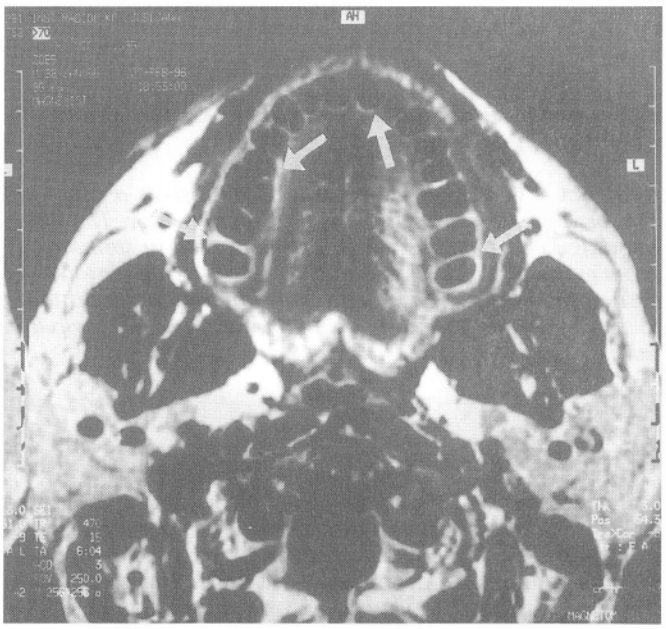

Fig. 3. *MR image of a 3 mm thick transversal slice through the maxilla. Contrast agent gadolinium-DTPA was used. The image is of the same slice as in Figure 2. The contrast between the inflamed and noninflammed tissue is increased.*

Sl. 3. *MR slika 3 mm debele transverzalne rezine skozi zgornjo čeljust,. Gadolinij-DTPA je bil uporabljen kot kontrastno sredstvo. Kontrast med vnetim in zdravim parodontalnim tkivom je povečan.*

Results

After the clinical examination and X-ray analysis *periodontitis marginalis chronica* was diagnosed. The assessment of probing depth was taken before and after the therapy to asses the pocket depth reduction.

On the MR images gingiva, periodontal ligament, dental pulp and the cancelous bone can be observed. The opposite is with the cortical bone of the jaws and with hard dental tissues like enamel, dentine and cementum. The inflamed tissue can be well resolved from the healthy tissue.

Signal intensity (SI) on MR images was evaluated for healthy (h) and inflamed (i) tissues. For the reference intensity, SI of fat tissue was taken. T_1 weighted images showed the ratio between inflamed and healthy tissues as $SI_i/Si_h = 1.3\pm0.2$. With the use of the contrast agent the SI increased in the inflamed tissues and we got the values of $SI_i/Si_h = 2.1\pm0.25$. T_2 weighted images lack such sensitivity.

After the periodontal therapy the amount of plaque as measured by P1 was reduced, and the bleeding sites on probing were reduced, and the probing depth decreased. That indicates the reduction of inflammation.

That was werified by SI measurements. T_1 weighted MR images showed SI ratio between inflamed and healthy tissues as $SI_i/SI_h = 0.9\pm0.23$. With the use of the contrast agent we got the ratio of $SI_i/SI_h = 1.5\pm0.31$

Discussion

The results of the study indicate the possibilities of MR imaging for the diagnosis of periodontal inflammation, and for monitoring of the course of the periodontal treatment (7). Different MR techniques offer different possibilities to differentiate the tissues. In our research the best contrast between

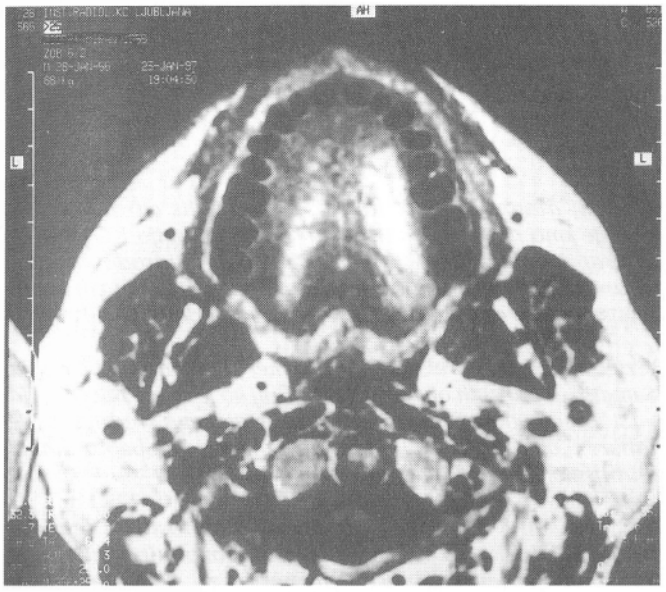

Fig. 4. *MR image of a 3 mm thick slice through the maxilla, made after the therapy. The image is of the same slice as in Figure 2. A decrease in inflammation of periodontal tissues and a decrease of SI in those tissues can be observed.*

Sl. 4. *MR Slika 3 mm debele transverzalne rezine skozi zgornjo čeljust, narejena po terapiji. Slika je narejena v isti legi kot slika 2. Opazimo zmanjšanje vnetja in tudi zmanjšanje SI v parodontalnih tkivih.*

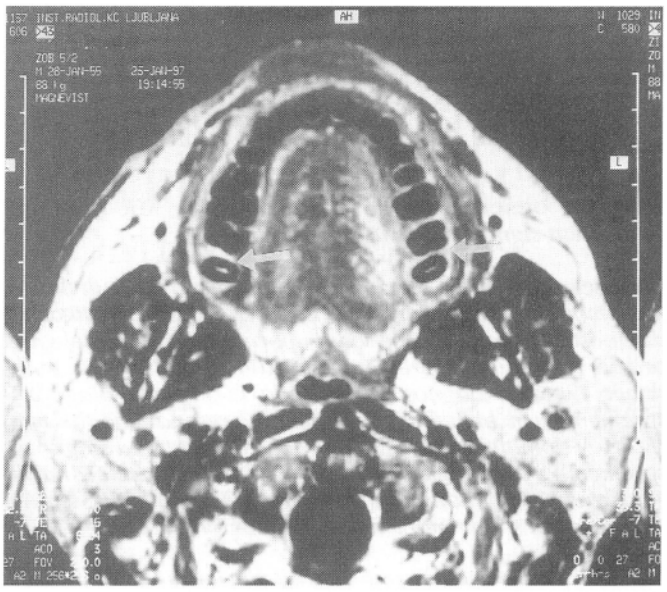

Fig. 5. *MR image of a 3 mm thick slice through the maxilla, made after the therapy. The contrast agent gadolinium-DTPA was used. The image is of the same slice as in figure 3. The contrast between the inflamed and noninflammed tissue is increased, indicating that there is still some inflammation present.*

Sl. 5. *MR Slika 3 mm debele transverzalne rezine skozi zgornjo čeljust, narejena po terapiji. Gadolinij-DTPA je bil uporabljen kot kontrastno sredstvo. Slika prikazuje isto rezino kot slika 4. Kontrast med vnetim in nevnetim tkivom je povečan, kar še vedno kaže na prisotnost vnetja.*

the inflamed and healthy tissue was obtained by T2 weighted images, but the resolution was inadequate for a good localization of the different tissues observed. The best resolution was observed in T1 weighted images, especially when the contrast of inflamed tissues was increased by the use of the contrast agent (8) and when Fat-suppressed T1 weighted images were performed.

MR images demonstrate an increased signal intensity in the gingival tissue around the teeth with gingival inflammation. This is in consent with the histological analysis in which this inflamed tissue is more edematous (9). This is also in consent with MR spectroscopy measurements done previously *in vitro* on dog gingival tissue samples (10). Proton spin lattice relaxation times T_1 and T_2 were measured by a pulsed MR fourier-transform spectrometer working at γ = 100MHz frequency. The relaxation times were compared for the samples from the same mandible. Relaxation times were the longest in the acute inflamed tissue, shorter in the chronic inflamed tissue, and the shortest in the normal tissue. The content of free protons in these tissues is higher and so is the signal intensity which depends on relaxation times (11, 12).

The therapy produced the reduction of pocket depth, and the reduction of bleeding upon probing. These results indicated the reduction of inflammation of periodontal tissues and were in consent with SI measurements of MR images made after the therapy which showed the reduction of the SI. That also indicated the reduction of inflammation.

By the MR imaging the extent of the inflammation could be determined, because it offers a three dimensional representation. The localization is easier with the use of the contrast agent which accumulates in the inflamed tissue (8). The molecules of the contrast agent penetrate more easily into the damaged inflamed tissues where they enhance the proton relaxation and, thus, increase the signal intensity (SI). Therefore, the contrast between the inflamed and noninflamed tissue is increased.

In the study the MR imaging was used to monitor the course of the healing process of periodontal tissues after the therapy. Clinical indexes taken after the therapy indicate an improvement of the periodontal tissue status and so do the results of the SI ratio measurements.

Conclusions

We have concluded that MR imaging could be used as a diagnostic mode for the assessment of the inflamed soft periodontal tissues in humans.

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Ključne besede: *vnetje obzobnih tkiv; MR tehnike; intenziteta signala; kontrastna sredstva*

Izvleček - Izhodišča. *Pri zdravljenju vnetja obzobnih tkiv je pomembna zgodnja, pravilna in natančna diagnoza. Slikovne diagnostične metode, ki se sedaj uporabljajo v stomatologiji, prikažejo predvsem spremembe v trdih zobnih in obzobnih tkivih. Slikovna diagnostična metoda, ki omogoča diagnozo sprememb v mehkih tkivih, je magnetno resonančno slikanje (MRS). V stomatologiji se MRI uporablja predvsem za diagnozo sprememb v čeljustnem sklepu, ustnem dnu in jeziku. V naših raziskavah nas je zanimala uporabnost različnih MRS tehnik pri diagnozi sprememb v mehkih obzobnih tkivih.*

Metode. *Za raziskavo smo izbrali pacienta, ki smo mu s kliničnim pregledom in analizo rtg-ortopanskega posnetka postavili diagnozo parodontitis marginalis chronica. MR slikanje smo naredili na inštitutu za radiologijo KC pred in po terapiji ter rezultate primerjali z drugimi parodontalnimi parametri. Za izboljšanje kontrasta med vnetimi in zdravimi tkivi smo uporabili kontrastno sredstvo gadolinij-DTPA.*

Rezultati. *Pri MR preiskavi smo izmerili intenziteto signala IS vnetih in zdravih obzobnih tkiv. Bolj ko je vneto tkivo, večja je IS. Ta se še poveča po uporabi kontrastnega sredstva, ki se nabere v vnetih tkivih. Po terapiji se je vnetje obzobnih tkiv zmanjšalo, kar so pokazale tudi meritve IS.*

Zaključki. *MRS kot nova dopolnilna diagnostična tehnika omogoča lokalizacijo vnetja v mehkih obzobnih tkivih in je zaradi svoje neinvazivnosti primerna za sledenje poteka parodontalnega zdravljenja.*

Research article/Raziskovalni prispevek

THE EFFECT OF SETTING MODE ON WATER DIFFUSION AND FLEXURAL STRENGTH OF RESIN MODIFIED GLASS IONOMER CEMENTS

VPLIV NAČINA STRJEVANJA NA DIFUZIJO VODE IN UPOGIBNO TRDNOST STEKLASTO IONOMERNIH CEMENTOV Z DODATKI KOMPOZITOV

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Key words: *glass ionomer cements; setting reaction; water; mechanical properties; magnetic resonance imaging*

Abstract – Introduction. *Glass ionomer cements are susceptible to water contamination in the early stages of the setting reaction. Resin modified glass ionomer cements (RM GIC) were developed, where photopolymerization should prevent early contact with water. It is not known how susceptible these materials are when they set without photopolymerization. The purpose of the present study was to visualize the water penetration into RM GIC samples prepared in two different setting modes as a function of time using MR microimaging. In addition we wanted to answer the question if the flexural strength of the cement is impaired when the photopolymerization is excluded.*

Materials and methods. *Resin modified glass ionomer cement Fuji II LC CAPSULE (GC, Japan) was used in this study. Half of the samples for MR microimaging were radially exposed to a light source and the other half were allowed to set without photopolymerization. Both groups of samples were then stored in distilled water at 37° C and imaged at different times. The same cement was used for the preparation of samples for flexural strength testing. Half of the samples for each test time were photopolymerized and the other half were stored in darkroom. All samples were subjected to the three-point bending flexural strength test.*

Results. *MR microimaging showed that the variation in the setting mode results in substantial differences in the water penetration dynamics into the resin modified glass ionomer cement. The water uptake was slower in VL cured than in only chemically cured material. The flexural strength of VL cured cement was significantly higher than of chemically cured cement. Water storage lowered the values in all test points and in both setting modes significantly ($p < 0,05$) except for the chemical cured samples after 7 days.*

Conclusions. *Flexural strength measurements of the cement set without photopolymerization confirmed our MR microimaging results and it is evident that the exclusion of photopolymerization leads to an inadequate matrix formation which results in lower strength and makes this material more prone for water diffusion.*

Introduction

The materials used in dentistry are required to have long term durability in the oral cavity. This is a complex environment where the materials are in contact with saliva, a fluid which contains a variety of organic and inorganic species, together with a bacterial flora complex. In consequence, the uptake of fluid and the solubility of these materials are of considerable clinical significance.

Glass ionomer cements have certain characteristics that make them attractive to the dentists. They bond adhesively to enamel and dentine (1), release fluoride ions over prolonged period of time (2) and are harmless to dental pulp (3). However, the handling properties of these materials make them technique sensitive because of their susceptibility to moisture contamination during the early stages of the setting reaction. Glass ionomer cements are water based materials with water playing an important role in the setting reaction. If water is lost from the cement during setting the cement forming reactions will slow down or even cease, resulting in poor physical properties. On the other hand, if water comes into contact with cement before it has hardened, the matrix forming ions such as calcium and aluminium will be washed out which leads to improper matrix formation (4).

Recently, resin-modified glass ionomer have become available that offer improved handling characteristics (5). The common factor for resin modified glass ionomer cements is that they undergo dual setting reaction. The primary reaction is similar to that in light cured dental composites and is initiated by visible light. The secondary one is cement-forming acid base reaction which continues after light activation has been terminated and is responsible for the maturation of cement. Two matrices are therefore being formed: (i) a matrix of metal poly acrylate salts and (ii) polymer matrix (6). In the absence of light no free radical polymerization takes place and no polymer matrix is established. The study of Cho et al. showed that

resin modified glass ionomer cements were less affected by moisture than conventional glass ionomer cements (7). The water contamination was studied by measuring the methylene blue stain penetration (8) and colorimetrically (9).
Magnetic Resonance Imaging (MRI) is a widely used diagnostic procedure in medicine. Its main advantages include excellent soft tissue contrast, non invasiveness, and absence of ionizing radiation. The use of MRI in dentistry has, however, been limited to the diagnostics of temporo-mandibular joint disorders (10) and imaging of pathological changes of the tongue and floor of the mouth (11). Recent studies (12-14) indicate that hard and soft dental tissues could also be imaged because of their porosity and high water content and in periodontology MRI may soon become routine diagnostic procedure. In the research of plastic dental materials, however, MR microimaging has been shown to be novel tool for characterizing the structure and the setting processes due to it's high resolution (15, 16).
Though the resin modified glass ionomer cements (RM GIC) are said to be more resistant to early contamination by water than conventional GIC, it is not known how susceptible these materials are when they set without photopolymerization. The purpose of the present study was to visualize the water penetration into RM GIC samples prepared in two different setting modes, i.e. with and without polymerization, as a function of time using MR microimaging. In addition we wanted to answer the question if the flexural strength of the cement is impaired when the photopolymerization is excluded.

Materials and methods

MR microscopy

Resin modified glass ionomer cement Fuji II LC CAPSULE (GC, Japan, Batch No. 140451) was used in this study. Encapsulated material was chosen to keep the powder/liquid ratio constant throughout the experiment. Capsules were activated, triturated as suggested by the manufacturer and mixed cement was syringed into the quartz tubes (4 mm ID × 10 mm long). Tubes were covered with Mylar matrix at both ends and a slight pressure was applied to avoid air bubbles entrapment. Half of the samples were radially exposed to a Translux Standard EC light source (Kulzer, Germany) for 120 sec, the other half were allowed to set without photopolymerization, in a darkroom. One hour after the start of mixing, samples were extruded from the quartz tubes, immediately immersed in distilled water, and stored at 37° C. Eight specimens were prepared with each setting mode and imaged at different times.
MR microimaging is a variation of conventional MR imaging with much enhanced resolution (17). It is achieved with special gradient coils, capable of generating magnetic field gradients of about 10 Gauss/cm in magnitude. MR microimaging was performed on a Bruker Biospec System equipped with micro imaging utilities. A 100 MHz horizontal bore Oxford Instruments magnet (Oxford Instruments Ltd., U.K.) was employed. Standard spin echo sequence with the repetition time (TR) of 400 ms and the echo time (TE) of 18 ms was used.
In the first series four samples (two VL cured and two where photopolymerization was excluded) were positioned and fixed in the micro MRI probe assembly manually. The transverse slice was set at the center of the sample. A special insert was constructed that enabled the identical slice positioning at later experiments. A small tube containing a mixture of normal and deuterated water in the ratio of 2 parts in 8 was added as a standard to which the signals from the samples were normalized. This way the comparability of measurements at different times was achieved. First images were taken 24 hours after the start of mixing and the data acquisition was repeated at 96, and 192 hours. Four series were imaged consecutively using the same time intervals.

Flexural strength

For the preparation of samples for flexural strength measurements a 25 mm × 2 mm × 2mm dismountable stainless steel mold was used. The mixed cement was syringed into the mold, covered with two matrix strips and pressed between quartz plates. For the VL cured samples the center of the sample was initially activated with the curing light for 20 sec and then light guide was moved towards both ends so that the irradiation areas overlapped. The same was repeated on the opposite side of the sample. Chemically cured samples where photopolymerization was excluded were immediatelly transferred to a darkroom. All samples were stored in distilled water at 37°C for the time periods indicated prior to testing. A sample size of 14 (7 VL cured and 7 without photopolymerization) was used for each of the time periods, 1h, 24h and 7 days. A control group was prepared for each time period and setting time where samples were kept in exicator to avoid moisture contamination. After the storage time specimens were subjected to the three-point bending flexural strength testing using an Instron universal testing machine type 4301(Instron Corp., Canton, USA) with a cross-head speed of 1 mm/min. Flexural strength was calculated from the equation $S = 3FL/2bh^2$ where F is the maximum force (N) exerted on the specimen, L is the distance (mm) between the supports, b is the width (mm) and h is the height (mm) of the specimen (18). The data were subjected to a one-way ANOVA followed by Tuckey's HSD test at the 5% level of significance.

Results

Water diffusion into Fuji II LC CAPSULE glass ionomer cement as seen by MRI micro images at different exposure times is demonstrated in Fig. 1. VL cured (lower two samples) and only chemically cured (upper two samples) are shown together to emphasize the differences. On the images the radial front of water penetrating the samples is clearly visible. After 24 hours of immersion (Fig. 1a), water has diffused approximately 1 mm into the chemically only cured material and considerably less in the VL cured samples. As immersion time is increased rings become wider and after 96 hours (Fig. 1 b) the water has reached the center of all chemically cured samples. At the same immersion time the water signal from a VL cured samples is still within a well defined ring of the cylinders cross section with the plane of the image.
After 192 hours water has reached the cylinders center of both groups of samples. In VL cured material the signal appears to be rather homogeneous throughout the sample, indicating a uniform water distribution. In the chemically cured samples, however, a few larger areas of high signal intensity appeared, implying the existence of large pores full of water. However the 3000 micrometer slice is relatively thick compared to a typical pore dimension so that significant overlapping takes place and it is not very likely that single pores could be visible on such an image.
The changes in flexural strength as function of storage time are listed in Tab. 1. Through the test time period the flexural strength of VL cured cement was significantly higher than of chemically cured cement where photopolymerization was excluded. Study design did not allow the measurement of the flexural strength of chemical cured samples stored in water 1 h after start of mixing, since at that time samples were removed from the molds, where they set only via acid base reaction.
Water storage lowered the values in all test points and in both setting modes significantly ($p < 0,05$) except for the chemical

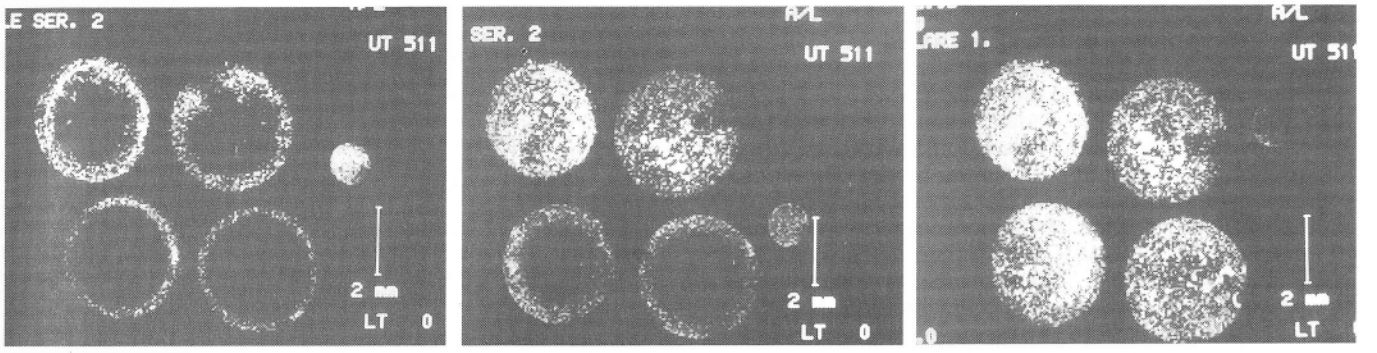

Fig. 1. *MR microimages of VL cured (lower two samples of each micrograph) and chemical-cured (upper two samples of each micrograph) resin modified glass ionomer cement taken after a) 24 h , b) 96 h and c) 192 hours of immersion in water.*

Sl. 1. *MR slike cementa strjenega z vidno svetlobo (spodnja dva vzorca na vsakem posnetku) in cementa, kjer smo izključili fotopolimerizacijo (zgornja dva vzorca na vsakem posnetku), ki smo jih hranili v vodi a) 24 urah, b) 96 urah in c) 192 urah.*

cured samples after 7 days. Dry VL cured samples showed significant increase in flexural strength between 1h and 24h as well as between 24h and 7d. On the other hand values for VL cured samples stored in water increased between 1h and 24h and than dropped at test time period of 7days.

Tab. 1. *Mean flexural strength in MPa + SD for the cement set with and without photopolymerization for wet and dry samples.*

Tab. 1. *Povprečna upogibna trdnost + SD cementa strjenega z fotopolimerizacijo in cementa ki se je strdil brez fotopolimerizacije. Vrednosti so za suhe vzorce in vzorce, ki smo jih hranili v vodni kopeli.*

	Photopolymerization Photopolymerization		Without photopolymerization Without photopolymerization	
	dry dry	wet wet	dry dry	wet wet
1 h	66.23 + 5.87	44.96 + 10.83	18.68 + 1.25	
24 h	87.75 + 6.88	85.72 + 5.73	37.60 + 11.39	19.35 + 9.24
7 days / dni	115.69 + 7.47	47.08 + 7.45	37.43 + 12.6	27.18 + 7.75

Discussion

It was demonstrated that the variation of the setting mode produces substantial differences in the water penetration dynamics into the FUJI II LC CAPSULE resin modified glass ionomer cement. It appears that VL cured material withstands water penetration better than its chemically cured variant. This would suggest that both setting reactions contribute to the final structure of the cement. In the absence of visible light no polymer matrix is contributing and the material is more prone to water penetration. As acid base reaction is susceptible to extrinsic water during the early stages only chemically cured material may be more affected by dissolving reactants. This situation which was in our case simulated in a dark conditions may happen clinically in deeper layers of fillings or underneath inlays and other cast restorations, where material may only set chemically due to inadequate light access and activation. Hegarty and Pearson showed that overall performance of such filling could be compromised (19).

After 192 hours of immersion in water a few larger areas of high signal intensity appeared in the chemically cured samples. Although there are differences in the structure seen on micro MRI images between VL cured and only chemically cured samples, the resolution of our experiment is not sufficient to get information about the size of individual pores. The slice thickness in our experiment was 3mm and was much larger than the average pore size in glass ionomer cements reported by Bertenshaw and Piddock (20). For this reason we do not see the pores but rather a projection of many pores in space onto the plane of the image (x,y plane). The z axis averaging occurs and many pores "shadows" overlap thus giving the impression that big pores exist in the structure. From such images information about pore size and their shape can be arrived only if the slice is thinner than the pore diameter.

From the results obtained we can conclude that MR microscopy is a good method for monitoring the water permeability of glass ionomer cements. The technique is nondestructive and provides a possibility to follow up the process on the same sample without the need to destroy it. No other presently available method can give as unambiguous visualisation of water front moving into the material. The evaluation of the results is straightforward and can be performed on a personal computer with any commercially available imaging processing software. In low magnetic field, as is 100 MHz in our case, low spatial resolution does not allow the visualization of the internal structure in the μm range but on a larger scale some structural changes are resolved. By using some special imaging techniques such as relaxation times mapping, diffusion imaging, magnetization transfer contrast, additional information about the internal structure could be acquired. Another possibility is to move to higher magnetic fields where enhanced intrinsic signal to noise ratio offers improved spatial resolution of the image. Further work in this direction is in progress.

The flexural strength of materials in transverse bending has been suggested as an appropriate method for assesing the mechanical properties of resin modified glass ionomer cements (21). Since tensile stress induced by bending should be highest at the outermost layer of the test specimen, failure will begin at its surface. Furthermore, the surface is the most susceptible to an influence from contact with water and a decrease in mechanical properties with time should be reflected in the results of the flexural strength (22).

A significant increase of flexural strength was observed for the VL cured samples stored in dry conditions. These materials undergo a dual setting reaction and both processes might contribute to the final structure formation, since it is known that it takes several weeks for the acid base reaction to be completed (23). It is also documented that polymerization induced by visible light does not cease when the light source is removed and the radical formation proceeds in the so called "dark phase" (24). The question remains whether the photopolymerization that is responsible for the fast initial set impairs the acid base reaction by reducing the mobility of its

reactants. Flexural strength measurement of the cement set without photopolymerization confirmed our MR micro-imaging results and it is evident that the exclusion of photo-polymerization leads to an inadequate matrix formation which results in lower strength and makes this material more prone for water diffusion. Water immersion lowered the flexural strength of the cement in both setting modes. This could be explained with the hydrophylic nature of the poly hydroxietil methacrylate poly(HEMA), which is an essential component of the RM GIC. Poly HEMA has a structure of a hydrogel (25) and it is well known that hydrogels take up water that acts as a plasticiser and lowers mechanical properties.

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Ključne besede: *steklasto ionomerni cementi; strjevanje; voda; mehanske lastnosti; slikanje z magnetno resonanco*

Izvleček – Izhodišča. *Steklasto ionomerni cementi (SIC) so sodobni dentalni materiali na vodni osnovi, ki se vežejo na trda zobna tkiva, sproščajo fluoridne ione in ne dražijo zobne pulpe. Strjujejo se preko acido bazne reakcije pri čemer so občutljivi na vodo v začetni fazi strjevanja. V želji, da bi to slabost odpravili, so razvili nov tip SIC z dodatkom plastičnih mas, ki imajo dvojni mehanizem strjevanja. Poleg stekel, polielektrolitov in vode, torej sestavin značilnih za klasične SIC, vsebujejo ti materiali še plastično maso v monomerni obliki, ki svetlobno polimerizira. Fotopolimerizacija, ki zagotovi hitro strjevanje na začetku naj bi preprečila prezgodnji vdor vode v material, ni pa znano kako se prodiranju vode med strjevanjem upira cement, če fotopolimerizacijo izključimo. V našem delu smo sledili prodiranju vode v SIC z dodatkom plastičnih mas pri dveh različnih načinih strjevanja, za kar smo uporabili magnetno resonančno mikroslikanje. Želeli smo tudi odgovoriti na vprašanje ali se cementu, ki se strjuje v temi, t.j. brez fotopolimerizacije, spremenijo mehanske lastnosti, kar smo preverjali s trotočkovnim upogibnim testom.*

Metode in materiali. *Za študij prodiranja vode v steklasto ionomerne cemente z magnetno resonančno mikroskopijo smo sveže namešan cement injicirali v valje iz kvarčnega stekla dolžine 10 mm in notranjega premera 4 mm. Pripravili smo dve skupini vzorcev (n = 8): Vzorce prve skupine smo krožno obsevali 120 s z dentalno polimerizacijsko lučjo, vzorce druge skupine pa smo takoj po injiciranju v kvarčne valje shranili v temnem prostoru, ter tako izključili fotopolimerizacijo. Vzorce obeh skupin smo med poskusom hranili v vodni kopeli pri temperaturi 37°C. MR meritve smo opravili na tomografskem sistemu Bruker Biospec (Bruker, Nemčija), ki je bil opremljen z dodatnimi gradientnimi tuljavami za mikro-slikanje. Uporabili smo tehniko slikanja spinskega odmeva s časom ponavljanja TR = 400 ms in časom spinskega odmeva TE = 18 ms. Cementne valje smo vložili v sondo za mikro-slikanje, katere premer je dovoljeval istočasno slikanje štirih vzorcev. Slikali smo rezino debeline 3mm na sredini cementnega valja in pravokotno na njegovo vzdolžno os. V sondo smo poleg vzorcev vložili kapilaro premera 1mm napolnjeno z D_2O/H_2O v razmerju 8: 2, ki nam je služila kot standard s katerim smo primerjali jakost signala v vzorcih. Vzorce smo prvič slikali 24 ur po namešanju in nato v 24 urnih časovnih intervalih 7 dni.*

Za študij upogibne trdnost steklasto ionomernega cementa z dodatki plastičnih mas (Fuji II LC CAP, GC, Japonska) smo izdelali vzorce dimenzij 25mm × 2mm × 2mm. Upogibno trdnost smo izmerili: 1 uro, 24 ur in 7 dni po začetku strjevanja. Za vsak merilni čas smo izdelali 2 skupini vzorcev (n = 14) Vzorce prve skupine smo obsevali z dentalno polimerizacijsko lučjo. S polimerizacijo smo pričeli na sredini vzorca in ga osvetljevali 20 sec. Optični vodnik premera 6mm smo nato izmenično prestavljali proti obema koncema vzorca, tako, da so se osvetljena področja delno prekrivala, postopek pa smo ponovili na drugi strani vzorca. Vzorce druge skupine smo takoj po injiciranju v modele shranili v temnem prostoru. Ker nismo uporabili fotopolimerizacije je strjevanje potekalo samo preko acido-bazne reakcije. Tako smo skušali oceniti prispevek polimerne komponente k upogibni trdnosti SIC. Za vsako meritev (1h, 24h, 7dni), smo posamezno skupino razdelili na dva dela. Sedem vzorcev smo shranili v vodni kopeli, ki ne prepušča svetlobe, pri temperaturi 37°C, drugo polovico (kontrolna skupina) smo hranili v eksikatorju in se tako izognili vplivu vlage v prostoru. Upogibno trdnost smo merili na univerzalnemu trgalnemu stroju Instron 4301 (Instron Corp., USA). Izvedli smo trotočkovni upogib, Vzorce smo obremenjevali do preloma in registrirali porušno silo. Dobljena vrednost nam je služila za izračun upogibne trdnosti.

Rezultati. *MR mikroskopski posnetki pokažejo razlike v prodiranju vode v SIC z dodatkom plastičnih mas glede na način strjevanja. Svetla področja na slikah so mesta z veliko intenziteto MR signala, t.j. signala zunanje proste vode, ki je prodrla v cement. Vezane vode, ki je strukturno vgrajena v cementno mrežje zaradi kratkih relaksacijskih časov ne vidimo. Po 24 urah v vodni kopeli se že nakazujejo razlike v hitrosti prodiranja vode v obe skupini vzorcev. Prodiranje je hitrejše v kemično strjene vzorce, kamor je voda prodrla 1 mm, v vzorce strjene z vidno svetlobo pa 0,5 mm. Podobno opazimo po 96 urah, ko je voda prodrla že do sredine kemično strjenih vzorcev, pri vzorcih strjenih z vidno svetlobo pa je vodna fronta napredovala počasneje. Po 192 urah je voda dosegla center valjev pri obeh skupinah vzorcev, vendar je bil signal pri vzorcih strjenih z vidno svetlobo homogen, kar kaže na enakomerno porazdelitev vode v materialu, pri drugi skupini pa so vidna področja z močnim MR signalom, torej z veliko vsebnostjo proste vode.*
Upogibna trdnost cementa strjenega z vidno svetlobo je bila v vseh merilnih točkah višja od kemično strjenega materiala. Pravtako je voda statistično značilno znižala upogibno trdnost v primerjavi z suhimi vzorci.

Zaključki. *Prikazali smo, da način strjevanja pomembno vpliva na hitrost prodiranja vode. Obe reakciji strjevanja prispevata k končni strukturi cementa in brez svetlobne polimerizacije je material bolj občutljiv na prodiranje vode. Z MR mikroskopijo lahko difuziji vode sledimo uspešneje, kot to omogočajo dosedanje posredne metode. Meritve upogibne trdnosti so podprle MR posnetke saj izključitev fotopolimerizacije vodi v nepravilno tvorbo mrežja, kar se kaže tudi v nižji upogibni trdnosti.*

Review article/Pregledni prispevek

THE ROLE OF BACTERIAL PROTEINASES IN PERIODONTAL DISEASE

VLOGA BAKTERIJSKIH PROTEINAZ PRI PARODONTALNI BOLEZNI

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Key words: *porphyromonas gingivalis; pathogenesis of periodontitis; host-defense system; gingipain; proteinase inhibitors*

Abstract – *The organism Porphyromonas gingivalis has been found to be a major factor in the development of periodontitis. This bacterium secretes significant quantities of proteinases which can specifically activate the kallikrein/kinin, coagulation, and complement cascade systems to produce both a source of host nutrients and host proteinases at or near the site of infection. It is through utilization of the host-defense system to its own benefit that P. gingivalis manages to grow and proliferate throughout the gingival tissues, while causing the tissue damage associated with periodontal disease. Therefore, a suitable target for therapy are proteinases which activate the systems described earlier, rather than bacterial pathogens themselves. Instead of the use of antibiotics, the data from the reviewed literature suggest that either synthetic proteinase inhibitors and/or vaccines directed against proteinases from these organisms might be an alternative for therapy.*

Introduction

Periodontitis is an infectious disease which causes the destruction of the connective tissue within the gingiva, resulting in gum resorption and tooth loss. The primary pathogen which initiates the development of adult onset periodontitis is believed to be Porphyromonas gingivals, an opportunistic anaerobe which grows beneath the gum line after the build-up of dental plaque (1). This bacterium has a number of intriguing properties which allow it to evade host-defense, including the presence of an antioxidant shield formed by the storage of hemin in the pericellular space, the secretion of antioxidants, including reducing agents and superoxide dismutase, and the release of proteolytic enzymes which act in a myriad of ways to ensure its growth and proliferation. It is the action of these latter enzymes which has been the subject of intensive studies in both ours and several other laboratories (2, 3).

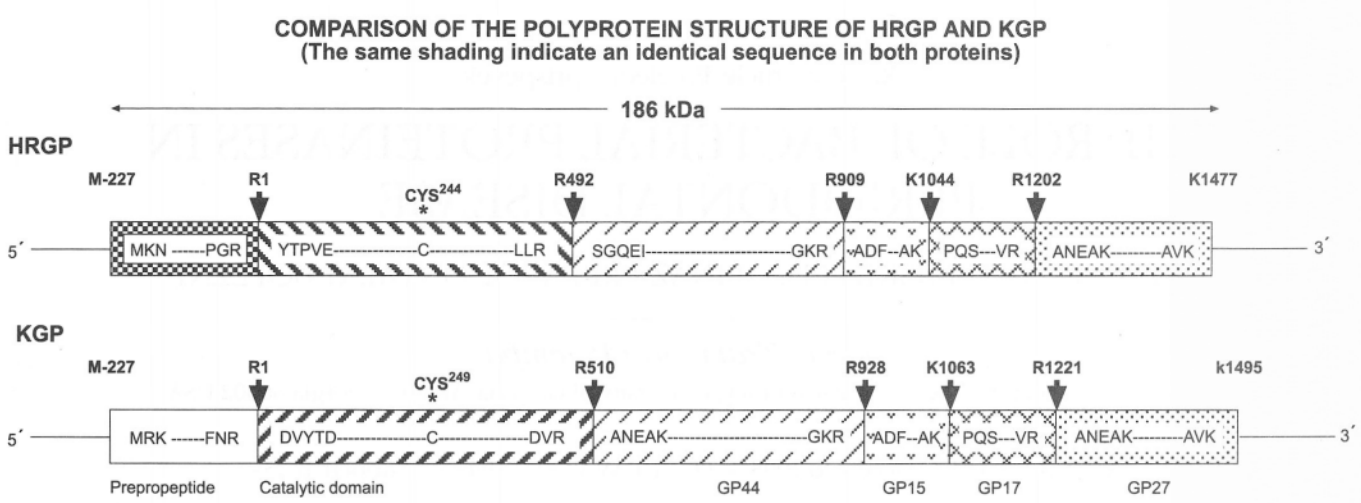
Proteolytic Enzymes from Porphyromonas gingivalis

For several years it was believed that the primary proteolytic activity present in and secreted by P. gingivalis was a singular "trypsin-like" proteinase (4, 5). However, it is now clear that there are a multitude of activities which are likely to be involved in ensuring the survival of this pathogen, including cysteine and serine proteinases. Furthermore, the "trypsin-like" activity which appears to play a major role in growth and proliferation has now been resolved into two specific cysteine proteinases (6, 7), each of which will be discussed in detail.

Gingipains

At least two of the major proteinases synthesized and secreted by P.gingivalis are members of the papain family, based on their requirement for reducing agents. For this reason, we refer to these enzymes as gingipains, reflecting both enzyme type and source. Each of these enzymes has been purified to homogeneity, and it has been found that one has a specificity for cleavage at arginyl-X residues (referred to as gingipain R or RGP) (6), while the other cleaved at lysyl-X residues (gingipain K or KGP) (7). Significantly, it was also noted that there were at least three forms of RGP, two secreted forms being of molecular weight approximately 50 kDa and differing in primary structure at only a few sites, and a third which was present primarily in vesicles shed by this organism and of much larger size (HRGP) (95 kDa) (8). In contrast, a single 105 kDa form of KGP has been isolated from vesicles and from culture media. Both of the low molecular weight forms of RGP exist as a single chain, while HRGP and KGP each exist as a complex containing 5 and 6 subunits, respectively. RGPs, HRGP, or KGP are not inhibited by human proteinase inhibitors.

The genes coding for both HRGP and KGP have been sequenced, and it is now known that each translated protein is synthesized as a precursor (186 kDa) which is then processed into active forms by proteolytic cleavage after four arginyl-X and one lys-X peptide bonds (Fig. 1) (9, 10). In gene knockout experiments it has been found that deletion of all RGP genes resulted in the improper processing of a number of important proteins required for the pathogeneicity of P. gingivalis, including KGP (11). We infer from these results that HRGP processing is an autolytic phenomenon occurring on the surface of this organism, and that KGP processing requires the activity of fully-functional HRGP or RGP. Finally, KGP must also be involved in its own maturation, as well as that of HRGP. These suggestions are in line with the specificity of cleavage during the maturation of HRGP and KGP to give the multiple polypeptide forms characteristic of each of these enzymes.



Detail examinations of the comparative structures of the polyprotein structures of HRGP and KGP gives some striking results. There are no homologies within either the prepropeptide or catalytic domains, the latter in agreement with the unique specificities of each proteinase. This includes the area surrounding the reactive site cysteine residue which has been identified for both enzymes. However, in the downstream regions there is considerable homology, with more than one-half of the total structures of each enzyme being identical to each other. Within this downstream region there are several repeat tandems, including three repeats which contain, internally two FEED sequences, typical of adhesion/hemagglutinin domains. Indeed, it has been clearly shown that both KGP and HRGP have the ability to bind to fibronectin, fibrinogen, and laminin, while the low molecular weight forms of RGP are unable to perform this function due to the lack of these domains in their structure (12).

The Role of Arginine-Gingipains in Periodontitis

Because the hallmarks of periodontitis include bleeding on probing, increased gingival crevicular fluid, and accumulation of neutrophils within the gingival crevice, it was suggested that the role of members of the RGP family might involve disregulation of the coagulation, kallikrein/kinin, and complement systems, since each of these cascades involves activation by arginyl-X cleaving proteinases. The results which were obtained in all three systems yielded amazingly similar results. In studies on the coagulation pathway it was found that RGPs could rapidly activate Factor × to Xa, thus also ensuring thrombin formation (13, 14). This latter enzyme has multiple functions and has been shown to activate systems which result in bone resorption. Clearly, it can also convert fibrinogen to fibrin, but this will be discussed separately with relation to functions of KGP. With regard to the kallikrein/kinin pathway, it was found that RGPs could activate both Hageman factor (weakly) and prekallikrein (strongly), resulting in the production of kallikrein which would then degrade kininogen to release the potent vasoconstrictor, bradykinin (15).

Finally, it was found that RGPs could degrade complement C5 to specifically produce C5a, a major chemotactic factor for neutrophils (16). In addition, a separate, as yet uncharacterized proteinase could remove the C5a receptor from the neutrophil surface, thus halting chemotaxis (17). How these processes are inter-related will be discussed later.

The Role of KGP in the Pathogenesis of Periodontitis

The complete function(s) of KGP is not totally understood. However, one major property is its ability to degrade fibrinogen, after adherence through its C-terminal adhesion domain (12). This would result in reduced clotting and is probably responsible for the bleeding on probing associated with the disease. Thus, even though thrombin is being produced by the activity of Factor Xa on prothrombin (a result of RGP activation of Factor X), its primary function in facilitating periodontal lesions is likely unrelated to its action as a clotting factor, since it is a highly pleiotropic enzyme. An alternative function for KGP may also involve the removal of C5a receptors from neutrophils, as described above, although inhibition studies of this process still suggest that other proteinases may also be involved in this process.

Hypothetical Role of Gingipains in Periodontitis

From the various hallmarks associated with the development of periodontitis, it was suggested that gingipains synthesized and secreted by P. gingivalis must play major roles in the pathogenesis of this disease. The proposed scenario is as follows:

1. Infection by P. gingivalis within the gingival pocket results in the release of either free or vesicle-associated RGPs and KGP.
2. RGPs activate the kallikrein/kinin cascade, resulting in increased availability of complement factors in periodontal tissue (edema and/or increased gingival crevicular fluid by the action of bradykinin on kininogen).
3. RGPs activate C5 present in the crevicular fluid to release the chemotaxin C5a, resulting in the infiltration of neutrophils.
4. Both free and vesicle-bound KGP and other uncharacterized proteinases degrade the C5a receptor on neutrophils in proximity to the infecting organism, resulting in attenuation of any neutrophil bactericidal activity.
5. Degranulation and/or death of neutrophils occurs near the site of infection, resulting in the release of host-derived oxidants and proteinases which inactivate host proteinase inhibitors by oxidation or proteolytic cleavage.
6. Uncontrolled degradation of host tissues by neutrophil-derived proteinases occurs, resulting in accelerated inflammation and tissue damage without the elimination of infection.

Host Evasion by Pathogens

The scheme described in the previous section suggests that pathogens can activate host-defense systems to their advantage. In the case of those organisms which cannot provide nutrients through the activity of their own systems, it appears that they have readily adapted to utilize host-nutrients and host-enzymes. In essence, by providing uncontrolled proteinases to activate the kallikrein/kinin pathway two requirements are immediately met. The first is a source of nutrients through increased crevicular flow, while the second is the local availability of complement proteins. This allows the use of the next system, whereby the pathogen-derived proteinases can now activate the complement system to release C5a which will act as a chemotaxin for neutrophil recruitment to the infected site. Clearly, this seems illogical; however, before the phagocytic neutrophil can ever reach its target it is stopped in its tracks by cleavage of receptors from its surface, again by pathogen-derived proteinases. Ultimately, the neutrophil dies and degranulates to release proteinases whose specificities are far broader than that of the pathogen-derived enzymes, so that they can readily degrade a broad class of proteins to peptides and free amino acids. In this manner, the host has provided all of the requirements for growth of the organism.

Conclusion

The premise that P. gingivalis-derived proteinases may function to take advantage of the host-defense response to infection is probably not unique to this organism. It is likely that other pathogens, not necessarily of bacterial origin, also utilize such pathways. However, such organisms probably could not survive without these proteolytic processes occurring within the infected host. Historically, most attempts to attenuate or destroy bacterial pathogens have involved the use of antibiotics. Thus, a logical target(s) for therapy is the proteinase(s). Synthetic proteinase inhibitors and/or vaccines directed against proteinases from these organisms would be an excellent alternative for antibiotic therapy.

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Ključne besede: *porphyromonas gingivalis; patogeneza parodontalne bolezni; obrambni mehanizem; gingipain; proteinazni inhibitorji*

Izvleček – *Porphyromonas gingivalis (P.g.) je ena od glavnih bakterij v zobnih oblogah, ki sodeluje pri začetku razvoja parodontitisa odraslih ljudi. Porphyromonas gingivalis ima mnogo mehanizmov, s katerimi se brani pred obzobnimi reakcijami organizma. Med temi mehanizmi so sposobnost obrambe pred oksidanti in možnost sproščanja proteolitičnih encimov, ki P.g. omogočajo rast in proliferacijo. Članek obravnava: vlogo dveh specifičnih cisteinskih proteinaz gingipain R in gingipain K, ki jih sprošča P.g. Gingipain R sodelujejo pri disregulaciji strjevanja krvi in sistemov kalikrein/kinina in komplementa, gingipain K pa ima sposobnost razgradnje fibrinogena.*

Avtorja predstavljata hipotezo, da P.g. s sproščanjem obeh gingipainov povzroči aktivacijo sistemov kalikrein/kinina in komplementa ter nastanek edema, krvavitev in povečanega izločanja gingivalne tekočine v obzobnih tkivih.

Avtorja predpostavljata, da bi s sintetičnimi proteinaznimi inhibitorji ali cepivom proti gingipainoma lahko učinkovito zdravili parodontalno bolezen.

Research article/Raziskovalni prispevek

LYSOSOMAL PROTEOLYSIS IN INFLAMMATION

LIZOSOMALNA PROTEOLIZA PRI VNETJU

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Key words: *lysosomal enzymes; cathepsins; proteinase inhibitors; inflammation*

Abstract – *Host response to pathogen invasion of gingiva involves the accumulation of neutrophils and macrophages at the site of acute inflammation, with the release, among other biological active molecules, of lysosomal proteolytic enzymes, including cathepsins G, D, B, L, H and possibly others. In addition, the expression of their endogenous inhibitors, produced locally or systemically, is altered resulting in an imbalance between proteinases and inhibitors. When the host proteolytic cascades are activated by the pathogens to their advantage, the disease becomes chronic, resulting in substantial loss of periodontal tissue and alveolar bone. Our investigations of patients with acute and chronic periodontitis have demonstrated that, as well as metalloproteinases, cysteine proteinases, cathepsins B, L and H and their endogenous inhibitors, such as tissue derived stefins A and B, cystatin C, kininogens, a variety of salivary cystatins (S, SN, SA) and alpha-2 macroglobulin, play an active role in the process. Biological components of the cysteine-dependent host proteolytic system, highly upregulated in inflamed tissue and secreted into gingival fluid, may provide a potential diagnostic tool for prognosis of advanced periodontitis. The inhibitors of cysteine proteinases may prove as therapeutic agents in later stages of the disease, possibly applied in combination with inhibitors of pathogenic agents.*

Proteinases – Characteristics and function

One of the initial events associated with the invasion of pathogenic bacteria, of which *Porphyromonas gingivalis* is the most abundant pathogen associated with dental plaque, is the release of various types of bacterial proteinases. In addition to bacterial metallo proteinases (1, 2) serine and cysteine proteinases have been found at the site of inflammation. In particular, the family of gingipains plays a crucial role in the initiation of physiologic and proteolytic cascades (3). The crucial physiologic event seems to be the recruitment of inflammatory cells to the infected site, degranulation of neutrophils and increased phagocytic activity of macrophages with concomitant release of lysosomal cathepsins. The results of increased host proteolytic activity is enhanced degradation of extracellular matrix proteins, presumably facilitating the invasion of bacteria into host tissue and providing the amino-acid nutrients, needed for growth. Furthermore, specific limited proteolysis by host proteinases may effect the cellular immune response and chemotaxis. Among the most investigated gingival tissue proteinases involved in inflammation are elastase, cathepsin G, aspartic and cysteine lysosomal proteinases.

Aspartic proteinase, cathepsin (Cat) D has a rather broad specificity at a slightly acidic pH range (3.5–5.5) and is present in high amounts in autophagic and heterophagic lysosomes and large acidic vesicles. It is found associated with various pathological conditions, such as tumor metastasis, Alzheimer disease and various types of inflammation. Ishikawa et al. (4) first reported increased Cat D levels in periodontitis. We have found a significant 9-fold increased Cat D activity in gingival extracts of patients with stage III (advanced) periodontitis (5) and enhanced secretion of Cat D into gingival fluid in these patients (6). In addition to direct degradation of extracellular matrix at the sites of acidic microenvironments, Cat D may also act indirectly by activating cysteine proteinases such as Cat B and Cat L, thereby initiating a proteolytic cascade, as proposed for tumor cell invasion (7). Cat D may also degrade a variety of biologically active peptides and proteins at inflammatory sites, including the inhibitors of cysteine proteinases (8), thereby furthe enhancing the activity of cysteine proteinases.

Lysosomal cysteine proteinases comprise a large family of papain-like enzymes, of which some, including Cat B, Cat L and Cat H, present in nearly all mammalian cells, while others, such as Cat S, Cat O, Cat K, etc. are expressed in specific cell types and/or are associated with various types of pathologic conditions including cancer (9) and inflammation (10). Cat L has collagenolytic and elastinolytic activities and Cat B was also found to degrade fibronectin and laminin at physiologic pH. These proteinases are secreted as precursors from tumor cells, similarly to Cat D, while active mature forms are found secreted in large amounts from macrophages. In addition cysteine proteinases mediate hormone processing, activation of proteinase precursors such as urokinase-type plasminogen activator, and are involved in antigen presentation (11–13). Increased activity and protein levels of Cat H, Cat B and Cat L in affinity purified extracts of human gingival tissues correlated with the stage of the disease (5), although the correlation was relatively poor for whole tissue homogenates (14, 15). Cat L secretion in gingival fluid was significant in advanced periodontitis. This may be associated with the increased alveolar bone resorbtion due to its collagenolytic activity and the enzyme was suggested as a diagnostic marker for chronic, stage III, periodontitis.

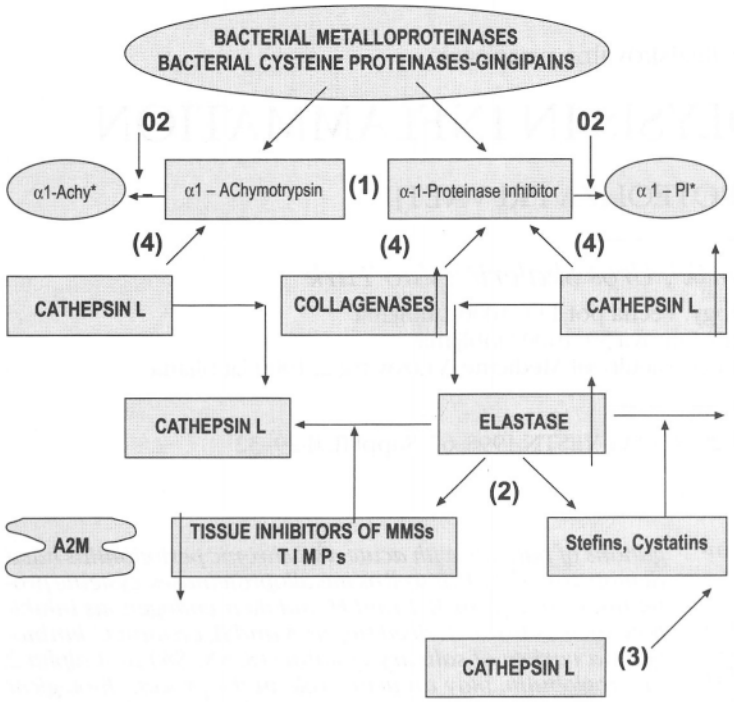

Fig. 1. *The scheme of proteolytic cascade during progression of periodontal disease. (a) Bacterial proteinases and oxidation inactivate alpha-1 antitrypsin and alpha-1 anti-chymotrypsin; (b) Human neutrophil elastase (HNE) is activated, possibly inactivating TIMPs and cystatins; (c) Degradation of cystatins is also enhanced by Cat D, released from macrophages; (d) Activated Cat D further activates cysteine proteinases; (e) Cat L and HNE inactivate serpins and TIMPs; further enhancing the activities of serine and metalloproteinases, leading to the "proteolytic burst". (f) All these proteinases are controlled and entrapped by alpha 2-macroglobulin.*

Sl. 1. *Shematski prikaz proteolitične kaskade pri napredovanju parodontalne bolezni. (a) Bakterijske proteinaze in oksidacija inaktivirajo alfa-1 antitripsin in alfa-1 antikimotripsin; (b) Elastaza nevtrofilnih granulocitov (Human neutrophil elastase-HNE) je aktivirana in verjetno inaktivira TIMP-e in cistatine; (c) Razgradnjo cistatinov pospešuje tudi Cat D, ki se sprosti iz makrofagov; (d) Aktiviran Cat D aktivira cisteinske proteinaze; (e) Cat L in HNE inaktivirata serpine in TIMP-e; povečata aktivnost serinskih in metaloproteinaz, kar vodi v povečano proteolizo; (f) Vse te proteinaze kontrolira alfa 2-makroglobulin.*

Inhibitors

Inhibitors of all four classes of proteinases are present in mammalian cells and body fluids. Serine proteinases are inhibited by the large family of serpins, while matrix metalloproteinases are inhibited by a family of TIMPS (Tissue Inhibitors of Metalloproteinases). Cysteine proteinase inhibitors (CPIs), comprising a superfamily of cystatins, comprise three families, *i.e.* stefins, cystatins and kininogens (11, 16). Numerous salivary cystatins (S, SA and SN) have been characterised (16, 17) and may have a protective role against host, but not against bacterial, cysteine proteinases. Presumably, this is also the major biological role of CPIs in inflamed human gingiva, from which three members of cystatin superfamily, stefin A, kininogen and cystatin C have been isolated (18). The high amounts of stefin A were expected due to the known specific association of this inhibitor with tissue of epithelial origin. We reported that stefins A and B also inhibit chemotaxis of neutrophils, indicating a role of cysteine proteinases in the process (19), although the mechanisms and regulation of stefins at various stages of periodontal disease have not been studied so far. Cystatin C concentration decreased significantly with pocket depths and it was proposed as a good diagnostic indicator for the activity of periodontitis (20). Serum inhibitors that have been investigated in gingival inflammation are alpha 2-macroglobulin, alpha-1 antitrypsin and kininogen, although these are also synthesized in a variety of tissues other than liver. Levels of total and complexed forms of alpha 2-macroglobulin from gingival fluid, collected at the sites of increased pocket depth (PD) and alveolar bone loss (AL), were lowered, presumably due to extensive binding of proteinases released into the sites of inflammation and clearance of the complexes by macrophages (21). In contrast, serum levels of alpha2-macroglobulin were increased and correlated significantly (r = 0.2–0.3) with pocket depth and gingival index. Increased synthesis of alpha 2-macroglobulin in the liver can be interpreted as a systemic response to constantly elevated local consumption at the numerous sites of gingival injury (22).

Proteolytic cascades – similarity to cancer progression.

Based on a large body of literature and on the above considerations, we have proposed the following scheme of proteolytic cascades occurring at the sites of inflammation (23, Figure 1). Initial proteolytic events are associated with the invasion of pathogens into the dentogingival crevice, releasing bacterial metallo- and cysteine proteinases which are not inhibited by endogenous host inhibitors. Bacterial proteinases have also the potential to inactivate serpins such as alpha-1 antitrypsin and alpha 1-antichymotrypsin (24), which control the elastinolytic activity of human neutrophil elastase (HNE) and Cat G, respectively, both of which are released by the degranulation of activated neutrophils at the site of inflammation. Alpha-1 antitrypsin is also inactivated by superoxide anions and hydrogen peroxide released during the so called "respiratory burst" (25), resulting in enhanced elastase activity which may be further responsible for the observed down-regulation of TIMPs and cystatins (10). Increased influx and maturation of macrophages cause the release of cathepsins into the extracellular milieu. It has been demonstrated (26) that the macrophage-elastin surface, similar to the osteoclast-bone surface, is rapidly acidified, allowing for cathepsin-mediated proteolysis. Although cystatins are also produced and released by macrophages, Cat D and HNE would rapidly inactivate this inhibitory potential at acidic and neutral pH, respectively, further facilitating the remodeling of extracellular matrix by cysteine cathepsins. Collective suppression of inhibitors by proteinases which are not their targets would therefore result in acceleration of general proteolysis, what we have termed "proteolytic burst" (23). This mediates not only tissue destruction, but also other physiologic events, such as chemotaxis and host immune response, both altered in acute and chronic inflammation. We conclude that the interplay between various proteinases and their endogenous inhibitors results in highly imbalanced conditions, facilitating the progression from acute and/or mild gingivitis to advanced periodontitis.

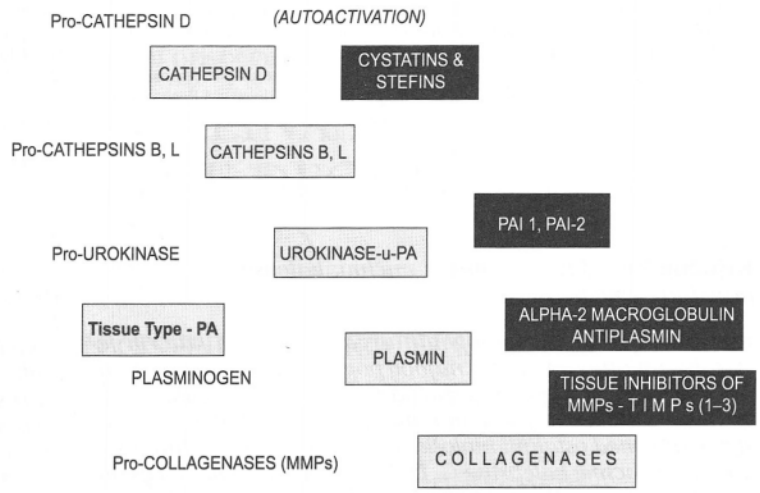

Fig. 2. *Proteolytic cascade in tumor progression as proposed by Schmitt et al. (7). (a) The cascade is initiated by the activation of proCat D, activating cysteine proteinases, Cat B and Cat L and possibly inactivating cystatins and stefins. (b) Cat B and Cat L activate prourokinase-type plasminogen activator (u-PA); (c) Activated urokinase (u-PA) activates plasminogen activator, which is also activated by tissue type plasminogen activator (t-PA); (d) Plasmin is a potent activator of pro-collagenases (matrix metalloproteinases. The endogenous inhibitors of theses proteinases, cystatins, plasminogen activator inhbitors (PAI-1 and PAI-2), TIMPs and alpha 2-macroglobulin, are also present in and/or around tumor tissues.*

Sl. 2. *Proteolitična kaskada pri napredovanju tumorjev (Schmitt in sod., (7)). (a) Kaskada se začne z aktivacijo proCat D v Cat D, ki aktivira cisteinski proteinazi Cat B in Cat L in verjetno inaktivira cistatine in stefine; (b) Cat B in Cat L aktivirata prourokinazni aktivator plazminogena (pro-u-PA); (c) Aktivirana urokinaza (u-PA) aktivira plazminogen aktivator, ki ga aktivira tudi tkivni aktivator plazminogena (t-PA); (d) Plazmin je možni aktivator pro-kolagenaz (matrix metaloproteinaz). Endogeni inhibitorji teh proteinaz, cistatini, inhibitorji aktivatorja plazminogena (PAI-1 in PAI-2), TIMP-i in alfa 2-makroglobulin so tudi prisotni v in/ali okoli tumorskega tkiva.*

Proteolytic cascades, such as the one described above, also play a crucial role in other pathophysiological conditions, such as blood coagulation and cancer progression. A large body of evidence has accumulated on the role of cathepsins in tumor invasion and the following cascades were proposed for this process (Figure 2, (8)). It has been demonstrated that malignant cells produce increased amounts of cathepsins and in this case, Cat D activation of procathepsins B and L was suggested as the initial step, possibly also associated with the inactivation of cystatins. Cat B and Cat L can activate the urokinase type of plasminogen activator, resulting in activation of plasminogen to plasmin, a serine proteinase which is abundant in plasma and body fluids surrounding tumor cells. Plasmin can accelerate its own production by activating prourokinase, but it can also activate various types of pro-collagenases, i.e. matrix metalloproteinases. This results in increased collagenolytic activity of matrix metalloproteinases, needed for extracellular matrix degradation, in addition to direct collagenolytic and elastinolytic activities of cysteine proteinases and plasmin.

In conclusion, cathepsins, normally localised mostly to lysosomes, are activated and either released or translocated to other subcellular compartments (9) during various pathologic conditions, including chronic inflammation and cancer progression. These processes are associated with an imbalance between cysteine proteinases and inhibitors, also due to proteolytic inactivation by other proteinases, which participate in the proteolytic cascades associated with these pathologic processes.

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Ključne besede: *lizosomalni encimi; katepsini; inhibitorji proteinaz; vnetje*

Izvleček – *Invazija patogenih organizmov, od katerih je Porphyromonas gingivalis najbolj pogosta bakterija v zobnih oblogah, sproži odgovor gostitelja v dlesni in vključuje dotok belih krvničk, nevtrofilcev in makrofagov, ki na mesto vnetja sproščajo, med ostalimi biološko aktivnimi proteini, tudi lisozomalne encime katepsine G, D, B, L in H. Poleg bakterijskih metalo in serinskih proteinaz, je bakterijska cisteinska proteinaza gingipain ključnega pomena tako pri sproženju proteoliznih kaskad, kakor tudi pri neposredni razgradnji thiv in pri proteoliznem moduliranju drugih proteinov, ki so vpleteni v vnetni proces. Aktivnosti proteinaze gostitelja regulirajo proteinazni inhibitorji, ki pa jih bakterijske proteinaze, superoksidni anioni in vodikov peroksid, ki se sproščajo v dlesen v začetni fazi vnetja, inaktivirajo. To močno poviša aktivnost nevtrofilne elastaze in katepsina G. Elastaza razgrajuej endogene inhibitorje gostitelja, tako tkivnih metalo-proteinaz (kolagenaz, gelatinaz), kakor tudi cisteinskih katepsinov B in L. Aktivnost teh se tako ob naraščajoči vsebnosti makrofagov, ki so glavni vir teh encimov, povečuje. Tudi katepsin D, ki se sprošča iz makrofagov pri procesih fagocitoze, razgrajuje inhibitorje cisteinskih katepsinov, cistatine, stefine in kininogene, kar še bolj zvišuje aktivnost katepsinov B in L. Spremembe v lokalni ali sistemski ekspresiji endogenih proteinaz in inhibitorjev vodijo do neravnotežja, ki močno poveča proteolizni potencial v dlesni. Ko torej patogeni organizmi uspejo aktivirati proteolizne sisteme gostitelja tako, da ti delujejo v njihovo korist, se bolezen iz akutne preusmeri v kronično, z naraščajočo razgradnjo dlesni in čeljustne kosti. Naše raziskave pacientov s paradontalno boleznijo so pokazale na pomembne spremembe vsebnosti katepsiov B, L in H ter njihovih endogenih inhibitorjev, stefina A in B, cistatina C, kininogena in vrste cistatinov v slini (cistatin S, SA in SN). Poleg lokalne spremembe proteoliznega ravnostežja, smo opazili tudi sistemske spremmbe v koncentraciji alfa-2 makroglobulina in alpha-1-antitrypsina v serumu pacientov z napredovano paradontalno boleznijo. Kaskadna proteolizna rekacija, ki jo predpostavljamo v napredovanju paradontalne bolezni je do neke mere podobna znanim kaskadnim reakcijam pri strjevanju krvi in predpostavljenim proteiznim rekacijam v invaziji tumorjev. Nadalje, nam močno povišane cisteinske proteinaze v vnetem tkivu in v gingivalni tekočini utegnejo služiti tudi kot možni diagnostični parameter za prognozo napredovanja bolezni. Nadalje se utegnejo inhibitorji cisteinskih proteinaz izkazati tudi kot zdravilne učinkovine pri preprečevanju napredovanja bolezni. Najverjetneje se bodo uporabljali v kombinaciji z inhibitorji patogenih organizmov in njihovih proteoliznih enimov.*

Review article/Pregledni prispevek

MATRIX METALLOPROTEINASES AND CYSTEINEPROTEINASES IN THE DEGRADATION OF THE BONE MATRIX

A REVIEW

VLOGA MATRIKSNIH METALO PROTEINAZ IN CISTEINSKIH PROTEINAZ V RAZGRADNJI KOSTNEGA MATRIKSA

PREGLEDNI ČLANEK

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Key words: *osteoclast; collagen; bone degradation; bone-lining cell; proteinases*

Abstract – *It is generally assumed that prior to osteoclastic bone resorption, the bone surface has to be freed of a thin layer of non-mineralized collagen fibrils. This would be followed by attachment of the osteoclast to the mineralized tissue and subsequent degradation of this calcified matrix. The osteoclast would then retract from the resorption pit, leaving non-digested demineralized bone collagen fibrils protruding from the wall of the lacuna. Subsequently these left-overs are to be digested. In the present paper we discuss (i) the cell types and proteolytic enzymes participating in the digestion of the layer of non-mineralized collagen covering the bone surface prior to and following bone resorption, and (ii) the enzymic armoury of the osteoclasts.*

Introduction

Degradation of bone is accomplished by osteoclasts. These cells have the capacity to digest mineralized matrices such as bone, mineralized cartilage and dentin (reviewed by 1, 2). In order to be able to digest the mineralized tissue the osteoclast has to attach to it. This process is probably facilitated by membrane-bound integrins and bone-associated proteins such as collagens and vitronectin (3-5). As a larger proportion of the bone surface is covered by a layer of non-mineralized collagen fibrils (6), attachment and subsequent degradative activity by osteoclasts is hindered to a considerable extent. So it appears that removal of these collagenous fibrils has to occur prior to the osteoclastic attack (7-9). The first question we address is which cell type is involved in this process and what enzymes participate (see 'Cleaning of the Bone Surface').

The next event involves attachment of the osteoclast to the 'cleaned' bone surface. Thereafter, they seal off an area from the extracellular environment by forming an attachment zone, the clear zone, and degrade the bone in the centre of this segregated site. Such a resorption area is characterized by deep invaginations of the osteoclast membrane, the alleged ruffled border. The osteoclast has the capacity to acidify this area (10, 11) which leads to dissolution of the mineral. Subsequently proteolytic enzymes are released and the proteins constituting the organic matrix are broken down. Enzymes involved in the latter process belong to the cysteine proteinases and probably also to the matrix metalloproteinases (12-21). The participation of both classes of enzymes is discussed in the second part (see 'Osteoclastic Breakdown of Bone').

Following digestion of mineral and the majority of matrix constituents the osteoclast retracts, leaving behind non-digested remnants of the demineralized bone matrix protruding from the bone surface. As in later stages the lacunae are characterized by the absence of most of such remnants, a large fraction of the collagenous matrix initially covering the resorption pit has to be removed. In the present paper we will address the question how the Howship's lacuna is cleaned in the final section of this review: 'Removal of Osteoclastic Left-overs'.

Discussion

Cleaning of the Bone Surface

One of the first indications that removal of non-mineralized fibrillar collagen protruding from the bone surface may indeed be an important event in the process leading to bone digestion came from studies performed by Chambers and coworkers (9). They demonstrated that treatment of the bone surface with collagenase resulted in osteoclastic attachment and degradation of the bone. They showed also that cells obtained from bone-associated tissue seeded on bone slices had the capacity to render the bone surface accessible to osteoclasts. These observations indicated (i) that prior to osteoclastic breakdown the bone surface has to be freed of adhering

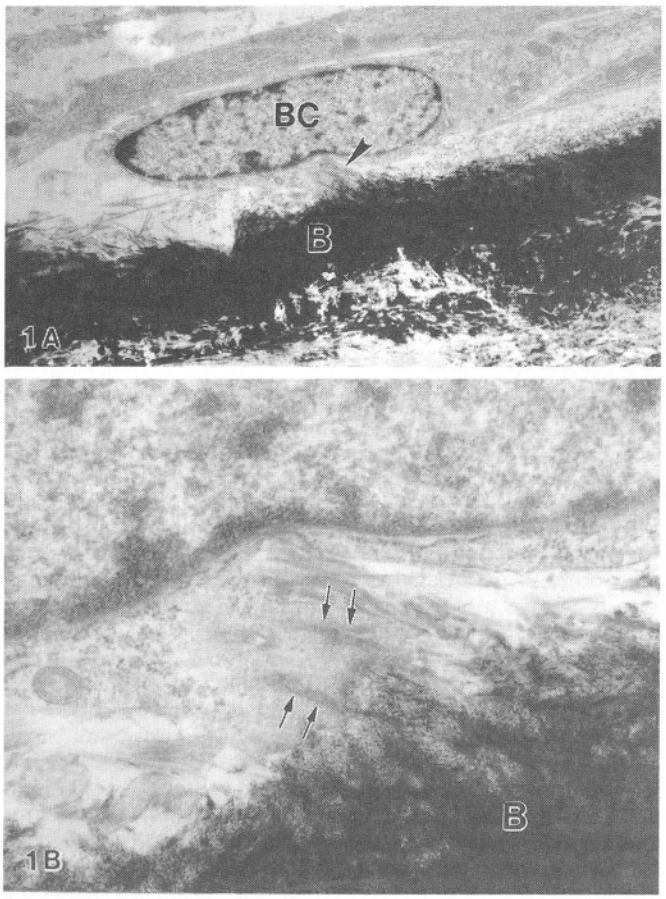

Fig. 1. *Osteoblast-like bone-lining cell (BC) adjacent to the bone surface. – 1A. Low magnification of the bone-lining cell. The arrowhead indicates the area shown at a higher magnification in 1B. – 1B. Note the cellular extensions surrounding collagen fibrils which protrude from the mineralized bone (arrows). Calvariae of five days old mice were cultured for 24 h in the presence of PTH (see 45) and prepared for electron microscopic examination as described previously (16). B: bone. A: × 10,000; B: × 20,000.*

Sl. 1. *Osteoblastom podobne celice (BC) na kostni površini. – 1A. Na kosti ležeče celice pri manjši povečavi. Puščica označuje površino, ki je na večji povečavi prikazana na sliki 1B. – 1B. Podaljšek celice obkroža kolagena vlakna, ki štrlijo iz mineralizirane kosti (puščice). Kalvarije pet dni starih miši so bile kultivirane 24 ur v prisotnosti PTH (glej 45) in nato pripravljene za elektronsko mikroskopsko analizo na predhodno opisan način (16). B: kost. A: × 10 000; B: × 20 000.*

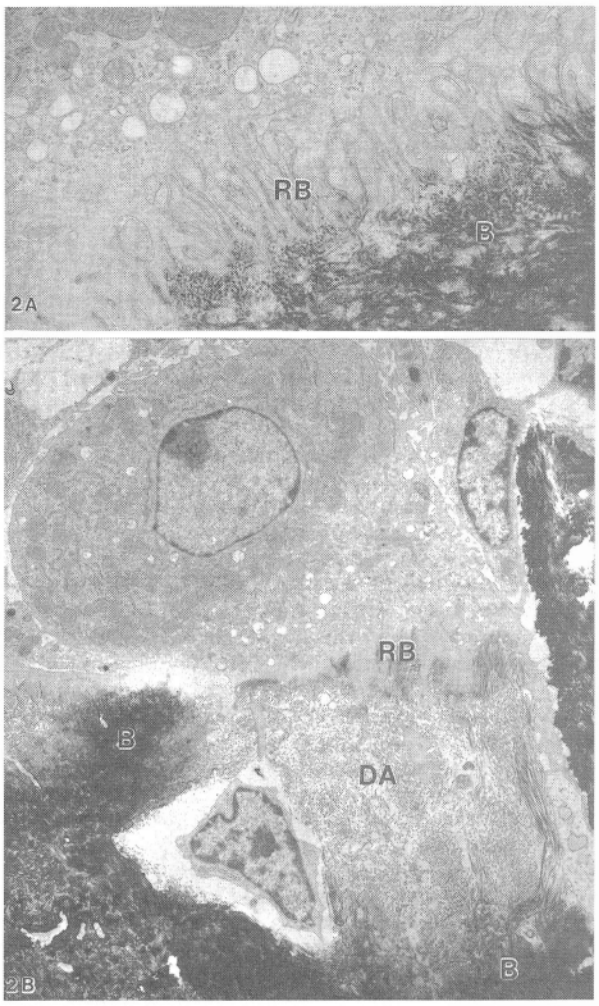

Fig. 2A. *High magnification of the resorption area of an osteoclast of a control explant. Note the presence of mineralized bone (B) adjacent to the ruffled border (RB). × 18,000. – 2B. The osteoclast of an explant treated for 24 h with the CP inhibitor E-64 (see 16). Note the large area of the demineralized bone (DA) adjacent to the ruffled (RB) of the cell. B: bone. × 5,300.*

Sl. 2A. *Večja povečava področja resorpcije osteoklasta na eksplantu kontrolne skupine. Ob resičasti površini osteoklasta (RB) je kost mineralizirana (B). × 18 000. – 2.B. Osteoklast z eksplanta v 24 urni obdelavi z inhibitorjem E-64 (glej 16). V bližini resic osteoklasta (RB) je obsežno področje demineralizirane kosti (DA). B: kost. × 5300.*

non-mineralized collagenous proteins, and (ii) that osteoblast-like cells have the capacity to clean the surface. Chambers and coauthors suggested that osteoblasts removed this non-mineralized collagenous material, probably by excreting the matrix metalloproteinase, collagenase. Numerous studies have indeed shown that cultured osteoblasts secrete high levels of this enzyme (22–27).

In line with this view are biochemical data, demonstrating that incubations with PTH resulted in a decreased amount of non-mineralized collagen (2, 28, 29), suggesting that a breakdown of this layer of collagen was stimulated by the hormone, and possibly due to osteoblastic activity (29, 30). Since similar data were obtained in the presence of the osteoclast-inhibiting compound calcitonin (see 2), the observations indicate that PTH-sensitive cells (e.g. osteoblasts) and not osteoclasts, were responsible.

In an attempt to identify the cell type and enzymes involved in this process we examined calvarial bone explants and studied the fate of the non-mineralized collagen protruding from the bone surface (31).The surfaces of these bone explants were covered by mononuclear cells (bone-lining cells and osteoblasts) and multinuclear cells (osteoclasts). Along the appositional surface of the explants typical osteoblasts were seen adjacent to a relatively thick and dense osteoid layer. The mononuclear cells bordering the surface characterized by a high resorbing activity, differed from the osteoblasts. They resembled bone-lining cells, having a somewhat flattened appearance. Both Golgi-apparatus and rough endoplasmic reticulum were present but not as pronounced as in the actively synthesizing osteoblasts. No signs of an osteoid layer were found on the resorption side of the calvarial bones. Between the cells and the mineralized bone surface a thin less compact

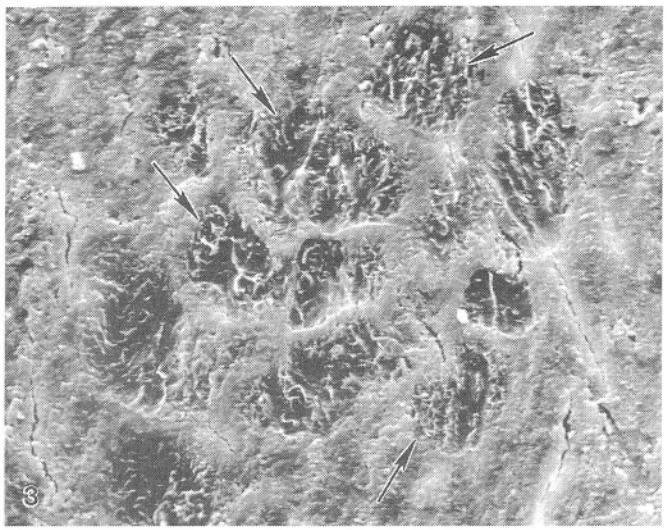

Fig. 3. *Scanning electron micrograph of resorption pits (arrows) created by isolated osteoclasts seeded on a bone slice. Note the presence of the fibrillar material protruding from the bottom of the pits. × 500.*

Sl.3. *Vrstično elektronsko mikroskopski posnetek resorpcijskih jamic (puščice), ki so posledica delovanja osteoklastov posejanih na kostni rezini. Opazne so štrleče fibrilarne strukture na dnu jamic. × 500.*

layer of non-mineralized collagenous fibrils was seen protruding from the mineralized bone. An interesting characteristic of these bone-lining cells was the presence of short cellular extensions which had engulfed non-mineralized collagen fibrils protruding from the bone surface (Fig. 1) (32, 33). In contrast, osteoblasts at the appositional side of the calvariae did not exhibit such a phenomenon.

Engulfment of collagen fibrils by bone-lining cells compares with a similar activity expressed by fibroblasts in most soft connective tissues (e.g. 34-36). The engulfed fibrils are taken up and degraded by the cells in their lysosomal apparatus (37). This pathway of collagen digestion is considered to be an important one during the normal turnover of soft connective tissue collagen (reviewed in 38).

The percentage of bone-lining cells demonstrating the engulfing activity was established in explants incubated with or without PTH (31). It was demonstrated that degradation by these cells of the collagen fibrils protruding from the mineralized bone surface was stimulated by PTH, compatible with an osteoblast-like cell type. In line herewith, we observed in an enzyme histochemical study, that the cells contained high levels of alkaline phosphatase. In this respect it is of interest to note that Rouleau and coworkers (39, 40) described the occurrence of PTH-target cells which expressed a phenotype different from mature osteoblasts.

Inhibition of the activity of cysteine proteinases with E-64 had no effect on the collagen-resorbing activity whereas blocking activity of matrix metalloproteinases (by the inhibitor CI-1) resulted in a significant increased number of collagen-engulfing cells (controls: 18± 2%; E-64-treated: 11±4%; CI-1 treated: 34*±3%, *$p < 0.05$; values represent mean % of collagen-engulfing cells±SD of six explants). This observation suggested to us that metalloproteinases, and not cysteine proteinases, are involved in the degradation of the collagenous fibrils. Our observations support the view that osteoblast-like cells are involved in freeing the bone surface of non-mineralized collagen (9, 29). This process seems to occur by engulfment, segregation and subsequent degradation of collagenous fibrils by these cells, and is likely to depend on the activity of metalloproteinases. Whether the metalloproteinase involved is collagenase or one of the other members of this class of enzymes has yet to be established.

It is tempting to speculate that following the 'cleaning' activity, the cells retract from the denuded bone surface (41, 42), thus creating space for the osteoclast to attach and exert its activity. Soluble components may be released locally (43) by which osteoclasts are attracted to these sites.

Osteoclastic Breakdown of a Bone

Degradation of a bone involves digestion of both mineral and matrix. Mineral dissolution is considered to occur prior to degradation of the matrix and to take place following acidification of the segregated area (10, 44). Most of the mineral is dissolved in the extracellular environment, although small amounts of mineral crystallites may be taken up by the osteoclasts (15). Mineral dissolution is followed by degradation of the matrix.

In establishing the possible involvement of proteolytic enzymes in the digestion of the matrix, several investigators (12-14, 17, 19, 20, 45) studied the effects of selective proteinase inhibitors. Of all the compounds tested, in particular those interfering with the activity of cysteine proteinases and metalloproteinases were shown to decrease osteoclastic bone digestion, thus strongly suggesting that members of both classes of enzymes are somehow involved in this process. At the electron microscopic level it was demonstrated that the inhibition of either cysteine proteinases (14-16, 46, 47) or metalloproteinases (16) resulted in the appearance of large demineralized areas bordering the osteoclasts (Fig. 2). The data indicated that demineralization continued whereas degradation of the matrix was inhibited. It was concluded that enzymes belonging to both classes of proteinases participated in matrix digestion. Since the inhibitors used affect the activity of all enzymes belonging to either cysteine proteinases or metalloproteinases, the enzyme(s) primarily involved could not be established. However, recent immunohistochemical studies have shown the presence of several cysteine proteinases, such as cathepsin B, L, and H, in both the resorption area and the osteoclast (48-50). Although each of these enzymes is capable of digesting one or more matrix components, the one primarily responsible remains to be established. Cathepsin L seems to be an attractive candidate as this enzyme is present in the resorption area (48, 49) and has a high potential activity against collagen (51). Hill and coworkers (19) postulated that the latter enzyme exerted its activity in the extracellular space, in the ruffled border area, whereas cathepsin B was active intracellularly. Recently it was shown that yet another cathepsin may be involved in digestion of the bone matrix, cathepsin K. The latter enzyme has been shown to be relatively abundant in the osteoclast (e.g. 52, 53). Moreover, pycnodysostosis a disease characterized by a decreased bone degradation and large areas of non-digested demineralized bone adjacent to the osteoclast (54), has recently been shown to have a defect in the cathepsin K gene (55).

With respect to the possible metalloproteinase involved, collagenase (MMP-1) has been frequently suggested as a likely candidate (e.g. 14, 45, 56-62). Although no conclusive evidence is available in support of the view that this enzyme indeed participates, Delaissé and coworkers (63) detected the enzyme in osteoclasts as well as in the resorption zone, and in situ hybridisation studies have demonstrated the messenger for the enzyme in odontoclasts (64). Moreover, it proved possible to extract the enzyme from bone tissue (65), the amount being influenced by compounds which affect bone resorption (66). Yet, it is still not clear whether this enzyme is indeed involved in the osteoclastic bone di-

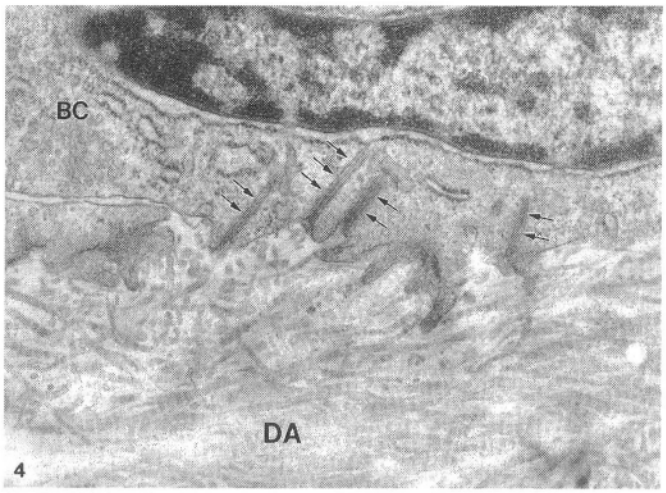

Fig. 4. *Osteoblast-like bone-lining cell (BC) engulfing demineralized bone collagen fibrils (arrows) protruding from a resorption pit (DA) vacated by an osteoclast. × 28,000.*

Sl. 4. *Osteoblastom podobne na kosti ležeče celice (BC) požirajo kolagene fibrile demineralizirane kosti (puščice), ki po umiku osteoklastov štrlijo iz resorpcijskih jamic (DA). × 28000.*

gestion. First, other members of this class of proteolytic enzymes may participate. Recent data presented by Hill *et al.* (67) have shown that bone degradation was inhibited by the natural occurring inhibitors TIMP-1 and TIMP-2. The latter inhibitor proved to have a stronger inhibitory effect on bone digestion than TIMP-1. Although both inhibitors block the activity of various MMPs, TIMP-2 has been suggested to be a more potent gelatinase inhibitor (68). Moreover, several recent studies have shown the presence and expression of gelatinase in osteoclasts (69–71). Although these observations do suggest that somehow metalloproteinases participate in the events leading to digestion of the bone matrix, Fuller and Chambers (47) shed doubt on their role in the degradation mediated by the osteoclast itself. These authors were unable to find an effect of inhibitors of this class of proteolytic enzymes on bone resorption by isolated osteoclasts seeded on bone slices. Neither could they demonstrate the presence of collagenase in or around the osteoclast. The lack of an effect of metalloproteinase inhibitors in this model system is in line with data presented by Delaissé and coworkers (14). In conclusion, so far participation of cysteine proteinases in osteoclast-mediated breakdown is generally recognized, the involvement of metalloproteinases is still not entirely clear. The latter enzymes may either be more important for the removal of the non-mineralized collagen fringe (see above) or for the migration of the osteoclast (72).

Sequential activity of enzymes?

As the inhibitors of both classes of enzymes used in our studies (16) clearly induced the appearance of demineralized bone collagen only at sites where osteoclasts were active, the calvarial model provided us the means to study the sequence in which the enzymes exert their activity and their substrate specificity.

The bone matrix contains a wide variety of non-collagenous components, among which proteoglycans. These structures are localized between and around the collagenous fibrils. Although members of both enzyme classes have the capacity to digest proteoglycans (reviewed in 73), it was unknown which enzymes are involved in their actual breakdown. In an attempt to investigate this in greater detail calvarial explants were cultured with enzyme inhibitors, and subsequently stained with a dye selective for proteoglycans, cuprolinic blue (74). The demineralized pits created by the osteoclasts showed differences in staining intensity depending on the inhibitor used. Blockage of the activity of cysteine proteinases resulted in a relatively high percentage (49%) of cuprolinic blue-positive resorption pits, whereas in the presence of the inhibitor of metalloproteinases the percentage of positive pits proved to be two-fold less (26%). These data suggest that the digestion of proteoglycans was hindered in the presence of the cysteine proteinase inhibitor, and more or less continued following blockage of metalloproteinases. We, therefore, tentatively conclude that the former class of proteolytic enzymes play an essential role in the proteoglycan degradation.

The observations offer an interesting possibility that a specific order exists in the events leading to digestion. Following demineralization, non-collagenous proteins like proteoglycans are removed due to the activity of cysteine proteinases, which is subsequently followed by metalloproteinase-mediated digestion of collagenous proteins. Such a sequence of enzyme activities would fit in with the proposed changes in pH in the resorption zone (see 2): at the onset of the degradation the area is characterized by a low pH, a situation optimal for the first enzymatic attack performed by cysteine proteinases. At a later stage the acidic environment is somewhat neutralized due to an increased level of the dissolved mineral (75), and degraded non-collagenous proteins. Under these conditions metalloproteinases exert their activity and digest the collagenous proteins.

Tab. 1. *The influence of proteinase inhibitors (E-64, CI-1) and osteoclast-inhibiting compound calcitonin on demineralization of bone surface.*

Tab. 1. *Vpliv proteinaznih inhibitorjev E-64 in CI-1 ter inhibitorja osteoklastov kalcitonina na demineralizacijo kostne površine.*

	DA/µm bone surface DP/µm kostne površine		DA/µm bone surface DP/µm kostne površine
24 h	**0.31±0.06**		
24 h E-64	**1.62±0.51***	+24 h E-64+CT	**0.10±0.08**
24 h CI-1	**1.76±0.99***	+24 h CI-1+CT	**1.68±0.72****

Calvarial explants of 5-day old mice were cultured for 24 h in the absence or presence of the cysteine proteinase inhibitor E-64 (42µM), or the matrix metalloproteinase inhibitor CI-1 (40 µM). PTH was added to the cultures in the concentration of 0.1 µM. After this period a series of explants was fixed and analyzed as described (16). Another series of these explants was cultured for an additional 24 h in the presence of the osteoclast-inhibiting compound, calcitonin (CT, 0.9 U/ml). Following this period the explants were analyzed as indicated.
The data are expressed as a mean demineralized area (DA) per bone length (n = 6 explants per treatment). *$p < 0.01$ vs controls (24 h); **$p < 0.01$ vs E-64 incubated explants (+ 24 h E-64 + CT).

Kalvarijski eksplanti pet dni starih miši so bili kultivirani 24 ur z dodatkom ali brez cisteinskega proteinaznega inhibitorja E-64 (42mM) oziroma inhibitorja matriksnih metaloproteaz CI-1 (40 mM). Po tem času je bila skupina eksplantov fiksirana in analizirana kot je bilo opisano (16). Druga skupina eksplantov je bila kultivirana nadaljnih 24 ur z dodatkom inhibitorja osteoklastov kalcitonina (CT, 0,9 U/ml). Po tem času so bili eksplanti analizirani na opisan način.
Podane so srednje vrednosti demineralizirane površine (DP) (n = 6 eksplantov za vsako obdelavo). *$p < 0.01$ vs. kontrole (24h); **$p < 0.01$ vs eksplanti inkubirani z E-64 (+24h E-64+CT).

Removal of Osteoclastic Left-overs

Following the excavation of the bone by the osteoclast, the cell leaves the resorption pit (76, 77) which is still containing non-degraded demineralized bone collagen fringes protruding from the bottom of the pit (Fig. 3). Recent findings in our group indicate that bone-lining cells are involved also in removal of this layer of the non-mineralized collagen.

In calvarial bone explants that were cultured for 24 h in the presence of the cysteine proteinase inhibitor E-64, or the metalloproteinase inhibitor CI-1 large demineralized areas were created by the osteoclasts (see above; Tab. 1; 16). When the explants were cultured for another 24 h, but now in the presence of the osteoclast-inhibiting compound calcitonin, the demineralized areas were completely removed in explants cultured with E-64. However, in those cultured with the metalloproteinase inhibitor the demineralized areas were still present. Mononuclear bone-lining cells proved to be present at these demineralized sites, and they had engulfed the non-mineralized collagen protruding from the wall of the Howship's lacunae (Fig. 4). A similar activity of such cells was seen in vacated Howship's lacunae of non-cultured explants and in explants cultured without inhibitors. Our data indicate that (i) the demineralized bone matrix present in the vacated Howship's lacuna following the osteoclastic activity is digested, (ii) resorption of this collagen fringe depends on the activity of mononuclear bone-lining cells, and (iii) metalloproteinases, and not cysteine proteinases are essential for the resorption of this fringe.

We thus propose that bone-lining cells not only clean the bone surface prior to the osteoclastic attack of the bone tissue but also degrade the 'left-overs' of the osteoclasts and that this degradative activity depends on the metalloproteinase activity.

Conclusion

We tentatively propose the following sequence of events during the bone resorbing process:

Non-mineralized collagen fringes covering the bone surface are digested by bone-lining cells, their activity being stimulated by PTH. The digestion process involves engulfment of the fibrils by cytoplasmic extensions and degradation of the enclosed fibrillar elements by metalloproteinases.

Osteoclasts attach to the 'cleaned' bone surface, form a ruffled border, dissolve the mineral crystallites and subsequently degrade the greater part of the matrix. This degradation of the matrix is supposed to depend on the following sequence of enzyme activities: first cysteine proteinases degrading non-collagenous proteins surrounding the collagen fibrils, followed by the activity of matrix metalloproteinases digesting the fibrillar collagen. The osteoclast then retracts from the resorption site, leaving undigested demineralized collagen fringes protruding from the wall of the Howship's lacuna. These fringes are subsequently engulfed and digested by bone-lining cells. This process too depends on the activity of matrix metalloproteinases.

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Ključne besede: *osteoklast; kolagen; razgradnja kosti; celice kostne površine; proteinaze*

Izvleček – *Za razgradnjo kosti so po splošnem prepričanju primarno odgovorni osteoklasti. V preglednem članku, ki povzema številne lastne in nekaj novejših tujih študij, avtorji ugotavljajo, da sta v procesu razgradnje kosti udeležena vsaj dva tipa celic in večje število proteolitičnih encimov. Na modelu kalvarijskega kostnega eksplanta so študirali časovno in prostorsko zaporedje aktivacije posameznih celic oz. proteolitišnih encimov.*

Na kostni površini je normalno nemineralizirana plast kolagenih vlaken, ki preprečuje pripenjanje osteoklastov in s tem razgradnjo kosti. Osteoblastom podobne celice, ležeče na kostni površini, imajo sposobnost endocitoze in razgradnje nemineraliziranih kolagenih vlaken, s čimer očistijo mineralizirano kostno površino. Omenjene celice se od zrelih osteoblastov razlikujejo po višji vsebnosti alkalne fosfataze in po kratkih celičnih podaljških, v katerih so vključki nemineraliziranih kolagenih vlaken s kostne površine. Endocitotični vezikli se združijo z lizosomi, v katerih so matriksne metaloproteinaze, ki razgradijo kolagena vlakna. Na očiščeno površino kosti se v naslednji fazi pripnejo osteoklasti in s sproščanjem kislih produktov lokalno demineralizirajo kost, manjše minerale pa razgradijo z endocitozo. Nizek pH v demineralizirani kosti aktivira cisteinske proteinaze, ki razgradijo nekolagene proteine (proteoglikane). Pri tem sodelujejo naslednje cisteinske proteinaze: katepsin B (ekstracelularno), katepsin L (intracelularno v resičastih podaljških osteoklastov) in katepsina K in H. Po razgradnji nekolagenih proteinov se zviša pH v demineraliziranem tkivu, kar aktivira osteoklastni matriksni metaloproteinazi, kolagenazo in gelatinazo, ki razgradita večji del kolagenega matriksa. Preostala demineralizirana kolagena vlakna v resorpcijskih jamicah, po odhodu osteoklastov, razgradijo osteoblastom podobne celice z matriksnimi metaloproteinazami.

Avtorji so na modelu kalvarijskega kostnega eksplanta študirali vlogo cisteinskih proteinaz in matriksnih metaloproteinaz v razgradnji kostnega tkiva. Nakazana je možnost terapevtske uporabe proteinaznih inhibitorjev, kar bi v veliki meri izboljšalo rezultate zdravljenja parodontalne bolezni, kjer je propad alveolarne kosti zelo pogost in resen problem.

Research report/Raziskovalno poročilo

INFLUENCE OF DEXAMETHASONE ON PROGRESSION OF EXPERIMENTAL PERIODONTITIS IN RATS

VPLIV DEKSAMETAZONA NA RAZVOJ POSKUSNO POVZROČENEGA VNETJA OBZOBNIH TKIV PODGANE

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Key words: *immunosuppression; ligature; bone resorption; morphometric analysis*

Abstract – Background. *Although several clinical observations have reported reduced inflammatory response to bacterial plaque in patients on the prolonged steroid therapy, the influence of these drugs on progression of the periodontal tissue breakdown is still unclear. The aim of this study was to further evaluate the influence of synthetic glucocorticoid dexamethasone on progression of the periodontal breakdown in rats.*

Methods. *In rats the periodontitis was induced by placing the silk ligatures around the cervix of the right second maxillary molar. Ten female rats, aged 8 weeks, were divided in two groups: five rats were daily injected with dexamethasone for a period of 14 days; the other five rats were injected with saline. Five μm thick sections of tissue blocks, stained with hematoxylin and eosin were used for the morphometrical analysis, performed on mesial and distal interdental regions of the experimental and control teeth. The measurement parameters included the surface of inflammatory infiltrate (SIN), the epithelial attachment loss (EAL), and the distance between the cemento enamel junction and the alveolar bone crest (DAB).*

Results. *The results showed a significantly reduced surface of inflammatory infiltrate (Student's t-test, $p < 0.01$), a significantly increased epithelial attachment loss (Student's t-test, $p < 0.05$), and a non significantly reduced distance between the cemento enamel junction and the alveolar bone crest around the experimental group animals teeth (SIN = 0.006±0.002 mm², EAL = 0.17±0.05 mm, DAB = 0.45±0.01 mm) as compared to the values of the control group (SIN = 0.038±0.08 mm², EAL = 0.12±0.05 mm, DAB = 0.53±0.01 mm).*

Conclusions. *We are concluding that dexamethasone reduces inflammatory reaction in periodontium, accelerates epithelial attachment loss, and reduces alveolar bone resorption in the case of experimental periodontitis in rats.*

Introduction

The major forms of periodontal disease are considered to be microbial infections in which certain bacteria play a significant role in inducing and maintaining the inflammatory process and the immunological host response in the periodontal tissue. The immune response besides being protective to a host could also have deleterious side effects on the periodontal tissue, especially in the case of the chronic bacterial plaque presence in the dento gingival area *(1)*.

Glucocorticoids have been shown to inhibit both the early and the late stages of the inflammatory process, and to have strong immunosuppressive effects. For these effects synthetic glucocorticoids are widely used in clinical praxis to improve different diseases and disorders with primary immunologic pathogenic mechanisms *(2)*. The present study was initiated to evaluate if antiinflammatory and immunosuppressive properties of these drugs may also inhibit the progression of periodontal disease.

Several authors studied the prevalence of periodontal disease in a group of patients on the prolonged corticosteroid therapy following renal transplant operations. Schuller et al. *(3)* found no correlation between periodontal disease, age, plaque or calculus in immunosuppressed patients receiving prednisone and azathrioprine systematically. They also suggested that these immunosuppressive drugs are inhibiting the clinical signs of periodontal disease *(3)*. A reduced inflammatory response of the periodontal tissue to bacterial plaque in immunosuppressed patients was confirmed by Kardachi and Newcomb *(4)*. On the other hand, Oshrian et al. *(5)* found similar levels of the plaque accumulation, gingival inflammation, and periodontal destruction in patients with and without the immunosuppressive therapy.

Similar were the results in patients suffering from multiple sclerosis, who had been on the glucocorticoid therapy for up to four years. When they were compared with patients without the steroid therapy, no differences were observed in the frequency and severity of periodontal disease between these two groups *(6)*.

Several histological changes were observed in periodontal tissues of rats and mice following the systemic cortisone administration. An alveolar bone osteoporosis, a reduced number

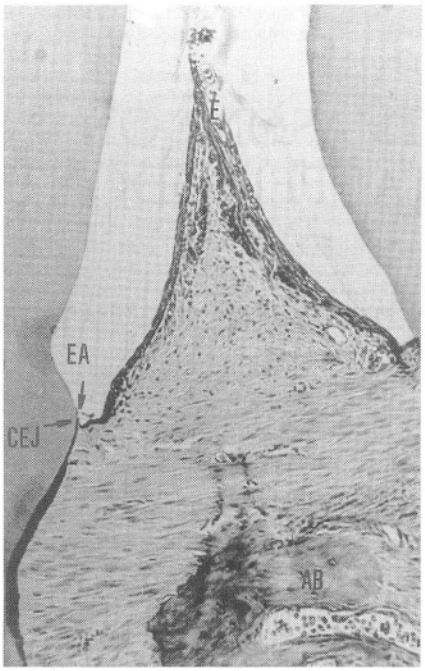

Fig. 1. *Interdental space on non-ligated side from the rat, receiving physiological solution: CEJ – cemento-enamel junction, EA – epithelial attachment, E – epithelium, AB – alveolar bone (H & E, 200×).*

Sl. 1. *Medzobni prostor na strani brez ligature pri podgani, ki je dobivala fiziološko raztopino: CEJ – skleninsko cementna meja, EA – epitelijsko prirastišče, E – epitelij, AB – alveolarna kost (H & E, 200-krat).*

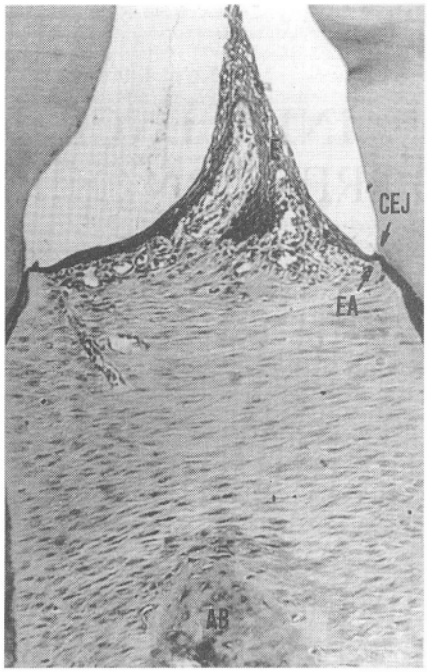

Fig. 3. *Interdental space on ligated side from the rat, receiving physiological solution: CEJ – cemento-enamel junction, EA – epithelial attachment, E – epithelium, AB – alveolar bone (H & E, 200×).*

Sl. 3. *Medzobni prostor na strani z ligaturo pri podgani, ki je dobivala fiziološko raztopino: CEJ – skleninsko cementna meja, EA – epitelijsko prirastišče, E – epitelij, AB – alveolarna kost (H & E, 200-krat).*

and size of osteoblasts, fibroblasts, collagen fibbers were found in corticosteroid treated animals *(7, 8)*.
In the present study the model of experimental periodontitis in rats was used to further evaluate whether a systemic administration of dexamethasone enhances or suppresses the periodontal tissue breakdown in rats.

Materials and methods

Experimental animals

Ten female Wistar rats, each weighing approximately 200g, were housed in cages, 2 per cage, and fed a standardized hard briquette diet and tap of water ad libitum. They were divided into two groups, receiving dexamethasone daily (Dexamethason®, Krka, Slovenia; 1mg/kg, intraperitonealy) (group 1) or physiologic solution (group 2).
Sterilized black braided 4/0 silk ligatures (Ethicon®, Edinburgh, Great Brittany) were placed around the cervix of the right second maxillary molar of rats, anaesthetized with anesthetic mixture of ketaminhydrochloride (Ketanest®, Parke-Davis, Berlin, Germany) xilasine (Rompun®, Bayer, Leverkusen, Germany), and atropine (Atropin, Belupo, Koprivnica, Croatia) in groups 1 and 2 according to the procedure of Sallay et al. *(9)*.

Morphometrical analysis

The animals of both groups were sacrificed after 14 days. Tissue samples, containing all three maxillary molars, the alveolar bone and the surrounding soft tissues, were taken from right and left sides of the animals (split mouth experimental design). They were fixed in 10% buffered formaldehyde (pH 7.4).
Tissue samples were decalcified in chloracetic acid. Samples were first put for 2 days in 10%, then for 2 days in 5%, for 2 days in 2.5% and finally for 2 days in 1.5% chloracetic acid. Afterwards the decalcified tissue samples were bisected in the parasagittal plane to obtain 5µm thick sections. Sections were stained with hematoxylin and eosin (H & E).
The morphometric analyses were performed in the interdental spaces mesially and distally of the examined tooth. In each interdental space the measurements were made on four different places, at least 100 µm apart from each other, and as the result the mean value of these four measurements was taken. The histological sections of one group were prepared from tissue samples of five animals, and for each tissue sample two values, for the mesial and distal interdental space was obtained, so that one experimental group consisted of 10 values.
The measuring equipment consisted of: microscope, color video camera, PC and MCID programme (The Microcomputer Imaging Device, Imaging Research Inc., 1995).
Measurements, performed on these H & E stained sections were:
1) evaluation of the inflammatory infiltrate by measuring the surface of the connective tissue, infiltrated with inflammatory cells (= SIN)
2) evaluation of the soft tissues destruction by measuring the distance between the cemento enamel junction and the most coronar border of the epithelial attachment (= EAL)
3) evaluation of the alveolar bone resorption by measuring the distance between the cemento enamel junction (CEJ) and the most coronar border of the alveolar bone (AB)(= DAB).

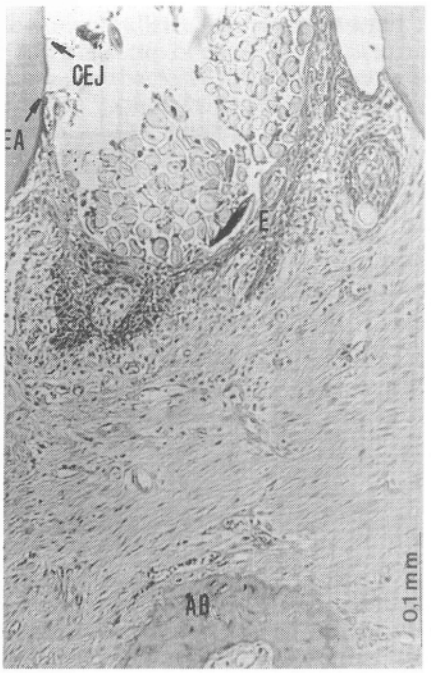

Fig. 2. *Interdental space on non-ligated side from the rat, receiving dexamethasone: CEJ – cemento-enamel junction, EA – epithelial attachment, E – epithelium, AB – alveolar bone (H & E, 200×).*

Sl. 2. *Medzobni prostor na strani brez ligature pri podgani, ki je dobivala deksametazon: CEJ – skleninsko cementna meja, EA – epitelijsko prirastišče, E – epitelij, AB – alveolarna kost (H&E, 200-krat).*

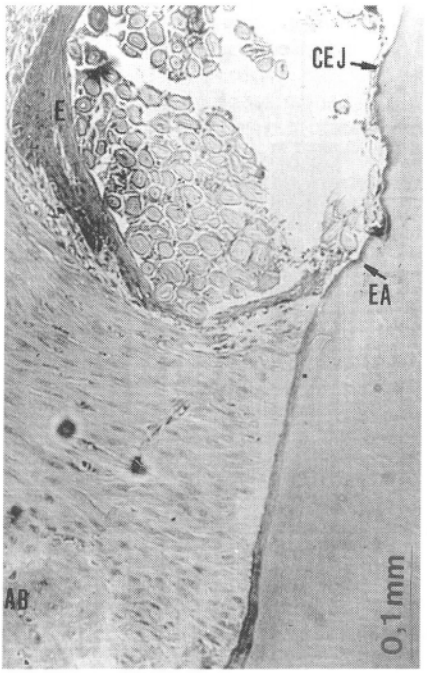

Fig. 4. *Interdental space on ligated side from the rat, receiving dexamethasone: CEJ – cemento-enamel junction, EA – epithelial attachment, E – epithelium, AB – alveolar bone (H & E, 200×).*

Sl. 4. *Medzobni prostor na strani z ligaturo pri podgani, ki je dobivala fiziološko raztopino: CEJ – skleninsko cementna meja, EA – epitelijsko prirastišče, E – epitelij, AB – alveolarna kost (H&E, 200-krat).*

The split mouth experimental design resulted in the production of four treatment groups: ligated molars in experimental (dexamethasone treated) animals (EL), non-ligated molars in the experimental animals (EO), ligated molars in the control (physiologic solution treated) animals (CL), and non-ligated molars in the control animals (CO).

Statistical analysis

Data were analyzed by using the Student *t*- test. The null hypothesis was rejected at $p < 0.05$.

Results

The surface of inflammatory infiltrate (SIN)

H & E staining of sections from sides without ligatures (CO and EO) showed that the inflammatory cell invasion and slight dilatation of capillaries in the connective tissue were confined to the area beneath the epithelium (Fig. 1, 2).

The same staining of sections from the ligated side of the control group animals (CL) revealed an inflammatory cell invasion and capillary dilatation in the connective tissue of the area immediately beneath the epithelium onto which the ligature was placed, and spread towards the alveolar bone crest on day 14 (Fig.3). The inflammatory cell infiltrates were mostly composed of polymorphonuclear leucocytes with a few monocytes. Under the ligatures of the experimental group animals (EL) the inflammatory reaction was limited to the area beneath the epithelium on day 14 (Fig. 4).

On the side without the ligature the SIN was significantly

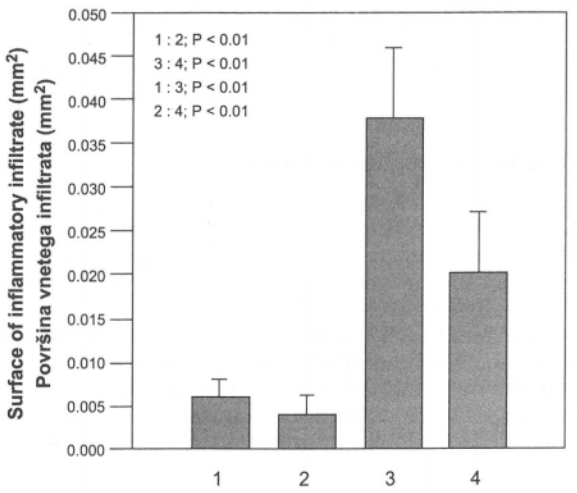

Fig. 5. *Surface of inflammatory infiltrate (SIN) in animals, receiving dexamethasone; side with ligature (1), side without ligature (2), and in animals, receiving physiological solution; side with ligature (3), side without ligature (4). Values are presenting the mean of 10 measurements with standard deviations.*

Sl. 5. *Površina vnetnega infiltrata (SIN) pri živalih, ki so prejemale deksametazon; stran z ligaturo (1), stran brez ligature (2), in pri živalih, ki so prejemale fiziološko raztopino; stran z ligaturo (3), stran brez ligature (4). Stolpec predstavlja povprečje desetih meritev s standardno deviacijo.*

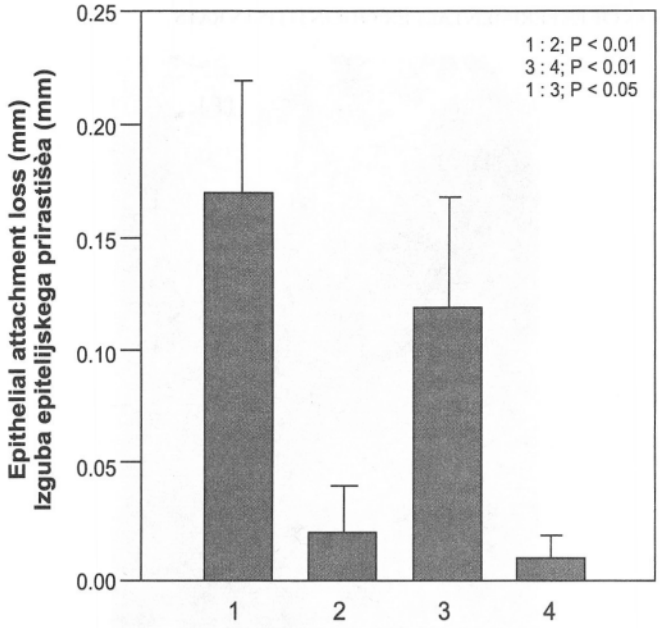

Fig. 6. *Epithelial attachment loss (EAL) in animals, receiving dexamethasone; side with ligature (1), side without ligature (2), and in animals, receiving physiological solution; side with ligature (3), side without ligature (4). Values are presenting the mean of 10 measurements with standard deviations.*

Sl. 6. *Izguba epitelijskega prirastišča (EAL) pri živalih, ki so prejemale deksametazon; stran z ligaturo (1), stran brez ligature (2), in pri živalih, ki so prejemale fiziološko raztopino; stran z ligaturo (3), stran brez ligature (4). Stolpec predstavlja povprečje desetih meritev s standardno deviacijo.*

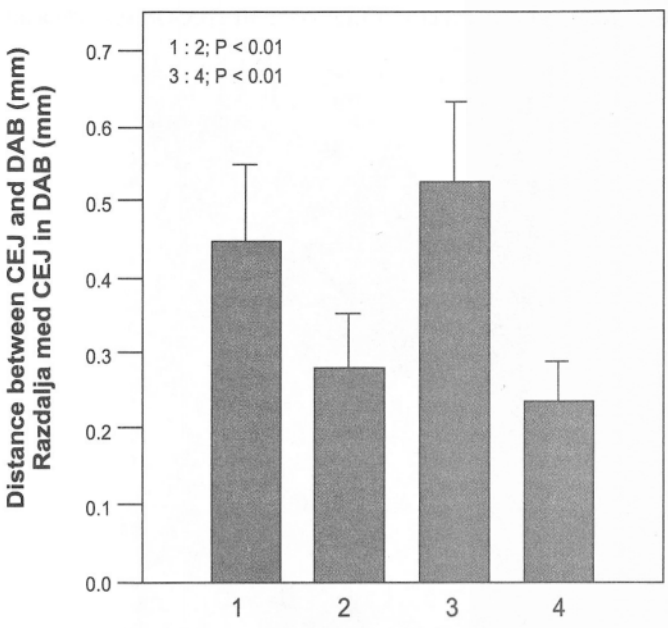

Fig. 7. *Distance between CEJ and AB (DAB) in animals, receiving dexamethasone; side with ligature (1), side without ligature (2), and in animals, receiving physiological solution; side with ligature (3), side without ligature (4). Values are presenting the mean of 10 measurements with standard deviations.*

Sl. 7. *Razdalja med CEJ in AB (DAB) pri živalih, ki so prejemale deksametazon; stran z ligaturo (1), stran brez ligature (2), in pri živalih, ki so prejemale fiziološko raztopino; stran z ligaturo (3), stran brez ligature (4). Stolpec predstavlja povprečje desetih meritev s standardno deviacijo.*

greater ($p < 0.01$) in the control group animals (0.020±0.007 mm^2) compared to the experimental group (0.004±0.002 mm^2). After placing the ligature the SIN increased significantly ($p < 0.01$), SIN was found significantly reduced ($p < 0.01$) in the animals receiving dexamethasone (0.006±0.002 mm^2), compared to the control group (0.038±0.008 mm^2) (Fig. 5).

The epithelial attachment loss (EAL)

In the animals with ligatures (CL and EL) the attachment loss occurred in the junction epithelium immediately beneath the cemento enamel junction where the ligature was placed (Fig. 3, 4). In rats without ligatures (CO and EO) no evidence of the attachment loss was observed (Fig. 1, 2).

The epithelium under the ligature spread toward the underlying connective tissue in the form of fingerlike extensions, termed rete pegs only in the animals of the control group. No such changes were noticed in animals of the experimental group. Instead, epithelial atrophy was observed in the ligated animals receiving dexamethasone.

On the side without the ligature the EAL in animals of the control (0.01±0.01 mm) and the experimental (0.02±0.02) group was minimal. After placing the ligature, the EAL was significantly greater ($p < 0.01$) compared with the non-ligated side. Comparing the sides with the ligature, a significantly greater ($p < 0.05$) EAL was found in animals that received dexamethasone (0.017±0.05 mm) than in animals that received physiologic solution (0.12±0.05 mm) (Fig. 6).

The distance between the CEJ and AB (DAB)

Under the ligature a moderate bone resorption occurred, but the resorption lacunes, containing osteoclasts, were observed only occasionally (Fig. 3, 4).

On the side without the ligature, the DAB was similar in the animals of the control (0.24±0.05 mm) and the experimental group (0.28±0.07 mm). After placing the ligature, the DAB was significantly greater ($p < 0.01$) compared with the non-ligated sides. The DAB was found to be slightly reduced in the animals receiving dexamethasone (0.45±0.01 mm) compared to the control group (0.53±0.01 mm) (Fig. 7).

Discussion

After two weeks of the experiment, the epithelial attachment loss, alveolar bone resorption and moderate inflammatory cell infiltrate reaching near the alveolar bone crest were observed in the control animals on the side with the ligature. In animals daily injected with dexamethasone an inflammatory reaction under the ligature was absent, and the epithelial attachment loss was significantly greater than that of the control group. It seems that local stimuli that appear due to the ligation (plaque accumulation and mechanical irritation) could become more harmful to the underlying soft tissues of gingiva after a dexamethasone injection. However, the alveolar bone resorption was slightly lower in rats recieving dexamethasone with ligatures that in rats receiving a physiological solution. Thus, our study indicates that the progressive alveolar bone resorption results from the synergistic effect between a bacterial plaque and the host response reactions. The alveolar bone resorption could be altered by dexamethasone probably because of the suppressed host response reactions that might contribute to the periodontal tissue destruction.

The influence of the suppressed host response on the alveolar bone resorption progression due to the immunosuppression has already been examined in several different studies. In rats a general immunosuppression can be achieved by the

whole-body-irradiation, or by the cyclophosphamide treatment. In one study a severe and rapid bone loss was described in the whole-body-irradiated rats monoinfected by Eikenella corrodens *(10)*. In a series of experiments an extremely rapid bone destruction and bacterial invasion of periodontal tissues was reported in cyclophosphamide treated rats with ligatures around the teeth *(9, 11)*. Findings in B lymphocyte deficient rats *(12)* or lymphocyte deficient rats *(13)* also demonstrated the essential alveolar bone loss. It is noteworthy that none of these studies reported less alveolar bone loss. Thus, we could hypothesize that the immunosuppression after a dexamethasone injection is not the cause for the reduced alveolar bone resorption, and that probably some other mechanism is involved in this process.

Gyofry et al. *(14)* have shown that neurogenic mechanisms play a crucial role in the development of the ligature induced inflammation in rats. According to literature data the substance P has a crucial role in the pathogenesis of such a neurogenic inflammation *(14)*. Glucocorticoids have been shown to reduce the substance P contents in neurons *(15)*, and increase the activity of the enzymes that degrade the substance P *(16)*. Therefore these drugs could prevent the development of the neurogenic inflammation after ligation, and thus the alveolar bone resorption. It might however be possible that the corticoid alters the periodontal tissue destruction by the neurogenic inflammation inhibition.

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Ključne besede: *imunosupresija; ligatura; kostna resorpcija; morfometrična analiza*

Izvleček – Izhodišča. *Pri pacientih, ki so dolgotrajno prejemali glukokortikoidna zdravila, so v obzobnih tkivih ugotovili zmanjšan vnetni odgovor, kljub prisotnosti bakterijskega plaka. Še vedno pa ni jasen vpliv dolgotrajnega jemanja teh zdravil na hitrost uničenja obzobnih tkiv pri obolelih s parodontalno boleznijo. Zato smo se namenili natančneje opredeliti vpliv sintetičnega glikokortikoida deksametazona na potek razgradnje obzobnih tkiv v primeru eksperimentalnega parodontitisa pri podganah.*

Metode. *V poskusu smo vnetje obzobnih tkiv pri podganah sprožili z namestitvijo svilenih niti okoli zobnega vratu drugega desnega zgornjega kočnika. Deset živali, starih približno osem tednov, smo razdelili v dve skupini: petim smo dva tedna vsak dan intraperitonealno vbrizgavali deksametazon (1mg/kg telesne teže), drugim petim pa fiziološko raztopino. Iz živali smo osamili tkivne vzorce, ki so vsebovali alveolarno kost, tri kočnike in okolna mehka tkiva ene strani čeljusti. Po postopku dekalcifikacije tkiv z razredčeno klorocetno kislino smo za morfometrično analizo pripravili histološke preparate 5 µm debelih rezin obzobnih tkiv, narezanih v parasagitalni ravnini, ter jih obarvali s hematoksilinom in eozinom. Iz vsakega tkivnega vzorca smo pripravili najmanj štiri rezine, ki so bile med seboj oddaljene 100 mm in kot en rezultat vzeli njihovo povprečje. Na njih smo v mezialnih in distalnih medzobnih prostorih izmerili površino vnetnega infiltrata (SIN), izgubo epitelijskega prirastišča (EAL) in razdaljo od skleninsko cementne meje do roba alveolarne kosti (DAB).*

Rezultati. *Histološka slika je pri živalih po vbrizgavanju deksametazona pokazala zmanjšanje vnetne reakcije v obzobnih tkivih in atrofijo epitelija dlesni. Pri živalih, ki smo jim vbrizgavali deksametazon, smo ugotovili statistično značilno zmanjšano površino vnetnega infiltrata (Studentov t-test, $p < 0,01$) (SIN = 0,006±0,002 mm^2), značilno povečano izgubo epitelijskega prirastišča (Studentov t-test, $p < 0,05$) (EAL = 0,17±0,05 mm), ter neznačilno zmanjšano razdaljo od skleninsko cementne meje do roba alveolarne kosti (DAB = 0,45±0,01 mm) v primerjavi s kontrolnimi živalmi (SIN = 0,038±0,08 mm^2, EAL = 0,12±0,05 mm, DAB = 0,53±0,01 mm).*

Zaključki. *V primeru eksperimentalnega parodontitisa pri podganah deksametazon zavira vnetno reakcijo v obzobnih tkivih, pospešuje izgubo epitelijskega prirastišča ter zmanjšuje resorpcijo alveolarne kosti.*

Research article/Raziskovalni prispevek

THE INFLUENCE OF APPROXIMATE AMALGAM RESTORATIONS ON ALVEOLAR BONE LOSS

VPLIV APROKSIMALNIH PLOMB NA IZGUBO ALVEOLARNE KOSTI

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Key words: *alveolar bone resorption; radiographs; amalgam fillings*

Abstract – Background. *Along with systemic factors it has been shown that local factors such as inadequate restorative dentistry may result in accelerated alveolar bone loss. The aim of the study was to examine the prevalence of overhanging approximate restorations and to quantify their effect on marginal alveolar bone in comparison with the effect of intact teeth surfaces and surfaces with well-fitting amalgam restorations.*

Materials and methods. *Panoramic radiographs of 671 randomly selected patients, aged 18–75 years (x= 43.8) were examined. Interproximal bone loss around teeth with overhanging amalgam restorations was measured and compared to bone loss around well-fitting amalgam restorations and adjacent to intact approximate surfaces.*

Results. *The prevalence of overhanging approximate amalgam fillings was 19%. Overall alveolar bone loss was greater around teeth with overhanging amalgam restorations than around teeth with well-fitting restorations. The lowest bone resorption was adjacent to intact approximate teeth surfaces (all statistically significant, $p > 0.01$).*

Introduction

Many factors may influence on the course of periodontal disease. Along with systemic factors it has been shown that local factors such as inadequate restorative dentistry may result in accelerated alveolar bone loss (1, 2).

Many studies evaluated the effect of restorative care on periodontal health by clinical, roentgenographic and combined studies (3–7). In evaluating of metal restorations, greater bone loss was found in areas associated with overhanging margins of restorations (3), but no distinction was made between different restorative materials. When the effect of both materials, amalgam and gold overhanging restorations was evaluated, greater severity of periodontal disease was around teeth associated with definite amalgam overhangs (4). When the size of the overhangs was considered in the analysis of radiographs (5), a greater periodontal destruction was found adjacent to large overhangs. Karlsen (8) found that overhanging subgingival restorations gave more pronounced gingival changes than the well-fitting ones. On the other hand, the cervical excess removal of the filling material was proved useful in preventing the onset and progression of periodontal disease (7).

Defective dental restorations providing local anatomic factors which promote plaque retention are, therefore, the major contributory factor of the periodontal disease progression. In daily practice overhanging margins of dental restorations present a very frequently observed problem which may greatly impinge on the maintenance of gingival and periodontal health. The objectives of the present study were to examine the prevalence of overhanging amalgam restorations in randomly selected patients, and to quantify the effects of inadequate approximate restorations on the marginal alveolar bone comparatively to the effect of well-fitting approximate fillings and intact approximate tooth surfaces.

Materials and Methods

Panoramic radiographs of 671 unreferred patients (325 males and 346 females), aged 18–75 years ($\bar{x}$ = 43.8±7.6), were drawn randomly at the Department of Dental Diseases and the Department of Oral Diseases and Periodontology, Dental Clinic, Ljubljana. Radiographs (Siemens) were placed on the x-ray viewer, grabbed by the camera, compressed and saved on the disk (PC/AT). Radiographic images were decompressed to a resolution of 720x512 pixels and displayed on the monitor.

In order to eliminate the effect of different restorative materials, only amalgam approximate fillings were studied. Mesial and distal approximate restorations of premolars and molars (except the third) were recorded. Approximate amalgam fillings with margins projected 0.5 mm and more away from the tooth surface were treated as overhangs. On the contrary, approximate fillings with no visible overhang were treated as flush-fitting (well-fitting) restorations.

Corresponding interproximal alveolar bone loss of premolars and molars in both jaws was determined. A cursor, controlled by the graphic tablet, was superimposed onto the image to mark the co-ordinates, anatomical landmarks for each tooth: the tip of the crown, the root apices and the alveolar bone margins on the mesial and distal surface of teeth. The amount of the alveolar bone supported each tooth, and was expressed as a fraction of a total tooth length. This technique provides

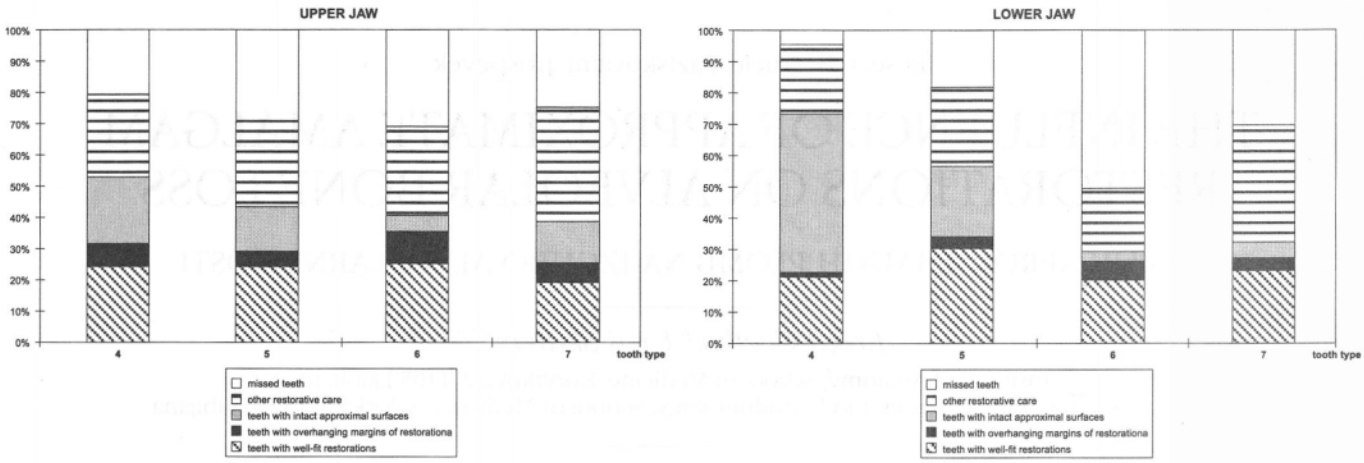

Fig. 1. *Prevalence of different restorative care according to the tooth type in the upper jaw (A) and in the lower jaw (B).*

Sl 1. *Pogostnost posameznih načinov zdravljenja zob zgornje (A) in spodnje (B) čeljusti.*

the basis for the comparison of bone loss on all the teeth, thus minimizing errors due to forshortening or elongation.

The optimum alveolar bone height assessed in the reference group of 20 patients, aged 18–25 years with clinically healthy periodontium was 62% of the total tooth length. Alveolar bone loss was determined in percent from this optimal alveolar bone height. Statistical significance was tested by t-test.

Results

The total of 8421 posterior teeth were examined in 671 panoramic radiographs. Percentage of missing teeth was determined. Out of the present teeth the proportion of teeth with intact approximate surfaces, overhanging and well-fitting approximate restorations were assessed (Fig. 1A, B).

The prevalence of approximate fillings for each particular tooth type ranged from 25% on lower first premolars to 55% on lower first molars. Among that fillings the greatest prevalence of overhanging fillings was on first molars in the upper jaw (27.5%), and the lowest on first lower premolars (5%). The lowest proportion of intact approximate tooth surfaces had first molars (5% in the lower jaw and 7.5% in the upper jaw). Out of the total sample the prevalence rate for overhanging fillings was 19%.

For the evaluation of interproximal bone loss the patients were ranged into five age groups. The height of the alveolar bone was compared among successive age groups. Alveolar bone loss increased by age (Fig. 2). Inside each particular age group a significantly higher bone loss was found adjacent to teeth with overhanging amalgam fillings compared to the bone loss adjacent to intact approximate tooth surfaces. Bone loss around teeth with well-fitting amalgam restorations was lower than around teeth with overhanging fillings, but statistical significance was proved in the ages up to 30 and over 50 years. For the total population alveolar bone loss was greater around the teeth with overhangs, lower around teeth with well-fitting restorations, and the lowest around intact tooth surfaces ($p < 0.01$). The bone loss was not significantly different between women and men.

Discussion

The findings suggest that there is a relationship between the quality of restorative care and periodontal health. Teeth with inadequate restorations had significantly greater alveolar bone loss than adequately restored teeth or teeth without approximate restorations.

The proportion of overhanging margins in this study 19% of restored approximate posterior surfaces is relatively low, but in accordance with other investigations. Several clinical, roentgenographic or combined studies have shown that the percentage of defective approximate restorations varies between 16.5% and 70%. The corresponding figure in the study by Gilmore & Sheiham (4) was 24%. Bjorn (3) found overhangs in 70% of approximate restorations with 55% of all defects being greater than 0.2 mm. Leon (11) found 16.5%, Hakkarainen and Ainamo (12) 50%, Rojs & Skalerič (13) 48.7% and

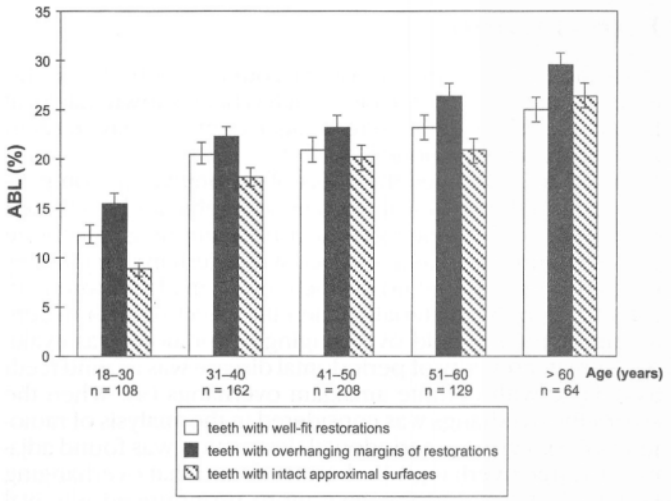

Fig. 2. *Mean bone loss (%) adjacent to approximate teeth surfaces by age groups.*

Sl 2. *Srednje vrednosti izgube kosti (%) ob aproksimalnih površinah zob v petih starostnih skupinah.*

Jenkins with co-workers (14) 65% of overhanging approximate restorations.

Differences in all these studies could be partly attributed to the definition of the overhang. In our study only clearly visible overhangs were recorded. Tooth surfaces that usually exhibit anatomical concavities in the cervical area such us upper premolars are particularly prone to overhangs, but can

not be demonstrated by the roentgenographic examination. Values for overhangs obtained by the roentgenographic examination are therefore lower comparing to values obtained by clinical studies. Differences in the investigated communities influence also on the prevalence number of the overhangs. At the Dental Clinic the dental care standard in patients included in our study is high, thus low prevalence of defective margins is reasonable.

The analysis of the particular tooth type has shown the lowest prevalence rate of overhanging restorations on approximate surfaces of mandibular premolars. This can perhaps be attributed to smaller cavities in more caries-resistant teeth, their circular cross-sectional root shape and smaller proximal contact surface areas. These are overall factors which would tend to simplify the placing of an amalgam restoration. On the other hand, the highest prevalence rate of overhangs found on molar teeth could be due to early eruption and low caries resistance of these teeth. The majority of them are restored soon after their eruption, and in the adult period these teeth most probably have repeatedly made restorations with great probability that their margins are placed subgingivally.

The tooth surface integrity disruption adjacent to gingival tissue increases plaque accumulations and sometimes inflammation, leading to the alveolar bone destruction. Our findings that interproximal bone loss increased with age lends credence to the assumption that our sample is a representative cross-section of the population. This report provides a quantitative evidence that overhanging amalgam restorations result in increased alveolar bone loss comparing to well-fitting ones. The results agree with those reported by Bjorn and coworkers (3), Gilmore & Sheiham, 1971 (4) and Leon, 1976 (11). It was assumed that general bone loss with the advancing age might even exclude the effect of overhanging restorations on the bone height. The results show that this is not the case. The destructive effect of the overhangs was maintained with age. It seems possible that the plaque retentive capacity of these restorations gives the apical migration of plaque a better start than offered by an intact surface, and that this differences persist over the years (12).

The comparison of the alveolar bone destruction performed for each particular tooth in the successive age groups showed similar differences such as for the total population, but differences were not significant. Several factors could influence on these results. First, no attention has been given to the duration of the overhangs. Many of them may have been relatively new and, therefore, may not have exerted their alleged effect on the alveolar bone over any considerable length of time. Considering the health status of the entire mouth rather than individual tooth surfaces alone the plaque accounted for a great part of variances. No information was available whether the restoration was extended subgingivally or not. Large supragingival overhangs cause less marginal bone resorption than subgingivally placed. Since radiographs were randomly selected, only few homologue pairs of teeth were found which did not permit any statistical analysis.

While both, the quality and the presence of restorations are significant factors in gingivitis and periodontitis, the amount of plaque is even more important. Patients can respond to the same stimulus at different rates, so unidentified differences exist among such patients. Subtle differences in the baseline microflora may effect the suspect of individual sites. Host factors, such as hormones (16, 17), immune status (18-20) and nutrition (21) are some of the many potential variables that are commonly offered to explain such differences.

Alveolar bone loss adjacent to restorations with no radiographically visible overhangs was lower than around teeth with overhangs, but higher than around teeth with intact tooth surfaces. The health of periodontium is thus adversely affected by the presence of the restoration. Data exist that even an adequately restored tooth leads to increase gingivitis and periodontal pocket formation (13). Subgingivally placed restorations irrespective of their quality had significantly more periodontal disease associated with them (11).

The mechanism underlying alveolar bone loss due to overhanging amalgam margins appears relatively clear. The roughness of restorative materials has been implicated in the gingival inflammation. It is believed that inflammation occurs due to the favourable environment for the plaque accumulation, rather than from the mechanical irritation (22). Adherence of bacteria to amalgam and tooth surfaces is resulted in the accumulation of plaque and calculus formation by the subsequent gingival inflammation. Creating of an altered ecological environment occur with the colonisation of more periodontopathic organisms in the subgingival flora (23). Also, the overhang may impinge on the embrasure space, and make the interproximal cleaning difficult. In the face of the continued inflammation, when the plaque irritation persists for a prolonged period of time, the alveolar bone destruction may result.

Since close association among iatrogenic factors such as overhanging restorations and the alveolar bone destruction has been recognised, several important clinical implications can be drawn from our study. Gingival cavo-surface margins of amalgam restorations are important determinants of periodontal health, and require careful attention of the dentists. Gingival sulcus should be avoided by any restorative materials; when clinical situations demand intrasulcular restorations, margins should be carefully contoured. If overhangs persist, they should be corrected to prevent the onset and progression of periodontal disease. The removal of the overhang permits more effective plaque control, resulting in the disappearance of the gingival inflammation and increased alveolar bone support.

The more restorations placed in the patient's mouth, the more adverse will be the effect on periodontal health, especially if the restoration quality is inadequate. The larger the number of restorations, the more important plaque control measures become the control of periodontal health. But without an effective plaque control, the quality of restorative care is relatively unimportant as a determinant of periodontal health.

Preferences

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Ključne besede: *resorpcija alveolarne kosti; rentgenski posnetki; amalgamske plombe*

Izvleček – Izhodišča. *Dokazano je bilo, da poleg sistemskih dejavnikov tudi neustrezne zobne plombe in prevleke pospešijo izgubo alveolarne kosti. Namen študije je bil ugotoviti pogostnost previsnih plomb in oceniti ter primerjati vpliv previsnih in dobro prilegajočih se plomb oziroma intaktnih zobnih površin na rob alveolarne kosti.*

Materiali in metode. *Pregledano je bilo 671 ortopantomografskih posnetkov naključno izbranih pacientov starih 18–75 let (x = 43,8). Izmerjeno izgubo kosti v interdentalnem prostoru ob previsni plombi smo primerjali z izgubo ob dobro prilegajočih se plombah in intaktnih zobeh.*

Rezultati. *Pogostnost previsnih aproksimalnih amalgamskih plomb je bila 19%. Izguba alveolarne kosti ob zobeh s previsnimi aproksimalnimi amalgamskimi plombami je bila statistično značilno večja kot ob zobeh z dobro prilegajočimi plombami ($p < 0,01$). Kostna resorpcija ob intaktnih zobeh je bila najmanjša ($p < 0,01$).*

Zaključki. *Previsne amalgamske plombe so pomemben dejavnik, ki vpliva na potek parodontalne bolezni. Nujna je pozorna modelacija aproksimalnih plomb, še zlasti predelov ob gingivalni stopnici. Obstoječe previse je potrebno odstraniti, kar prepreči nastanek oz. zmanjša hitrost napredovanja parodontalne bolezni.*

Research article/Raziskovalni prispevek

PLAUSIBLE HISTOPATHOLOGICAL CHANGES IN LIVER AND KIDNEY BASED ON EXPERIMENTAL PERIODONTITIS IN BEAGLE DOGS

MOŽNE HISTOPATOLOŠKE SPREMEMBE NA JETRIH IN LEDVICAH PO EKSPERIMENTALNO POVZROČENEM PARODONTITISU PRI PSIH

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Key words: *periodontal disease; mesangial thickening in glomerulus; dog*

Abstract – Background. *The aim of the study was to establish the possible histopathologic changes on the liver and kidneys in fifteen Beagle dogs who had one year long experimentally induced periodontitis.*

Methods. *Statistical analysis was used to determine if there were a relationship between the extent of periodontal disease and histopathologic changes in the tissues examined. In the fifteen Beagle dogs studied, there was an association found between periodontal disease and histopathological changes in the kidney and in the liver.*

Results. *The results of this study indicate a relationship between periodontal disease (PI = 2.8, GI = 2.5, PD = 3.7 mm, AL = 3.5 mm, all in average), morphological alterations in the kidney (mesangial thickening in glomerulus, tubule's degeneration, lymphoplasmacytic inflammation in interstitium) and in the hepatic parenchyma (multifocal inflammation and some focal fibrosis).*

Conclusions. *These results eventually support the hypothesis that the chronic periodontal inflammatory process is the potential cause for many pathological changes in the vital organs.*

Introduction

Periodontal disease is a common problem in dogs, and may affect their health and quality of life. A greater than 85% incidence of periodontal disease has been reported in dogs (1). The presence of systemic disease in dogs with chronic periodontal disease has been attributed to bacteremia and absorption of bacterial toxins from the oral cavity (1-9). Some problems said to be associated with chronic bronchitis, pulmonary fibrosis, endocardiosis, endocarditis, interstitial nephritis, glomerulonephritis, and hepatitis (1-9).

Periodontal disease in human beings may affect their general health and lead to systemic disease (10). A significant relationship in human beings between dental disease, cardiovascular disease, and total mortality has been reported by Loesche and Tzukert (10, 11). Bacteremia is sometimes attributed to poor dental hygiene, periodontal infections, and periapical infections in human beings (12). Additionally, infective endocarditis can be associated with tooth brushing and mastication, as well as with the dental procedure (13, 14). During mastication, bacteria enter the blood stream via the lymphatics at a predicted cumulative rate which is about 1,000 times greater than the rate occurring in a single tooth extraction (15). Additional concern in human beings with an oral infection is the potential development of the acute bacterial myocarditis, brain abscesses, uveitis, "fever of unknown origin", and other problems (16).

The aim of the present study was to evaluate the relationship between one year long experimentally induced periodontitis and plausible histopathologic changes in liver and kidneys in dogs.

Materials and methods

Fifteen male Beagle dogs, aged three and weighing approximately 13kg were involved in this study. At the end of a year long experimental periodontitis with elastic ligatures, the dogs were euthanised with a lethal injection of T61 after deep anesthesia (7, 17, 18). Before euthanasia, a periodontal disease score was determined for each dog. The individual tooth periodontal disease score was the sum of the scores of the following criteria for each tooth: plaque index - PI, probing depth - PD, attachment loss - AL and gingival index - GI. The animal periodontal disease score was the sum of the tooth periodontal disease scores. Criteria used to determine the plaque index, probing depth, attachment loss and gingival index are listed in Tab. 1 (17, 18).

All collected tissues were analysed for evidence of the gross pathologic change. Liver and kidneys were microscopically examined organs. Histopathologic scores were determined for each liver and kidney based on the criteria listed in Tab. 2. Scores from zero to five were assigned for each criterion, with zero equal to normal and five equal to the most severely af-

Tab. 1. *Scoring Criteria for experimental induced periodontal disease in dogs.*

Tab. 1. *Vrednotenje parodontalnega statusa pri eksperimentalno povzročenem parodontitisu pri psih.*

Score točke	PI	GI GSmm	PDmm IEPmm	ALmm
0	no plaque ni plaka	normal gingiva normalna gingiva	2	1.5
1	thin film of plaque at gingival margin visible when checked with an explorer tanka plast plaka ob gingivalnem robu	mild inflammation, slight change in colour, slight oedema, no bleeding on probing rahlo vnetje in edem gingive z rahlo spremembo barve, brez krvavitev	> 2.5	> 1.5
2	moderate amount of plaque, interdental space free of plaque zmerna količina plaka, interdentalni prostori so brez plaka	moderate inflammation redness, oedema, bleeding on probing zmerno vnetje, rdečina, edem gingive s krvavitvijo	3-5	2-4
3	heavy plaque at gingival margin, interdental space filled with plaque velika količina plaka ob gingivalnem robu, vključno z inter/ dentalnimi prostori	severe inflammation with marked redness and oedema ulcerations, tendency toward spontaneous bleeding močno vnetje z izrazito rdečino in edemom, ulceracijami in nagnenostjo k spontanim krvavitvam	5.5-8	4.5-7

Tab. 2. *Criteria and categories used for kidneys and liver histo/ pathologic scores.*

Tab. 2. *Vrednotenje histopatoloških sprememb v ledvicah in jetrih.*

Score Točkovanje	kidney - ledvici	liver - jetra
0	normal normalni	normal normalna
1	glomerulus mesangial thickness mezangialne zadebelitve na glomerulih	parenchyma: focal inflammation, focal fibrosis lokalna vnetna žarišča in lokalne fibroye v parenhimu
2	tubules degeneration degeneracije tubulov	portal inflammation portalno vnetje
3	interstitium lymphoplasma/ cytic inflammation, pyelitis limfoplazmocitno vnetje intersticija, vnetje ledvičnega meha	
4 and 5	severe histopathologic changes težje histopatološke spremembe	and in liver in v jetrih

fected. Kidney scores were based on the sum of the score for both kidneys from each Beagle dog (17, 18).
The pathologist collected organ specimens and performed histopathology by herself. She was unaware of the periodontal disease score.
Multiple regression analysis was carried out to determine if the experimentally induced periodontal disease score was a predictor of histopathologic changes - organ histopathologic score. The statistical significance was set forth at $p < 0.05$.

Tab. 3. *Periodontal Disease Score as a Predictor of Kidney Histopathologic Scores in Dogs.*

Tabela 3. *Vrednosti histopatoloških sprememb na ledvicah v povezavi s parodontalnim statusom.*

Tissue Tkivo	Criterion Ocenjevanje prizadetosti tkiva	Multiple Regression (p value) Multipla regresija
Kidney 1 Ledvica 1	glomerulus glomeruli	0. 001*
Kidney 2 Ledvica 2	tubules tubuli	0.30
Kidney 3 Ledvica 3	interstitium intersticij	0.42

* significant association $p < 0.05$
* značilnostna povezava $p < 0.05$

Tab. 4. *Periodontal Disease Score as a Predictor of Liver Histo/ pathologic Scores in dogs.*

Tab. 4. *Vrednosti histopatoloških sprememb na jetrih v povezavi s parodontalnim statusom.*

Tissue Criterion Ocenjevanje prizadetosti tkiva	Multiple Regression (p value) Multipla regresija
Liver 1 - parenchyma Jetrni parenhim 1	0.35

* significant association $p < 0.05$
* značilnostna povezava $p < 0.05$

Results

Chronic experimental periodontitis was evaluated by plaque index PI = 2.8, gingival index GI = 2.5, probing depth PD = 3.7, attachment loss AL = 3.5 in average.
Eight of the fifteen dogs studied had the initial changes of glomerulus like mesangial thickening on kidneys (Fig. 1).
Three of them had some focal inflammation in the liver parenchyma, and focal fibrosis (Fig. 2, Fig. 3).
Two of them had a lymphoplasmacytic inflammation on the kidneys interstitium. These two changes could not be associated with periodontal disease - experimental periodontitis.
Statistically significant relationships were present between the periodontal disease score (Tab. 1) and histopathologic scores for the criteria included into the category "kidney - glomerulus" $p = 0.001$ (Tab. 3). The periodontal disease score was not a statistically significant predictor for the criteria included into the categories "kidney tubules" $p = 0.30$ and "kidney interstitium" $p = 0.42$ (Tab. 3). It was also not a statistically significant predictor for the criteria included into the category "liver parenchyma" $p = 0.35$ (Tab. 4).

Discussion

In dogs the kidney disease, particularly glomerulonephritis, is considered to be a potential consequence of chronic low-grade bacteremia associated with periodontal disease (1, 4, 6, 8, 19, 20). Pyelonephritis and interstitial nephritis may also result from bacteremia or sepsis related to an oral infection (5, 9).
The results of this study demonstrate an association between periodontal disease and morphologic changes in renal glomeruli and interstitium. Glomerular changes were mild , variable thickening of the mesangia, and were interpreted as being non-specific alterations suggestive of immune complex-mediated damage. The interstitial change showed was an increase in lymphocytes and plasma cells in the interstitium of the medulla (Fig. 4).
These findings suggest that periodontal disease may contribute to the development of chronic lesions compatible with

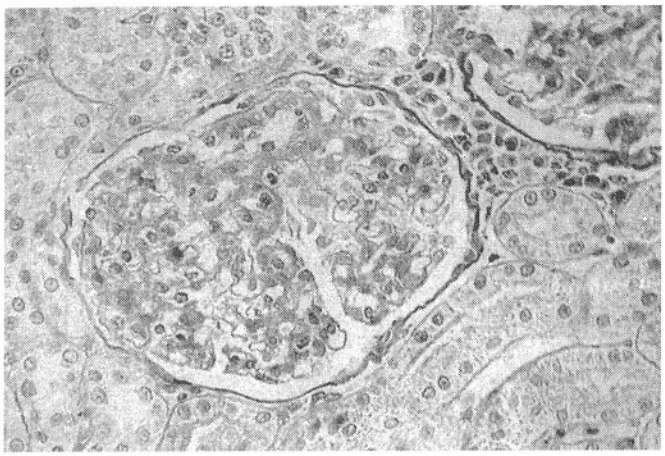

Fig. 1. *Mesangial thickening in the kidney glomerulus (original magnification × 200, stain PAS).*

Sl. 1. *Mezangialne zadebelitve v ledvičnem glomerulu (200-kratna povečava, PAS barvanje).*

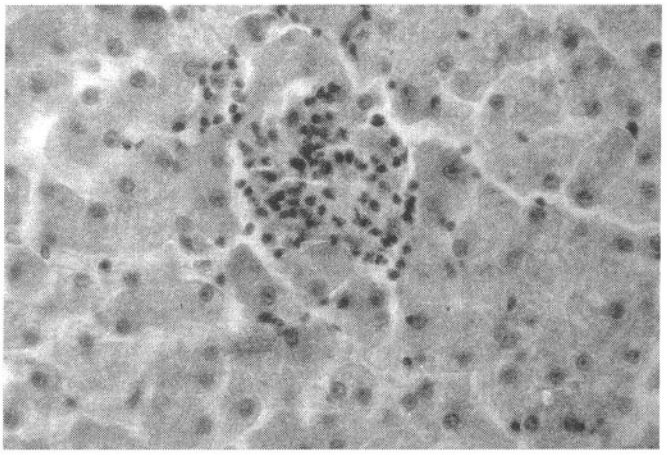

Fig. 2. *Focal inflammation in the liver parenchyma (original magnification × 200, stain HE).*

Sl. 2. *Omejeno vnetje jeternega parenhima (200-kratna povečava, HE barvanje).*

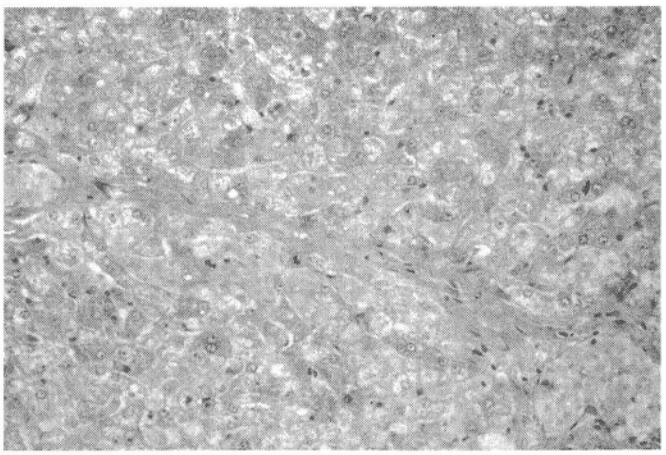

Fig. 3. *Local fibrosis in the liver parenchyma (original magnification × 200, stain HE).*

Sl. 3. *Lokalna fibrozacija jeternega parenhima (200-kratna povečava, HE barvanje).*

Fig. 4. *Mononuclear cells in the interstitium of the kidney medulla (original magnification × 100, stain HE).*

Sl. 4. *Mononuklearne celice v intersticiju sredice ledvic (100-kratna povečava, HE barvanje).*

low-grade but persistent damage insult to the kidney. In extreme cases, where a disease process sufficiently injures the glomeruli and interstitium, a chronic renal failure may result (21).

Bacteremia associated with periodontal disease is a suspected cause of some hepatic disorders, including hepatitis in dogs (1, 3, 4, 6, 8, 22, 23).

There was not a significant relationship between the periodontal disease score and the hepatic parenchymal inflammation score in this study, but the inflammation was generally mild, multifocal, and of minimal clinical significance with elevated liver enzyme activity AST - aspartate aminotransferase and ALT - alanine aminotransferase (22, 23).

The results of this study indicate a significant relationship between periodontal disease and morphologic alterations in the kidney (glomerulus) and a non significant relationship between periodontal disease and hepatic parenchyma. These results support the hypothesis that chronic periodontal inflammatory process is the eventual potential cause of many pathological changes in vital organs.

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Ključne besede: *parodontalna bolezen; mezangialne zadebelitve na glomerulih; pes*

Izvleček – Izhodišča. *Kronična parodontalna bolezen prizadane več kot 85% psov. Njen potek lahko velikokrat prizadane tudi življensko pomembne organe kot so jetra in ledvica. Lahko jo povzročimo eksperimentalno. Zato smo v naši raziskavi pri eno leto trajajočem eksperimentalnem parodontitisu skušali ugotoviti histopatološke spremembe na omenjenih organih.*

Metode in rezultati. *V raziskavi smo uporabili 15 psov moškega spola, pasme beagle, starih tri leta, s povprečno težo 13 kilogramov. Pri njih smo z elastičnimi tegi povzročili parodontitis in ga vzdrževali eno leto. Na koncu poskusa smo pse po globoki anesteziji eutanazirali in opravili patološko sekcijo ter histopatološki pregled ledvic in jeter.*
Kronični parodontitis smo ocenili s stopnjo gingivalnega indexa GI ki je bil v povprečju 2,5, plak indeksa PI = 2,8, globino sondiranja GS = 3,7 mm in izgubo epitelijskega prirastišča IEP = 3,5mm .
Patologinja, ki je opravila sekcijo in histopatološki pregled, z rezultati kroničnega parodontitisa ni bila seznanjena.

Rezultati. *Rezultati histopatološkega pregleda jeter so pokazali vnetna žarišča v parenhimu in lokalizirane fibroze ter posamezna vnetna žarišča v portalnem sistemu, v ledvicah pa mesangialne zadebelitve na glomerulih, limfoplazmocitna vnetna žarišča v intersticiju in tudi degeneracijo tubulov.*

Zaključki. *Rezultati kroničnega poteka eksperimentalnega parodontitisa in histopatoloških sprememb jeter (parenhim) in ledvic (glomeruli, intersticij) govorijo v prid hipotezi o vplivu parodontalne bolezni kot potencialnem vzroku mnogih patoloških sprememb na vitalnih organih. Vsekakor pa bi bilo potrebno za dokončno potrditev hipoteze opraviti večjo klinično in patološko študijo.*

Letter to the editor/Pismo uredniku

REGENERATION OF PERIODONTAL TISSUES AFTER LOCAL DELIVERY OF ANTIOXIDANTS

OBNOVA OBZOBNIH TKIV PO LOKALNEM DOVAJANJU ANTIOKSIDANTOV

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Key words: *periodontal treatment; liposomes; superoxide dismutase; catalase; beagle dogs*

Abstract – Background. *Bacteria from plaque activate gingival phagocytes to produce oxygen-derived free radicals which can cause an extensive tissue damage. The aim of our study was to evaluate the use of local administration of antioxidants in periodontal pockets, and the regeneration of periodontal tissues.*

Methods. *After the experimental periodontal breakdown followed in 15 beagle dogs, all the teeth were scaled supragingivally and animals were divided into three groups. The first group received the liposome-encapsulated superoxide dismutase (SOD), the second group the liposome-encapsulated catalase (CAT), and the third group received both enzymes. The efficiency of the periodontal treatment by scaling and root planning, as well as the same treatment followed by the subgingival application of liposome encapsulated antioxidant enzymes was evaluated. The drugs were applied subgingivally with a syringe on a daily basis for the period of 6 weeks. Gingival index (GI), probing depth (PD), and epithelial attachment level (EAL) were measured. Radiographs were evaluated at the baseline and at the end of the experiment. An analysis of radiographs was performed by using the digital subtraction radiography (DSR). A statistical analysis of data was performed by ANOVA and Student t-test.*

Results. *The greatest regeneration of periodontal tissues was found around the teeth by scaling and root planning followed by the SOD application. In this group a significant reduction of PD (1.1±0.1 mm), gain of EAL (0.6±0.1 mm), and the greatest alveolar bone apposition (10±4.6%) was observed.*

Conclusion. *Scaling and root planning followed by the subgingival application of the liposome encapsulated, the SOD suppressed periodontal inflammation, and stimulated the regeneration of periodontal tissues.*

Introduction

The primarily cause of periodontitis is considered to be a long standing bacterial infection. Bacteria from plaque activate gingival phagocytes to realize inflammatory mediators and oxygen-derived free radicals, such as superoxide radical and hydroxyl radical. Oxygen-derived free radicals are highly reactive and can cause an extensive damage to cells close to stimulated phagocytes (1, 2). Two enzymes are the most important to protect cells and tissues from the oxygen free radicals. The superoxide dismutase (SOD) removes superoxide and the catalase (CAT) removes hydrogen peroxide (3). Extracellular fluids have a very low SOD and CAT activity and small amounts of free radicals can cause an extensive damage in this compartment (2). SOD has a moderate anti-inflammatory activity which could be greatly improved by the encapsulation in liposomes (4). Free radicals play an important role in modulating the extent of the inflammatory response and the consequent tissue damage.

In this study the suppression of the periodontal inflammation and regeneration of periodontal tissues after a local administration of SOD and/or CAT was evaluated.

Animals and treatment procedure

The experimental periodontitis was induced by elastic ligatures around the second, third, fourth premolars, and the first molars on each side of the upper and lower jaw in 15 beagle dogs (5). Nine months after the beginning of the experiment the ligatures were removed. After the stabilization period of 3 weeks the treatment of inflammation was initiated. All teeth were scaled supragingivally. The animals were divided into three groups of five dogs. Each animal's dentition was divided into four quadrants in which different therapy modes were carried out:

1 - group of teeth with supragingival scaling only,
2 - group of teeth with supragingival scaling followed by a subgingival application of the liposome-encapsulated antioxidant enzymes,
3 - group of teeth with supra, subgingival scaling and root planning,
4 - group of teeth with supra, subgingival scaling and root planning followed by an subgingival application of the liposome-encapsulated antioxidant enzymes.

Scaling and root planing, as well as all the recordings were performed in the dogs anaesthetized by the Thiopental sodium (8 mg/kg) injection, and prolonged by the inhalation

anesthesia. During the experiment period the dogs were fed by a soft diet. The efficiency of the therapy mode 3, and the therapy mode 4 according to different enzymes was evaluated and compared with the therapy modes 1 and 2. By the therapy modes 2 and 4 the first group of dogs received the liposome encapsulated-superoxide dismutase (SOD) into the periodontal pockets, the second group the liposome encapsulated-catalase (CAT), and the third group both enzymes. Antioxidants were incorporated into the liposomes and mixed by the bioadhesive ointment-neutralized polymethyl methacrylate (10). The formulation was administrated subgingivally by a syringe and a blunt cannula on a daily basis for the period of six weeks. Gingival index (GI), probing depth (PD, and epithelial attachment level (EAL) were measured at the baseline and every two weeks of the treatment period. Radiographs were taken at the beginning and at the end of the experimental period. Analyses of these images were performed by using the digital subtraction radiography (DSR) (6-9). A statistical analysis of data was performed by ANOVA and Student t-test.

Results

Clinical measurements

Treatment by the supragingival scaling only has reduced PD for 0.3±0.1 mm from 3.0±1.1 mm at the baseline to 2.7±1 mm measured at the end of the treatment, EAL for 0.2±0.1 mm from 2.9±1.5 mm to 2.7±1.3 mm, and GI returning near to the beginning score GI = 1.8±0.2 (baseline GI = 1.9±0.1) (Tab. 1). In the therapy mode 2 the catalase had no effect on periodontal tissues. A significant suppression of the gingival inflammation was observed around teeth by the therapy mode 2 when SOD was applied GI = 1.3±0.4 (baseline GI = 1.8±0.1) ($p < 0.05$). A comparison between scaling and root planning, when the baseline score was GI = 1.8 ±0.2, and the therapy mode 4, based on the SOD application with the baseline score of GI = 1.8 ±0.1, showed the greatest suppression of the gingival inflammation (GI scores at the end of the treatment were: scaling and root planning GI = 1.5±0.3; the therapy mode 4 with SOD GI = 1.2 ±0.2, $p < 0.003$). In addition, the statistically significant reduction of PD from 3.2±1 mm at the beginning of the treatment to 2.1±0.7 mm at the end of the treatment period ($p < 0.043$), and EAL gain from 3.0±1.7 to 2.4±1.1mm ($p < 0.05$) was found.

Digital subtraction radiography

The therapy mode 1 with the supragingival scaling notified only the progress of the bone loss for 3.6% (Tab. 1). The therapy modes 3 and 4 evaluated the bone apposition, while no changes were observed in the group with the therapy mode 2. A statistically significant greater bone apposition was found in the group of teeth treated by scaling and root planing followed by the subgingival application of the liposome encapsulated SOD as compared with the therapy mode 1 ($p < 0.001$), therapy mode 2 ($p < 0.006$), and therapy mode 4 when CAT only was applied subgingivally ($p < 0.05$).

Discussion

A major source of oxygen derived free radicals are phagocytes (11). These cells are present in abundance in the inflammatory periodontal disease (12, 13). It was hypothesed that the highly reactive oxygen derived free radicals might be responsible for the initial degradation of extracellular matrix components seen in periodontal disease (14).

Tab. 1. *Gingival index score (GI), reduction of pocket depth (PD), and gain of epithelial attachment level (EAL) at the end of the treatment and percentage of the bone loss or gain according to different therapy modes.*

Tab. 1. *Gingivalni indeks (GI), zmanjšanje globine sondiranja (GS) in izboljšanje nivoja epitelijskega prirastišča (NEP) ob koncu zdravljenja ter odstotek izgube ali obnove čeljustne kosti glede na različne načine zdravljenja.*

Therapy mode Način zdravljenja	GI GI	Reduction of PD (mm) Zmanjšanje GS (mm)	Gain of EAL (mm) Izboljšanje NEP (mm)	Bone loss/ gain (%) Izguba/obnova kosti (%)
1,N	1.8±0.2	0.3±0.1	0.2±0.1	-3.6±4.3
2,S	1.3±0.4*	0.7±0.1	0.2±0.1	0
2,C	1.5±0.4	0.4±0.1	0.2±0.2	0
2SC	1.5±0.3	0.6±0.1	0.3±0.1	0
3,N	1.5±0.3	0.8±0.1	0.2±0.1	5.4±4.3
4,S	1.2±0.2*	1.1±0.1*	0.6±0.1*	10±4.6*
4,C	1.5±0.3	0.7±0.1	0.3±0.1	3.0±3.5
4,SC	1.3±0.2*	0.8±0.1	0.4±0.2	9.6±6.9

Legend / Legenda:

1,N - group of teeth with the supragingival scaling only
1,N - skupina zob, kjer so bile odstranjene obloge nad dlesnijo

2,S - group of teeth with the supragingival scaling followed by the subgingival application of the liposome-encapsulated superoxide dismutase
2,S - skupina zob, kjer so bile odstranjene obloge nad dlesnijo in v obzobne žepe dovajana, v liposome vključena superoksidna dismutaza

2,C - group of teeth with the supragingival scaling followed by the subgingival application of the liposome-encapsulated catalase
2,C - skupina zob, kjer so bile odstranjene obloge nad dlesnijo in v obzobne žepe dovajana, v liposome vključena katalaza

2,SC- group of teeth with the supragingival scaling followed by the subgingival application of the liposome-encapsulated both enzymes
2,SC- skupina zob, kjer so bile odstranjene obloge nad dlesnijo in v obzobne žepe dovajana oba encima vključena v liposome

3,N - group of teeth with the supra, subgingival scaling and root planning
3,N - skupina zob, kjer so bile odstranjene obloge nad dlesnijo in narejeno luščenje in glajenje zobnih korenin

4,S - group of teeth with the supra, subgingival scaling and root planning followed by the subgingival application of the liposome-encapsulated superoxide dismutase
4,S - skupina zob, kjer so bile odstranjene obloge nad dlesnijo ter narejeno luščenje in glajenje zobnih korenin z v obzobne žepe dovajano superoksidno dismutazo, vključeno v liposome

4,C - group of teeth with the supra, subgingival scaling and root planning followed by the subgingival application of the liposome-encapsulated catalase
4,C - skupina zob, kjer so bile odstranjene obloge nad dlesnijo ter narejeno luščenje in glajenje zobnih korenin z v obzobne žepe dovajano katalazo, vključeno v liposome

4,SC- group of teeth with the supra, subgingival scaling and root planning followed by the subgingival application of the liposome-encapsulated both enzymes
4,SC- skupina zob, kjer so bile odstranjene obloge nad dlesnijo ter narejeno luščenje in glajenje zobnih korenin, sledilo pa je dovajanje obeh encimov v obzobne žepe

In our study the greatest suppression of the gingival inflammation was observed around the teeth with scaling and root planning followed by the subgingival application of SOD (GI = 1.2±0.2). In addition, we evaluated the greatest reduction of PD, and gain of EAL around these teeth. The anti-inflammatory activity of SOD was seen even in the group of teeth treated by the supragingival scaling followed by the application of the liposome-encapsulated SOD (GI = 1.3±0.4).
Chronic inflammatory diseases such as periodontal disease are associated with the accumulation of chronic inflammatory cells occurring adjacent to the bone (15, 16). The radical production of activated phagocytes could be responsible for the activation of osteoclasts (17). The role of oxygen free radicals in the bone resorption was demonstrated by the *in vitro* study, when the bone resorption was prevented while SOD was added to isolated osteoclasts (18).

The DSR image analysis showed a progress of the alveolar bone loss when only the supragingival scaling was done. Probably the effect of SOD on free radicals from osteoclasts prevented the bone resorption and increased the bone apposition when added into periodontal pockets after scaling and root planning. The catalase used in concentration did not show any improvement in the observed parameter. Therefore, we could conclude that scaling and root planning followed by the subgingival application of the liposome-encapsulated SOD suppress the periodontal inflammation, and stimulate the regeneration of periodontal tissues.

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Ključne besede: *zdravljenje vnetja obzobnih tkiv; liposomi; superoksidna dismutaza; katalaza; psi pasme beagle*

Izvleček – Izhodišča. *Bakterije v zobnih oblogah aktivirajo fagocite v dlesni, da proizvedejo proste kisikove radikale. Kisikovi prosti radikali lahko poškodujejo tkivo. Namen naše raziskave je bil ugotoviti možnost lokalnega dovajanja antioksidantov v obzobne žepe in obnove obzobnih tkiv.*

Metode. *Po eksperimentalno povzročenem vnetju obzobnih tkiv na 15 psih pasme beagle so bile okrog vseh zob odstranjene zobne obloge nad dlesnijo. Živali smo razdelili v tri skupine. Prva skupina je v obzobne žepe dobivala v liposome vključen encim superoksidno dismutazo (SOD), druga katalazo (KAT) in tretja skupina oba encima hkrati. Primerjali smo zdravljenje vnetja obzobnih tkiv z luščenjem in glajenjem zobnih korenin, luščenje in glajenje korenin ter v obzobne žepe danimi encimi. Zdravljenje je trajalo 6 tednov. Ob zob smo vsakodnevno dajali 0,2ml učinkovine. Merili smo gingivalni indeks (GI), globino sondiranja (GS) in nivo epitelijskega prirastišča (NEP). Ob začetku in na koncu zdravljenja smo naredili rentgenske posnetke zob in jih analizirali z računalniško metodo prekrivanja. Rezultate smo statistično primerjali z ANOVA in Student t-testom.*

Rezultati. *Ob zobeh, kjer smo odstranili le zobne obloge nad dlesnijo se je GS zmanjšala le za 0,3±0,1 mm, NEP za 0,2±0,1 mm, GI pa se je vrnil na začetno vrednost (GI = 1,8±0,2). Iz rentgenskih posnetkov smo ugotovili 3,6% propad čeljustne kosti. Z luščenjem in glajenjem zobnih korenin se je GS zmanjšala za 0,8±0,1 mm, medtem ko se je NEP izboljšal enako kot pri zobeh, kjer smo odstranili le obloge nad dlesnijo. GI ob teh zobeh je ob koncu zdravljenja znašal 1,5±0,3. Z računalniškim prekrivanjem rentgenskih slik smo ugotovili 5,4% obnovo čeljustne kosti. Največja obnova obzobnih tkiv je bila ugotovljena ob zobeh, kjer smo luščili in gladili zobne korenine in v obzobne žepe dajali encim SOD. Izmerili smo statistično značilno zmanjšanje GS (1,1±0,1 mm), izboljšanje NEP (0,6±0,1 mm) in največjo obnovo čeljustne kosti (10±4,6 %). Vnetje dlesni je bilo ob teh zobeh najmanjše GI = 1,2±0,2 ($p < 0,003$).*

Zaključki. *Luščenje in glajenje zobnih korenin ter dovajanje v liposome vključene SOD zmanjša vnetje in pospeši regeneracijo obzobnih tkiv.*

Research article/Raziskovalni prispevek

INFLUENCE OF Er: YAG LASER PARAMETERS ON ABLATION OF ENAMEL AND DENTIN

VPLIV PARAMETROV Er: YAG LASERSKEGA SNOPA NA ABLACIJO SKLENINE IN DENTINA

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Key words: *laser, Er: YAG; enamel; dentin; ablation*

Abstract – Background. *The use of laser for hard dental tissue treatment was proposed in the early sixties. In last few years several studies were carried out with the Er: YAG pulsed laser (2.94 mm) due to its high efficacy of hard dental tissue removal in comparison to other laser types. It removes enamel and dentine by a thermo-mechanical ablation mechanism, which is based on the absorption of erbium laser light in a very thin tissue layer.*

Materials and methods. *The ablation process during a single laser pulse and the ablation speed at different repetition rates (from 2 to 20 Hz) were studied on extracted human teeth. The Er: YAG laser with pulses of adjustable length between 50 to 1000 ms was used.*

Results. *The ablation started at 4 J/cm²; at the pulselength of 50, 100, 300 ms it initially increases with applied fluence faster than at longer pulse durations up to 15 J/cm². In addition at short pulses only superficial modifications of dentine were observed by SEM in contrast to the overheating and melting at longer pulse irradiation times. The study of energy and repetition rate influence on ablation speed showed that carbonisation was observed at energies of 400 and 500 mJ/pulse above 4 Hz and at 200 mJ/pulse above 18 Hz.*

Conclusions. *Therefore, we conclude that single laser pulse ablation depends strongly on the pulse duration, while the ablation threshold is independent. The ablation rate increases near ablation threshold faster for short pulses in contrast to the ablation at high energy densities where the ablation is higher at longer pulse durations.*

Introduction

The use of laser for hard dental tissue treatment and as replacement for the conventional dental drill was proposed in the early sixties (1-3). The applicability of lasers in dentistry turned out to be more difficult as was expected in the beginning. The problem involves a relatively difficult processing of enamel and dentine, which are compound materials consisting of hydroksiapatite crystals and organic components with different optical, thermal and mechanical and material properties (4, 5). These are optical systems with wave absorption, scattering and guiding elements (6, 7). The absorption and transmission of laser light in human teeth strongly depends on the wavelength of the laser light. All the UV laser light is well absorbed, but in the mid-IR spectral range, the absorption in water and HA changes tremendously depend on the laser wavelength. The absorption of water at a wavelength of 3 mm for example is very high compared to the low absorption at 1 mm (Nd: YAG λ = 1.064 mm) and 10 mm (CO_2 λ = 10.6 mm). At 3 mm the absorption is even 10,000 times higher than at 1 mm (8, 9). Therefore, early attempts with lasers of improper wavelength were not satisfactory due to thermal side effects, such as carbonisation, cracks and melting of hard dental tissue and pulp necrosis.

In recent years several experimental investigations were carried out with the Er: YAG pulsed laser (10-15). Its high effectiveness is attributed to the extremely high absorption of its radiation (2.94 mm) in tissue water which removes enamel and dentine by a thermo-mechanical ablation process, the so-called microexplosions (16). As previous investigations have shown the Er: YAG laser ablation is caused by a special type of thermal photoablation (16, 17). The mechanism is based on the absorption of erbium laser light in a very thin tissue layer, and predominantly in one component of the tooth structure; the water. Therefore, this laser efficiently ablates hard dental tissues and most of the pulse energy is used for the ablation process, and not for heating of surrounding tissue. After the Er: YAG laser treatment almost no thermally induced changes in adjacent tissues can be seen, except a slight brownish rim around the crater in dentine. This slight carbonisation can be avoided by cooling the tooth surface with a water spray (18). The pulse duration of flash-lamp pumped Er: YAG lasers is usually from 200 to 400 µs. When longer laser pulse durations, or higher laser repetition rates are used, the temperature can increase. For that reason, a water spray cooling must be used, just like in the conventional high speed drilling procedure. In spite of that, the laser drilling speed is inferior compared to standard mechanical procedures. The laser drilling speed can be improved by increasing the laser power. However, it is very important to determine the laser parameters above which the laser irradiation can cause thermal damage to the surrounding dental tissue or dental pulp.

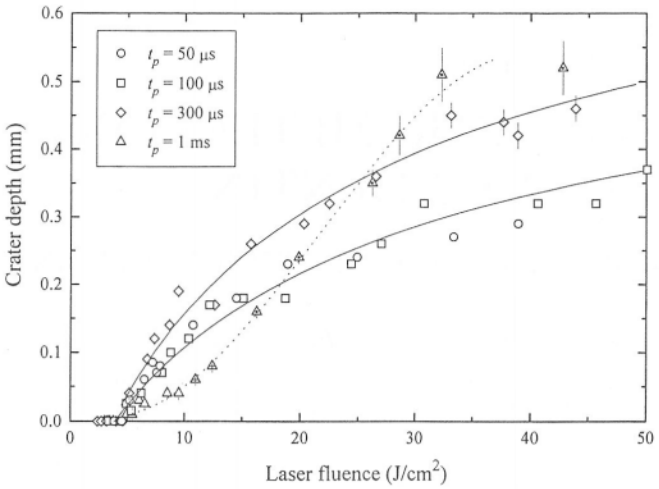

Fig. 1. *Crater depth in dentine after ten consecutive pulses of varying duration and energy as a function of energy density.*

Sl. 1. *Globina kraterja v dentinu po desetih zaporednih laserskih sunkih glede na gostoto energije laserskega žarka. Primerjamo različno dolgo časa trajajoče laserske sunke.*

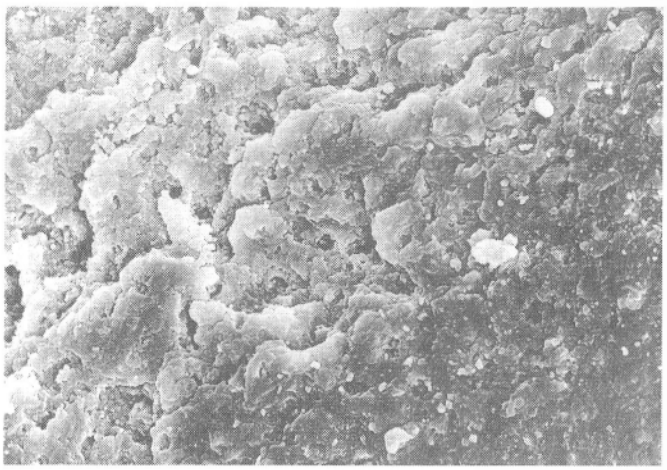

Fig. 2. *Scanning electron microscopic picture of the surface modification in dentine after short Er: YAG laser pulse duration. Only surface modifications are observed and no melting effects.*

Sl. 2. *Vrstično elektronska mikroskopska slika površinskih sprememb dentina po delovanju erbijevega laserja v zelo kratkih sunkih. Vidimo le površinske spremembe, ni opaznih razpok ali taljenja.*

In the present study the attention was focused onto the ablation process of hard dental tissues during a single laser pulse, and on the ablation speed at different repetition rates.

Materials and method

All drilling experiments were performed on extracted human teeth, cut parallel to longitudinal axis in 2 mm thick slices, and stored in 4% formaldehyde solution. In the first part of our study a free generating Er: YAG laser with pulses of adjustable length between 50 ms and 1 ms (Fotona d.d., Ljubljana, Slovenia) was used in the experiment. Ten consecutive laser pulses were applied to the same spot on the dental surface with no water spray cooling applied to the interaction site. Depths and diameters of the resulting craters were determined by optical microscopy. In the second part of the experiment the standard Twinlight dental laser system (Fotona d.d., Ljubljana, Slovenia) that incorporates two pulsed lasers Er: YAG and Nd: YAG was used. The laser pulse energies from 100 to 500 mJ, pulse duration 250 ms, repetition rates from 2 to 20 Hz, was focused perpendicularly to the tooth slice surface, and the spot area was approximately 0.8 mm^2. The water spray cooling system that is incorporated in the Twinlight laser system was applied during this experiment to prevent the temperature increase and desiccation of the tissue during the laser ablation process. The ablation efficiency was studied as a function of the pulse energy and pulse repetition rate. In order to detect structural alterations, scanning electron microscope (SEM) measurements of the irradiated samples were carried out.

Results

The Er: YAG drilling process and the influence of the pulse duration on ablation: depths of craters, drilled into dentine following ten consecutive pulses of varying duration and energy, are presented in Fig. 1 as a function of the applied energy density. Results of our previous studies (19) show that the ablation starts at a well defined fluence value which is independent of the pulselength and is approximately 4 J/cm^2. With pulselengths between 50 and 300 ms the ablation of the dental tissue initially increases with applied fluence faster than at longer pulse durations.

This difference is observed up to approximately 15 J/cm^2 where the situation is reversed, and the ablation rate at long pulses is more efficient compared to short pulses. The carbonisation of dentine was found to occur much more readily with longer laser pulses. At a pulselength of 1 ms, for example, a brownish rim of the craters develops at higher fluences around 10 J/cm^2, and turns into black at approximately 15 J/cm^2. With 300 ms pulses, however, colouration of craters was observed only above 100 J/cm^2, while heavy charcoaling develops at 150 J/cm^2. With even shorter laser pulses no carbonisation has been obtained in our previous experiments with fluence values up to 150 J/cm^2.

In addition to the above measurements the influence of pulselengths on laser effect in dentine and enamel were observed also by SEM. The thermo-mechanical process of ablation at short pulse duration produced only superficial modifications of dentine (Fig. 2).

Therefore, retentive surface modifications with no melting effect were produced at short pulse durations (200 ms) and lower energies, in contrast to the overheating and melting effect at longer irradiation times. At longer pulse durations (400 ms), where the interaction time of laser light with the tissue is longer, overheating and melting spots are observed due to overheating of the hard dental tissue (Fig. 3). We attribute this thermal effect to the diffusion of heat from the interaction site during the laser pulse.

The dependence of the ablation speed on energy and repetition rate in enamel is shown in Fig. 4. A microscopic observation showed no visible damage in enamel under all experimental conditions. In dentine (Fig. 5) the carbonisation was observed only at higher laser powers. At energies of 400 and 500 mJ/pulse the carbonisation was observed at repetition rates above 4 Hz, for pulse energies 300 mJ/pulse above 14 Hz, and at the laser energies of 200 mJ above 18 Hz. All experiments were performed with the water spray cooling of the interaction site to reduce the temperature increase and the desiccation of the tissue. Contrary to our expectations the thermal damage occurred more readily at higher single pulse energies than at higher repetition rates. For example, the car-

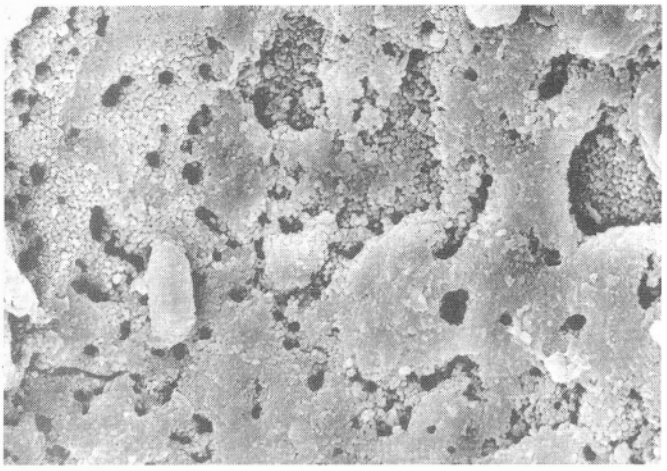

Fig. 3. *Scanning electron-microscopic picture of the surface modification in dentine after longer Er: YAG laser pulse duration. There are some melting effects observed in dentine.*

Sl. 3. *Vrstično elektronska mikroskopska slika površinskih sprememb dentina po delovanju erbijevega laserja v dolgih sunkih. Vidimo površinske spremembe in posledice taljenja površine.*

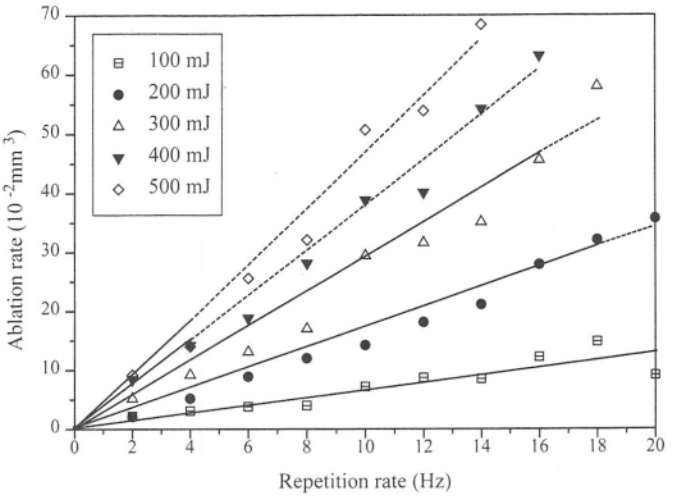

Fig. 5. *The dependence of the ablation speed on the energy and repetition rate in dentine. Thermal side effects and the carbonisation were observed and presented by dashed lines.*

Sl. 5. *Odvisnost hitrosti odstranjevanja dentina od energije in repeticije laserskih sunkov. Termične poškodbe so označene na grafu s prekinjenimi črtami.*

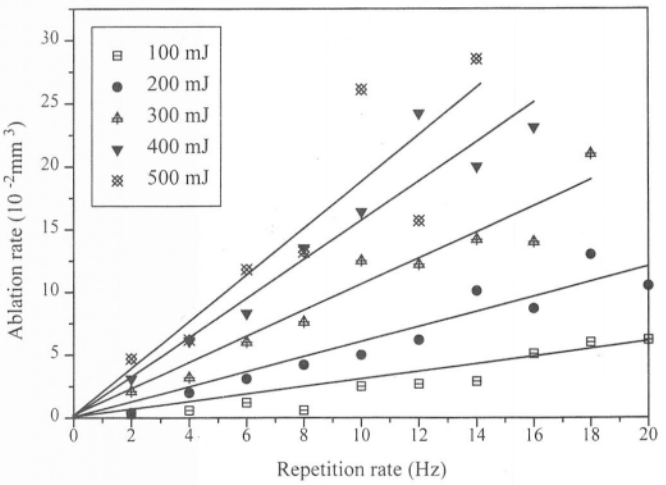

Fig. 4. *The dependence of the ablation speed on the energy and repetition rate in enamel. Thermal side effects were not observed.*

Sl. 4. *Odvisnost hitrosti odstranjevanja sklenine od energije in repeticije laserskih sunkov. Termičnih poškodb in karbonizacije nismo opazili.*

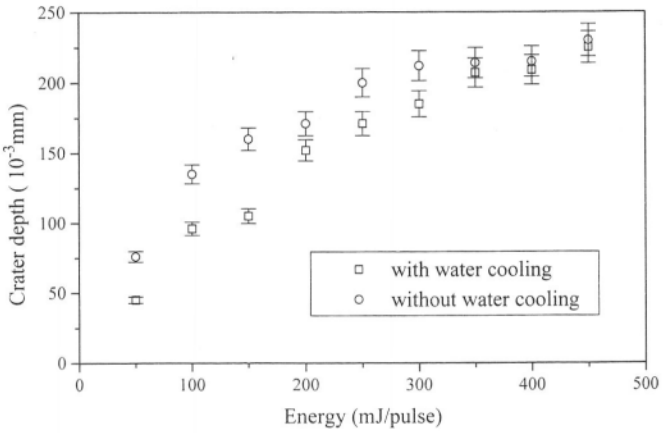

Fig. 6. *The effect of water cooling during the laser irradiation on the hard dental tissue ablation efficacy by laser.*

Sl. 6. *Vpliv vodnega hlajenja tkiva med laserskim obsevanjem na učinkovitost odstranjevanja trdih zobnih tkiv.*

bonisation occurred at 400 mJ, 4 Hz, i.e. at the average laser power of 1.6 W. However, no thermal damage was observed at 200 mJ, 16 Hz, i.e. at the average laser power of 3.2 W. Our study also shows that the application of the external water spray cooling has a negligible effect on the ablation efficacy (Fig. 6). In spite of the fact that Er: YAG laser light is strongly absorbed in water.

Discussion

When no water spray cooling is applied, the ablation efficiency of a single laser pulse saturates at high laser energies. This behaviour can be explained by an analytical model which takes into account the absorption of light in the ejected debris (20). The results of the calculation are in Fig. 1 presented by full lines. It is important to note that for longer pulse durations the saturation occurs at higher ablation crater depths. This is due to the fact that the debris density is lower and therefore the absorption of laser light is smaller, at longer pulse durations. Therefore at higher energy densities the ablation efficiency is higher when longer pulses are applied.

The situation is reversed at low energy densities. Near the ablation threshold the ablation is smaller for longer pulses. We attribute this observation to the interplay of ablation and thermal diffusion. For longer pulse durations, and therefore, lower laser powers the speed of ablation is small compared to the speed by which the heat diffuses into the tissue away from

the ablation area (20). This leads to the loss of energy which is used up for heating of surrounding tissue instead of for ablation.
Our investigations have shown that the thermal effect can be effectively reduced by cooling the ablated area with a water spray. No thermal increase is observed up to 20 Hz repetition rate which is indicated by the linearity of the ablation speed with the repetition rates in Fig. 4 and Fig. 5. When pulse durations of 200 to 400 ms are used, water cooling is more efficient for lower laser pulse energies and higher repetition rates. We attribute this observation to the fact that water spray cooling is relatively slow compared to the laser pulse durations. For this reason, cooling is more effective on the longer time scale, i.e. between laser pulses and not during a single laser pulse.

Conclusions

The results of our in vitro study in real conditions indicate that when no water cooling is applied, the single Er: YAG laser ablation depends strongly on the laser pulse duration. While the ablation threshold is independent of the pulse duration the ablation rate increases near the threshold faster for pulses of short duration. In contrast, at high energy densities, the ablation is stronger for longer pulses. We attribute these two observations to the thermal diffusion and debris screening. In practice, this observation seems to indicate that when the Er: YAG laser is used for the surface modification without external cooling long laser pulses of low energies must be applied in order to obtain low ablation and the tissue modification without melting. But when the Er: YAG laser is used for the cavity preparation, the intermediate laser energies up to 350 mJ/pulse must be used in combination with the external water spray cooling.

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Ključne besede: *laser; Er: YAG; sklenina; dentin; ablacija*

Izvleček – Izhodišča. *Prvi poskusi uporabe laserja namesto klasičnih zobozdravniških vrtalk za delo na sklenini in dentinu segajo v 60-ta leta. Vendar so šele z novo lasersko tehnologijo razvili laserje, od katerih na področju zobozdravstva veliko pričakujemo. Konec osemdesetih let so se začele poglobljene raziskave sunkovnega erbijevega laserja (Er: YAG), saj so začetni rezultati pokazali, da je zaradi valovne dolžine 2,94 mm svetlobni snop tega laserja primeren za odstranjevanje trdih zobnih tkiv. Je bolj učinkovit in povzroča manj termičnih poškodb okolnih tkiv od drugih poznanih laserjev. Razlog za to je specifični termično-mehanski način odstranjevanja zobnega tkiva (t.i. ablacija), ki je posledica močne absorpcije laserske svetlobe v tkivni vodi in njene nenadne uparitve. Razumevanje procesa absorpcije laserske svetlobe v trdih zobnih tkivih in načina njihovega odstranjevanja je pogoj za razvoj in klinično uporabo dentalnega laserja. To je tudi bil namen naših raziskav o delovanju erbijevega laserja na trda zobna tkiva, kjer smo želeli preiskati vpliv različnih parametrov na odnašanje tkiva.*

Metode. *Vse meritve smo opravili na ekstrahiranih človeških zobeh, ki smo jih razrezali v vzdolžni smeri na v 2 mm debele rezine in jih hranili v 4% formaldehidu. V prvem delu raziskave smo študirali proces ablacije in termalne difuzije v času sunka. Zato smo uporabili erbijev laser, ki omogoča nastavitev dolžine (od 50 ms do 1 ms). Meritve globine in premera kraterjev smo opravili po desetih zaporednih laserskih sunkih na isto mesto. V drugem delu naše študije smo študirali vpliv repeticije in hlajenja na modifikacijo površine in odnašanje zobnega tkiva. Uporabili smo komercialni dentalni laserski sistem Twinlight (Fotona d.d., Ljubljana), ki vsebuje erbijev in neodimov laser (Nd: YAG). Svetlobne sunke erbijevega laserja z energijo od 100 do 500 mJ, dolžino okoli 250 ms in repeticijo od 2 do 20 Hz smo usmerili pravokotno na skleninsko in dentinsko površino vzorcev, ki smo jo hladili z mešanico vode in zraka.*

Rezultati. *Naše predhodne meritve so pokazale, da se ablacija trdih zobnih tkiv začne, ko preseže gostota energije prag okoli 4 J/cm². Globina kraterjev z naraščajočo energijo sunkov raste, vendar se pri visokih energijah učinkovitost ablacije zmanjšuje in doseže plato. Hitrost ablacije se zmanjša zaradi absorpcije laserske svetlobe v delcih, ki z zvočno hitrostjo odletavajo z mesta odstranjevanja tkiva. Zato na hitrost odstranjevanja vpliva, razen energije posameznega sunka, tudi dolžina oziroma moč laserskega sunka. Pri daljših sunkih*

je gostota delcev, ki se odstranjujejo v smeri žarka v vsakem trenutku laserskega sunka manjša od gostote delcev pri kratkih sunkih. Zato se manj energije sunka absorbira in saturacija nastopi pri večjih globinah kraterjev. Učinkovitost ablacije je zato pri visokih energijah večja pri daljših dolžinah sunkov. Nasprotno pa je pri nizkih energijah. Pri nizkih gostotah energije (od 4 do približno 15 J/cm^2) globina kraterjev narašča hitreje pri kratkih sunkih (od 50 do 300 ms) kot pa pri dolgih sunkih (od 600 do 1000 ms). To razliko smo razložili s fizikalnim modelom, kjer smo upoštevali razliko v hitrosti ablacije in termalne difuzije v tkivu. Pri dolgih sunkih nizke energije se velik del te energije porablja za segrevanje okolnega tkiva in ne za ablacijo. Razliko v termalnih učinkih smo opazovali z elektronskim mikroskopom. Pri kratkih sunkih na površini tkiva nismo opazili pregrevanja tkiva taljenja ali razpok. Nasprotno pa je pri dolgih sunkih, kjer je moč laserskega sunka pri enaki energiji manjša, pa smo opazili taljenje in karbonizacijo tkiva na površini. Segrevanju tkiva, še posebno pri ponavljajočih se sunkih, se lahko izognemo s hlajenjem med laserskim delovanjem. Predhodne študije so pokazale, da je uporaba hlajenja z mešanico vode in zraka zelo primerna, saj na hitrost odstranjevanja dentina in sklenine skoraj ne vpliva, zmanjša pa termične poškodbe trdih zobnih tkiv v okolici delovanja laserja. Zato smo v študiji vpliva repeticije na hitrost odnašanja sklenine in dentina tkivo hladili z mešanico vode in zraka kot ga poznamo pri delu s turbinsko vrtalko. Ugotovili smo, da pri delu z repeticijo do 20 sunkov v sekundi, ob uporabi vodnega hlajenja, ni termičnih poškodb. Ablacija posameznega sunka ni odvisna od frekvence, zato laserski sunki odnašajo tkivo neodvisno eden od drugega.

Zaključki. *Na osnovi našega dosedanjega raziskovalnega in kliničnega dela menimo, da je erbijev laser primeren za obdelavo trdih zobnih tkiv. Z njim lahko odstranjujemo sklenino, dentin in kariozno tkivo, razen tega lahko z njim povečamo retencijsko površino zoba. Ugotovili smo, da moramo pri nižjih energijah uporabiti laserski sunek, ki je dovolj kratek (od 50 do 300 ms) in dovolj velike moči, da povzročimo površinske spremembe in ne gretja tkiva. Pri visokih energijah pa so učinkovitejši daljši sunki. V študiji vpliva repeticije na hitrost odnašanja sklenine in dentina v realnih pogojih s hlajenjem tkiva pa smo ugotovili, da hlajenje z mešanico vode in zraka, podobno kot pri turbinski vrtalki, učinkovito zmanjša pregrevanje tkiva. Prav tako smo ugotovili, da kljub absorpciji laserske svetlobe v vodi, hlajenje na hitrost odstranjevanja tkiva le malenkostno vpliva. Pri delu z repeticijo do 20 sunkov v sekundi ablacija posameznega sunka ni odvisna od frekvence in zato laserski sunki odnašajo tkivo neodvisno eden od drugega. Na osnovi teh rezultatov sklepamo, da lahko večjo hitrost odstranjevanja tkiva dosežemo s povečevanjem repeticije laserskih sunkov.*

Research article/Raziskovalni prispevek

EFFECT OF ND- AND ER: YAG LASERS IRRADIATION ON THE ROOT SURFACE STRUCTURE

STRUKTURA KORENINSKE POVRŠINE PO OBSEVANJU Z ND- IN ER: YAG LASERJEMA

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Key words: *dental root; laser light; morphology; thermal alterations*

Abstract – Introduction. *The Nd: YAG laser has been recommended and widely used as an adjunctive therapy in the treatment of periodontal diseased root surfaces. The aim of this "in vitro" study was to answer whether Nd: YAG or Er: YAG lasers alter the root surface structure.*

Materials and methods. *To determine root surface alterations we used thirty-five samples (3.0 × 4.0 × 1.0 mm), prepared from approximate root surfaces of periodontally involved human upper incisors. Samples were divided into seven groups, and irradiated for one minute by the Nd: YAG laser at power settings of 0.5W, 1.0W and 1.5W and by the Er: YAG laser at energies of 60 mJ, 80 mJ and 100 mJ per pulse, respectively. Five non-lased samples served as a control. Lased and non–lased samples were examined under scanning of the electron-microscope at x170 to x4600 magnification.*

Results. *We found that Nd: YAG laser irradiation altered the root surface structure. Alterations ranged from the fissure formation at 0,5W to the crater formation and meltdown of the root mineral at 1,5W of power. Er: YAG laser irradiation removed the entire cement layer and exposed dentinal tubules in all the irradiated groups.*

Conclusions. *In conclusion, both lasers altered the root surface structure, however, the Er: YAG laser caused less thermal damage in surrounding tissues.*

Introduction

High power Nd: YAG laser irradiation has been shown to decrease the fibroblast attachment to the root surface and to occlude dentine tubules (1). Furthermore, IR spectroscopy showed that protein/mineral ratio in cement samples had decreased after Nd: YAG laser irradiation. Thus the decreased protein/mineral ratio and the potential surface contamination with protein by-products may decrease the fibroblast attachment to the cement surface (2). Various surface changes such as charring and carbonisation of the cement surface, randomly distributed pitting and crater formation, and melting of the root mineral phase with subsequent resolidification as porous globules were observed on Nd: YAG irradiated surfaces (3). On the other hand, it has been demonstrated that the low power Nd: YAG laser had removed the smear layer efficiently without closing the dentine tubules (4). In *in vitro* conditions the Er: YAG laser effectively removes dental calculus an hard dental tissues (5). The aim of this *in vitro* study was to answer whether Nd: YAG or Er: YAG lasers alter the root surface structure.

MATERIALS AND METHODS

Sample preparation

Thirty-five specimens (3.0 × 4.0 × 1.0 mm) were prepared from approximate root surfaces of the extracted periodontally involved human upper incisors. The specimens were removed from areas of 2 mm below the cement-enamel junction. Specimens were randomly divided into seven groups of five specimens. Groups 1 to 6 were irradiated, while group 7 with non-lased specimens served as a control.

Laser treatment

A combination of the Er: YAG and Nd: YAG solid state lasers in the Twinlight® dental laser system (Fotona, Ljubljana) was used. The laser system operated at the following parameters (Tab. 1).
Samples were irradiated by the following power or energy settings, according to the random distribution into seven experimental groups (Tab. 2).
Samples were irradiated for 1 minute. The delivery contact optic fibre (600 μm) or hand-piece were held parallel to the sample surface, and continuously moved back and forth in an attempt to cover the entire root segment with overlapping strokes.

Scanning electron microscopy

All specimens were prepared for the SEM examination. Specimens were dehydrated in a series of graded ethyl alcohol solutions (50–100%).The final dehydration step was accomplished in hexamethyldisilazane. The specimens were mounted on aluminium stubs, sputter-coated with gold and examined by the scanning electron microscope (JEOL-JXA 840A). Micrographs were taken from lased and non-lased control surfaces at ´170 to ´4600 magnifications.

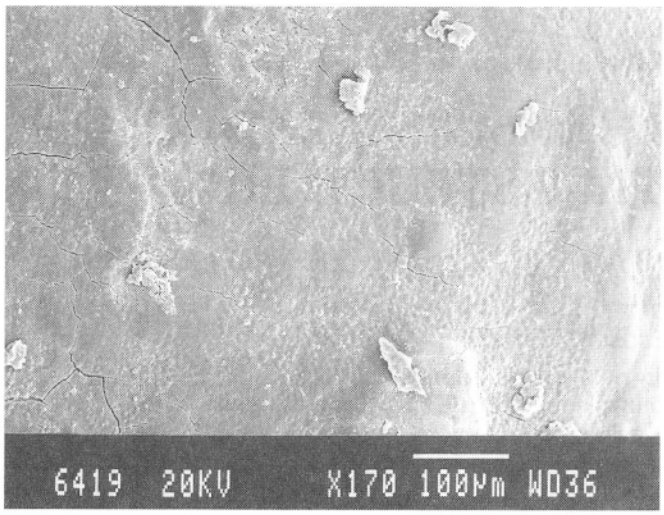

Fig. 1. *SEM picture of non-lased root surface showing normal granular texture. Bar = 0.01mm at magnification of ×170.*

Sl. 1. *SEM slika neobsevane koreninske površine kaže normalno granulirano strukturo. Merilo = 0,01mm pri 170-kratni povečavi.*

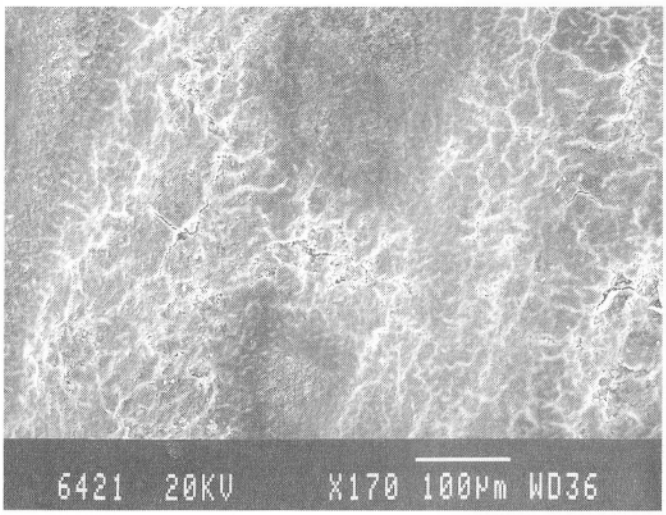

Fig. 2. *Root surface treated with an Nd: YAG (0.5W) laser showing fissures and cracks. Bar = 0.01 mm at magnification of ×170.*

Sl. 2. *Na koreninski površini obsevani z Nd: YAG (0,5W) laserjem so vidne fisure in poke. Merilo = 0,01mm pri 170-kratni povečavi.*

Tab. 1. *Properties of the Twinlight® laser system.*

Tabela 1. *Lastnosti laserskega sistema Twinlight®*

Laser type Tip laserja	Nd: YAG	Er: YAG
Wavelenght Valovna dolžina	1064 nm	2940 nm
Repetition rate Repeticija	10 p/s	10 p/s
Pulse duration Dolžina pulza	150 µs	250 µs
Spot size Premer pike	0.6 mm	0.8 mm
Delivery system Prenosni sistem	optic fibre optično vlakno	mirror system in articulated arm sistem zrcal v artikulirani roki

Tab. 2. *Power and energy settings of the laser beam in seven experimental groups.*

Tabela 2. *Nastavitve moči in energije laserskega žarka v sedmih poskusnih skupinah.*

Group Skupina	Laser Laser	Power/energy Moč/energija
1	Nd: YAG	0,5 W
2	Nd: YAG	1,0 W
3	Nd: YAG	1,5 W
4	Er: YAG	60 mJ/p
5	Er: YAG	80 mJ/p
6	Er: YAG	100 mJ/p
7	non-lased neobsevana	

Results

Scanning electron microscopy of the irradiated specimens showed various degrees of root surface alterations. Nd: YAG laser irradiation thermally damaged the root surface accompanied by minimal tissue ablation. On the contrary, Er: YAG laser irradiation effectively removed the cement layer and dentin without thermal side effects.

The smear layer and rests of dental calculus were seen on the control non-lased root surfaces (Fig. 1). Irradiation by 0,5 W Nd: YAG laser beam caused fissuring and cracking of the root surface (Fig. 2). An increase in power of the laser beam to 1,0 W resulted in ablation of the cement layer and dental calculus accompanied by the crater formation. Crater walls were partially covered by melted inorganic parts of the root cement (Fig. 3). Deep craters, entirely covered by spheres of the resolidified mineral were seen on root surfaces after irradiation by 1,5 W laser beam (Fig. 4).

Er: YAG laser irradiation entirely removed the root cement. Single craters, without deposits of melted minerals, and exposed dentinal tubules were seen on root surfaces lased by 60 mJ Er: YAG laser beam (Fig. 5, 6). Root surfaces lased by 80 mJ Er: YAG laser beam were covered by numerous confluent craters. Ejected parts of dentine and exposed dentinal tubules were seen on irradiated root surfaces (Fig. 7). Large ablation defects with partly rounded edges and exposed and opened dentinal tubules were seen on SEM microphotographs after irradiation by 100 mJ Er: YAG laser beam (Fig. 8.).

Discussion

The aim of our study was to evaluate alterations of the root surface lased by Nd: YAG laser irradiation at the lowest available power settings. We have found out that the Nd: YAG laser thermally alters the root surface, and that the Er: YAG laser removes the entire cement layer.

The effect of the laser beam in the tissue depend on physical properties of the laser light (wavelength, power, pulse duration, spot size and irradiation time), and on optical properties of the irradiated tissue (optical density, structure, absorption maxims) (6). Hard dental tissues are composed of hydroxyapatite crystals, organic parts and water. In the infrared part of the electromagnetic wavelength the absorption maximum for hydroxyapatite is at 10000 nm, and for water at 3000 nm (7). The wavelength of the Nd: YAG laser light is far from absorption maxims for water and hydroxyapatite. The Nd: YAG laser beam is therefore poorly absorbed in healthy dental tissues and penetrates deep into the irradiated tissue. On the contrary the wavelength of the Er: YAG laser of 2940 nm corresponds to the absorption maximum of water and to the minor absorption maximum of hydroxyapatite. The Er: YAG laser beam is thus entirely absorbed in the irradiated

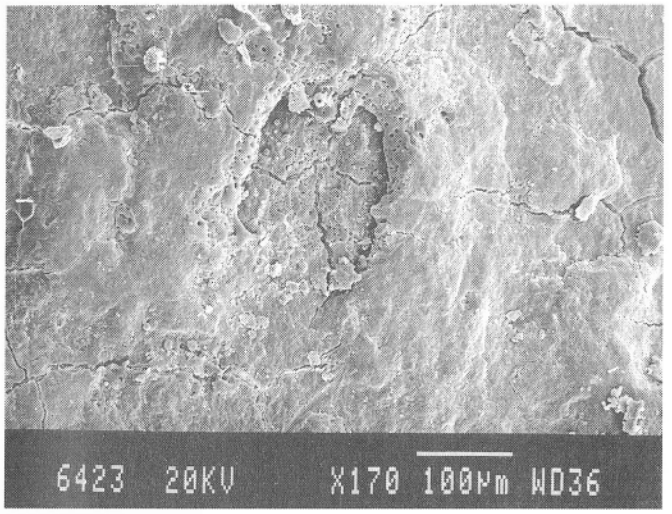

Fig. 3. *Root surface treated with an Nd: YAG (1.0W) laser showing cratering effect and meltdown and resolidification of the root mineral. Bar = 0.01 mm at magnification of ×170.*

Sl. 3. *Na koreninski površini obsevani z Nd: YAG (1,0W) laserjem so vidni kraterji in raztaljeni anorganski del korenine. Merilo = 0,01mm pri 170-kratni povečavi.*

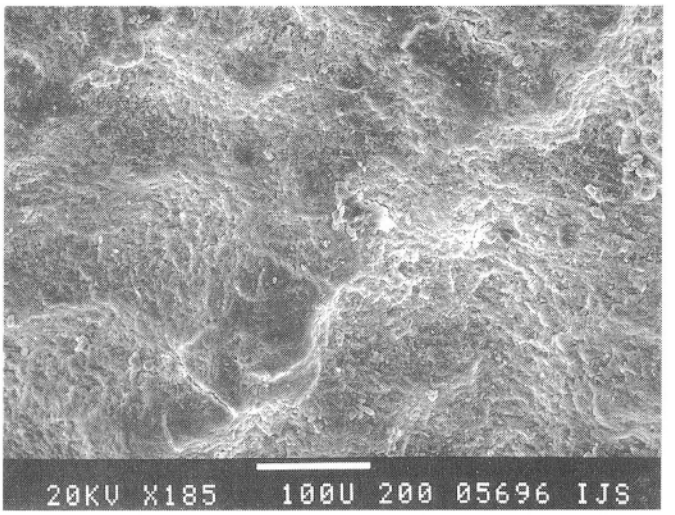

Fig. 5. *Root surface treated by an Er: YAG laser using 60 mJ/pulse showing the cratering effect without any meltdown or resolidification of the root mineral. Bar = 0.01 mm at magnification of ×185.*

Sl. 5. *Na koreninski površini obsevani z Er: YAG (60mJ/pulz) laserjem so vidni kraterji brez taljenja anorganskega dela korenine. Merilo = 0,01mm pri 185-kratni povečavi.*

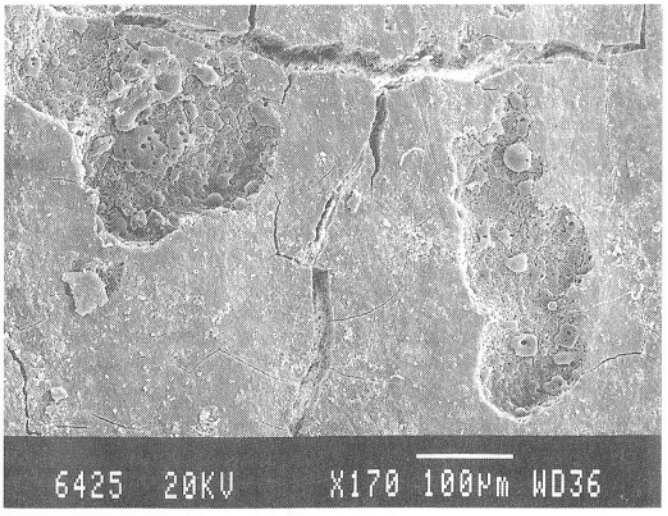

Fig. 4. *Root surface treated by an Nd: YAG (1.5W) laser showing globules of the resolidified root mineral. Bar = 0.01 mm at magnification of ×170.*

Sl. 4. *Na koreninski površini obsevani z Nd: YAG (1,5W) laserjem so vidne kroglaste mase raztaljenih mineralov koreninske površine. Merilo = 0,01mm pri 170-kratni povečavi.*

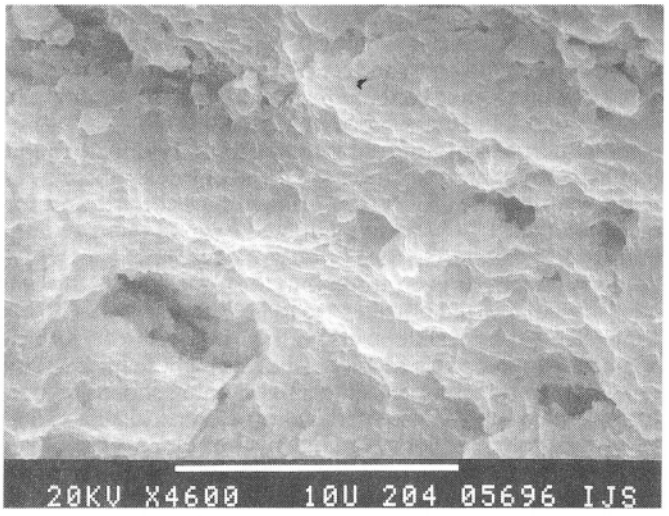

Fig. 6. *High magnification view of the crater wall from a root surface treated by an Er: YAG (60 mJ/p) laser showing dentinal tubules. Bar = 0.01 mm at magnification of ×4600.*

Sl. 6. *Na večji povečavi kraterske stene na koreninski površini obsevane z Er: YAG (60mJ/p) laserjem so vidni odprti dentinski kanali. Merilo = 0,01mm pri 4600-kratni povečavi.*

tissue superficial layer. Energy of the absorbed light determines the effect of laser light in tissue. Since the Nd: YAG laser light penetrates deep into the tissue, the beams energy is absorbed in a large volume of tissue. Energy of photons is thus converted into the thermal energy, and the final result is heating of the irradiated tissue which is a base for photothermal effects (8). Local increase in tissue temperature may break weak non-covalent chemical bonds. Energetically reach covalent chemical bonds might be broken at higher temperatures. The photoablative effect is due to a single or simultaneous multiphoton absorption (2). In the case of the Er: YAG laser the energy of laser beam is also converted into the thermal energy, however this conversion take place in a small volume of tissue. The final result is therefore the explosive heating of tissue which is a base for photomechanical effects.

Surface alterations after Nd: YAG laser irradiation include the smear layer ablation, fissuring and cracking. The power of the laser beam 0.5W is not sufficient to ablate the cement layer, however it is sufficient to increase the cement temperature locally, and therefore thermally damage the irradiated tissue. Thermal damage include desiccation, denaturation of protein and rapid thermal expansion followed by a slow cooling process. After lasing by the Nd: YAG laser at 1.0W of power the

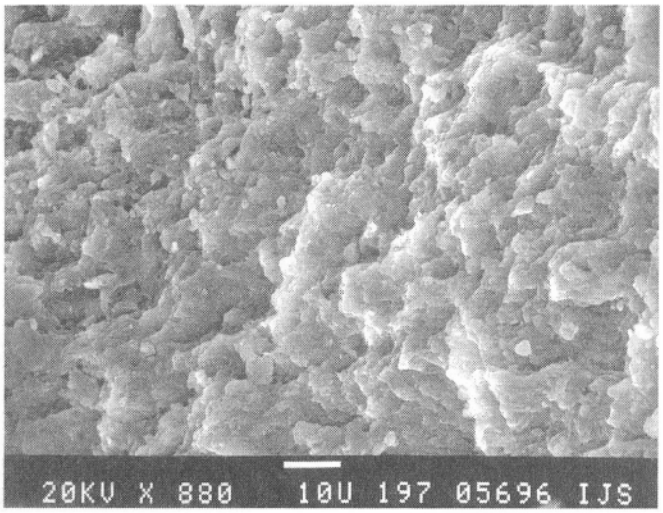

Fig. 7. *Root surface treated by an Er: YAG laser using 80 mJ/pulse showing deep craters. Bar = 0.01 mm at magnification of ×880.*

Sl. 7. *Na koreninski površini obsevani z Er: YAG (80 mJ/p) so vidni globoki kraterji. Merilo = 0,01mm pri 880-kratni povečavi.*

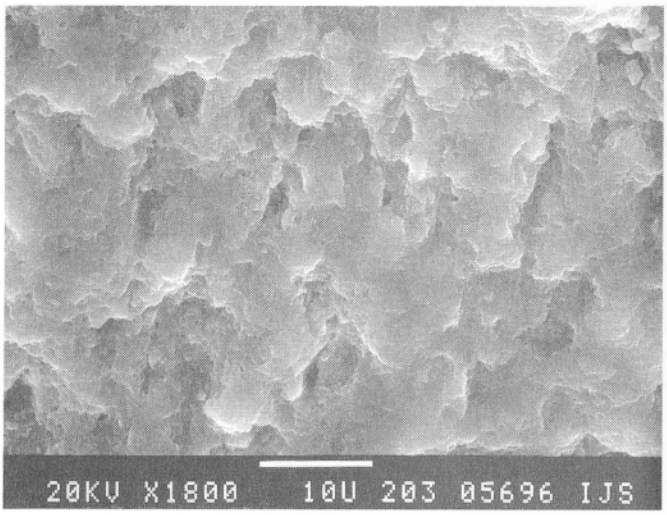

Fig. 8. *Root surface treated by an Er: YAG laser using 100 mJ/pulse showing the crater surface. Note numerous open dentinal tubules. Bar = 0.01 mm at magnification of ×1800.*

Sl. 8. *Na koreninski površini obsevani z Er: YAG (100 mJ/p) laserjem so vidni kraterji. Opazni so številni odprti dentinski tubuli. Merilo = 0,01mm pris 1800kratni povečavi.*

cement ablation by melting down of inorganic substances accompanied by the smear layer removal is seen in the SEM picture. The smear layer removal, the cement layer ablation, or the cement thickness reduction, fissure, cracks and exposure of dentin tubules are findings after the irradiation of the root surface at the highest power setting of 1.5W.

Similar findings are reported by *Morlock et al. 1992*, who found that Nd: YAG laser irradiation at 1.25 W of power ablated cement and dental calculus from root surfaces. Irradiation for one minute at 1.75 W resulted in meltdown of the root mineral around craters. On the contrary *Wilder-Smith et al. 1995* demonstrated that even three minutes of lasing by the Nd: YAG laser at 5 W did not alter the root surface.

Craters and spherical deposits on the irradiated root surface are ideal conditions for recolonisation of pathogenic bacteria (10). Conventional root scaling and planning should be performed after Nd: YAG laser irradiation. Dental calculus is due to its dark colour easier to remove by the Nd: YAG laser than healthy dentin or cement (11), but thermal side effects might cause irreversible changes in the dental pulp.

The Er: YAG laser beam is highly absorbed in hard dental tissues. The irradiated tissue is thus photomechanically removed in microexplosions. *Aoki et al. 1994* found out that 30 mJ/p laser beam effectively removed dental calculus from root surfaces without removing the entire cement layer (5). The lowest energy setting in our study was 60 mJ/p. Since the entire cement layer is ablated in all samples we concluded that even this lowest energy setting is too high for the calculus removal. In conclusion, both lasers altered the root surface structure, however the Er: YAG laser caused less thermal damage in surrounding tissues.

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Ključne besede: *zobna korenina; laserska svetloba; morfologija; toplotne spremembe*

Izvleček – Izhodišča. *Kot dodatno sredstvo pri zdravljenju parodontalne bolezni se uporablja neodimijev (Nd: YAG) laser. Obsevanje zobne korenine z Nd: YAG laserskim žarkom moči več kot 3W spremeni morfologijo, biokompatibilnost in kemično sestavo koreninske površine. Obsevana koreninska površina je posuta z globokimi kraterji in raztaljenimi anorganskimi delci, ki skupaj s produkti toplotne razgradnje organskega dela tkiva preprečujejo vzpostavitev novega vezivnega prirastišča. V literaturi ni podatkov o morebitnih strukturnih in kemičnih spremembah po obsevanju z Nd: YAG laserskim žarkom moči nižjih od 1,25 W. V zadnjem času je*

vedno več rezultatov "in vitro" raziskav o delovanju erbijevega (Er: YAG) laserja na trda zobna tkiva. Zaradi učinkovitega odnašanja tkiva brez toplotnih poškodb so te raziskave usmerjene pretežno na področje zdravljenja kariesa, le redke na področje zdravljenja parodontalne bolezni.
Zato je bil namen naše "in vitro" študije odgovoriti na vprašanje ali Nd: YAG ali Er: YAG lasersko obsevanje pri nižjih močeh (P < 1,5W) ali energijah (E_p < 100mJ) žarka morfološko spremeni koreninsko površino.

Materiali in metode. *Uporabili smo petintrideset dentinsko cementnih vzorcev velikosti 4mm × 3mm × 1mm, ki smo jih odvzeli iz stranskih ploskev korenin zgornjih sekalcev. Vzorci so bili naključno razdeljeni v sedem skupin po pet vzorcev. Prve tri skupine vzorcev smo obsevali z Nd: YAG laserskim žarkom valovne dolžine 1064 nm in moči 0,5W, 1,0W in 1,5W, naslednje tri skupine vzorcev smo obsevali z Er: YAG laserskim žarkom valovne dolžine 2940 nm in energijami 60 mJ/p, 80 mJ/p in 100 mJ/p. Oba laserja smo uporabili v pulznem načinu delovanja pri frekvenci 10 laserskih pulzov v sekundi (10 Hz). Vzorce smo obsevali tako, da smo eno minuto vodili optično vlakno (Nd: YAG) ali ročnik (Er: YAG) po celi površini vzorca. Sedma skupina vzorcev je bila neobsevana in je bila uporabljena za kontrolo. Vseh 35 vzorcev smo pripravili za elektronsko mikroskopsko analizo. Vzorce smo dehidrirali s potapljanjem v raztopine alkoholov naraščajočih koncentracij (50%–100%) in nazadnje v heksametildisalazanu. Sledilo je napraševanje z zlatom in slikanje z vrstično elektronskim mikroskopom (JEOL-JXA 840A) pri 170 do 4600 kratnih povečavah.*

Rezultati. *V študiji z Nd: YAG laserjem smo ugotovili, da obsevanje koreninske površine z Nd: YAG laserjem morfološko spremeni koreninsko površino. Na vrstično elektronskih posnetkih so vidne poškodbe koreninske površine od pok pri 0.5W do kraterjev pri 1.5W. Obsevanje koreninske površine z Er: YAG laserjem odnese celotno plast zobnega cementa. Na vrstično elektronskih posnetkih so vidni odprti dentinski kanalčki po obsevanju z laserskim žarkom energije 60 mJ/pulz. Zaradi intenzivne absorpcije Er: YAG laserskega žarka ni toplotnih poškodb preostalega tkiva.*

Zaključki. *Dobljeni rezultati so dokaz, da obsevanje z Nd: YAG laserjem že pri najnižjih močeh povzroči mikroskopske spremembe koreninske površine. Nasprotno se Er: YAG laserju zaradi njegove učinkovitosti in "hladnega" načina delovanja odpirajo nova področja aplikacije na trdih in mehkih tkivih ustne votline.*

Research Article/Raziskovalni članek

SYSTEMIC HEALTH IN PATIENTS WITH PERIODONTAL DISEASE

SISTEMSKO ZDRAVJE PACIENTOV S PARODONTALNO BOLEZNIJO

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Key words: *periodontal disease/epidemiology; systemic diseases; medical history; diabetes mellitus; smoking*

Abstract – Background. *Systemic diseases may modify a host response to bacteria in dental plaque, and influence on the development and progression of periodontal disease. A periodontal patient is most frequently middle aged or older. This life period is frequently accompanied by different diseases which can influence on the periodontal treatment outcome. In order to avoid unnecessary complications the periodontist must be aware of the patient systemic diseases and impairments. The aim of our study was to find out whether systemic diseases and conditions are more frequently present in periodontal patients in comparison with subjects with healthy periodontal tissues.*

Methods. *Periodontal tissues of the randomly selected 1210 inhabitants of Ljubljana were checked by The Community Periodontal Index of Treatment Needs Method. Out of all of 25- to 65-yr-old subjects two study groups were formed. In the first 195 persons with periodontal disease were chosen, and in the second 186 persons with relatively healthy periodontal tissues. Presence of systemic diseases were assessed by answers to a health questionnaire (Cornell Medical Index Health Questionnaire).*

Results. *Patients with periodontal disease had more frequent appearance of cardiovascular diseases among which the most frequently reported was high blood pressure (22.1%), followed by rheumatoid arthritis (20.0%), asthma/hay fever (10.3%) and diabetes mellitus (8.7%). In the "healthy" group hives (31.2%) and allergy to drugs (24.7%) were more frequently reported than in the "periodontal" group. In addition, among patients with periodontal disease moderate and heavy smokers were more frequently significant, and had less teeth than "healthy "subjects.*

Conclusions. *In the present study periodontal patients have systemic diseases more frequently than healthy persons without periodontal diseases. More than a quarter of patients also takes drugs currently. We conclude that a precise assessment of the dental patients medical history, especially of periodontal patients is an important part of the oral diagnosis and treatment planning.*

Introduction

Periodontitis belongs to a group of multifactorial chronic diseases, and is generally considered to be a consequence of an unfavourable host-parasite interactions. The bacteria in dental plaque are predominately responsible for the initiation of periodontal disease, but systemic diseases modify the host response to bacteria and are responsible for the progression of periodontal disease (1).

Systemic risk factors have recently been identified by large epidemiologic studies. Grossi and co-workers demonstrated that out of a large number of systemic diseases only diabetes mellitus was associated with a more severe destructive periodontal disease (2). Both type I and type II diabetes mellitus are risk factors for periodontal disease (3, 4).

Smoking is one of the most important risk factors for periodontal disease, increasing the risk 2 to 7-fold, depending on the level of smoking (2, 5).

Recent studies have shown that periodontal diseases which are chronic G-negative infections also effect systemic health of patients. Periodontal infection represents a risk factor for arteriosclerosis and thromboembolic events, for coronary heart disease and stroke (6). In their recent study Offenbacher and co-workers found out that periodontal diseases represent a previously unrecognized and clinically significant risk factor for preterm low birth weight (7).

Patients with periodontal disease are most frequently middle aged or older. Most frequently they have different systemic diseases or conditions like hypertension, ischemic heart disease or diabetes mellitus. These conditions may modify the potential response to periodontal treatment (8). Since periodontal therapy frequently involves surgical procedures, a periodontist has to know patient's medical history in order to provide treatment safely. (8). Using a health questionnaire helps the dentist to establish guidelines about prevention of the subacute bacterial endocarditis, and enables the patient to present information concerning difficulties which might have been otherwise overlooked (9).

The aim of the present study was to determine whether the frequency of systemic diseases and conditions is more prevalent in periodontal patients in a selected population of Ljubljana citizens in comparison with individuals with healthy periodontal tissues. We also wanted to find out whether there was a difference in smoking habits and in number of missing

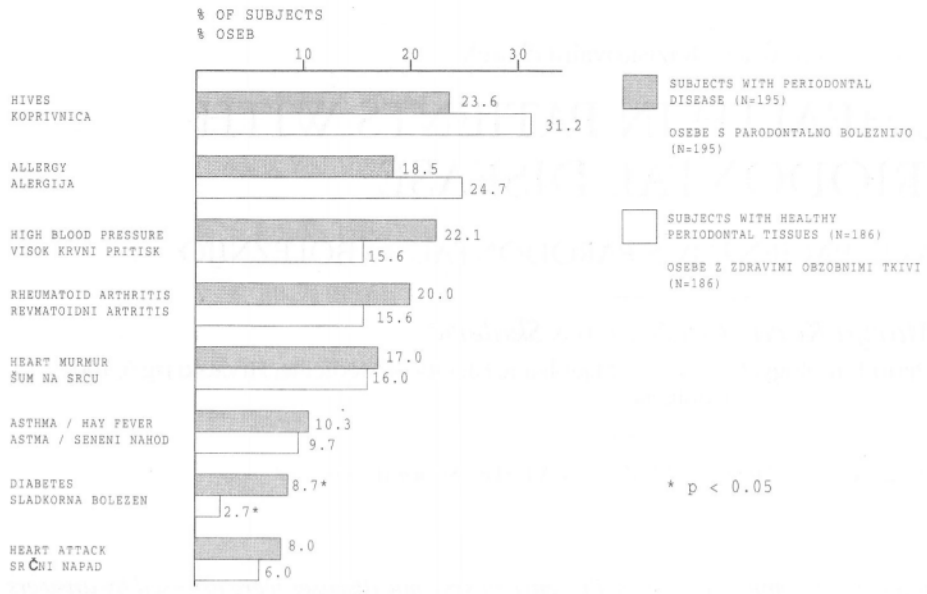

Fig. 1. *Frequency distribution of systemic diseases in 25- to 65-yr-old subjects.*

Sl. 1. *Frekvenčna porazdelitev sistemskih bolezni pri osebah starih od 25 do 65 let.*

teeth between the periodontal diseased group and the healthy group.

Subjects and methods:

Periodontal tissues of 1210 randomly selected citizens of Ljubljana 25-, 35-, 45-, 55- and 65-yr-old (585 males and 625 females) were examined by the methodology of The Community Periodontal Index of Treatment Needs (10).

According to CPITN the teeth are divided into 6 sextants. Findings are recorded according to the following four clinical evaluations in each sextant: healthy periodontal tissue, bleeding after gentle probing of pockets, calculus, shallow periodontal pockets (4-5 mm), or deep periodontal pockets (6 mm or more). Only the highest code number per sextant is recorded. Williams periodontal probe was used to evaluate the above mentioned periodontal parameter. Subjects were examined at the Department for Oral Medicine and Periodontology at the Dental Clinic in Ljubljana.

All subjects were divided into "diseased" and "healthy" groups according to the following criteria: the first group consisted of 195 "diseased" subjects with the signs of periodontal disease. 35-yr-old subjects had deep periodontal pockets in one or more sextants, 45-, 55- and 65-yr-old in 2 or more sextants. In the 25-yr-old group subjects had shallow or deep periodontal pockets in one or more sextants. The second group consisted of 186 "healthy" subjects with relatively healthy periodontal tissues according to their age. With the age there are less and less sextants with healthy periodontal tissues. Therefore our criterion for "healthy" sextants depended on the age of the examinees. 25-yr-old subjects had healthy periodontal tissues in 3 or more sextants, 35- and 45-yr-old examinees in 2 or more sextants, 55- and 65-yr-old in one or more sextants. Other sextants were coded with the code for bleeding or calculus. There were no sextants with periodontal pockets in "healthy" groups.

In both groups there were totally 381 persons, what represents 31.5% of all the examined population, among them there were 202 males and 179 females.

The frequency distribution of systemic diseases or conditions were assessed by the use of the Cornell Medical Index Health Questionnaire (11) dental modification. The questionnaire includes general data about the patient, instructions how to fill it in and confidential statement. Answers to 77 questions concerning diseases, states of diseases and symptoms are dichotomous. The study focused only on the affirmative answers.

Statistical analysis was performed by χ^2 test and Student t-test.

Results

Table 1 presents the number and the percentage of subjects in the "diseased" and in the "healthy" group according to the age.

The frequency distribution of the most prevalent systemic diseases and systemic conditions is shown in Fig. 1.

The most prevalent self-reported diseases were: hives, allergy to drugs, high blood pressure, rheumatoid arthritis, heart murmur, asthma/hay fever, diabetes mellitus, heart attack.

Tab. 1. *Number and % of subjects in "diseased" group and "healthy" group according to the age.*

Tab. 1. *Število in odstotek oseb v "parodontalni" skupini in" zdravi" skupini glede na starost.*

Age starost	Total N število oseb	"Diseased" group "Parodontalna" skupina N % štev. %	"Healthy" group "Zdrava" skupina N % štev. %
25	240	24 (10.8)	43 (17.9)
35	291	65 (22.4)	38 (13.1)
45	244	37 (15.1)	37 (16.8)
55	237	38 (16.0)	37 (15.6)
65	198	31 (15.6)	31 (15.6)
Σ	121	195 (16.1)	186 (15.4)

From Fig. 1 it can be seen that most diseases are more frequently present in the "periodontal" group with the exception of hives and allergy which appeared more frequently in subjects of the "healthy" group.

Differences in presence of most diseases between both study groups were not statistically significant, however, some of them approached statistic significance, for instance in the case of hives ($c2 = 2.8$; $p < 0.10$). Diabetes mellitus was significantly more prevalent in periodontal patients in comparison with the healthy ones ($c2 = 6.0$; $p < 0.05$).

The frequency of diseases according to the age:

The frequency of self-reported diseases in our study population was being increased by the age. In 65-yr-old subjects 48% have high blood pressure in the "diseased" group, and only 35 % in the "healthy" one. Prevalence of rheumatoid arthritis increases with the age in both groups. Heart attack is most frequent in 65-yr-old subjects in both study groups. Hives is most frequent in 45-yr-old patients in the "diseased" group (27.6%) and in 55-yr-old subjects in the "healthy" group (40%). Rheumatoid arthritis is most frequent in 55-yr-old subjects in both study groups. Heart murmur is approximately evenly present in all the age groups. Diabetes mellitus increases with the age. 25.8% of 65-yr-old periodontal patients have diabetes in comparison with 12.9% of "healthy" persons of the same age.

Tab 2 presents the percentage of 25-to 65-yr-old subjects with systemic diseases and smoking habits in both groups.

Tab. 2. *Medical history and smoking habit of subjects in "diseased" and in "healthy" group.*

Tab. 2. *Medicinska anamneza in navade kajenja oseb v "parodontalni " in "zdravi" skupini.*

	% of subjects % oseb	
	"Diseased group" "Parodontalna" skupina	"Healthy group" "Zdrava" skupina
difficulty in chewing food težave z zvečenjem hrane	33.8	20.4
severe headaches močni glavoboli	21.0	17.0
mouth breathers dihanje na usta	26.2	22.0
bleeding gums krvavitev iz dlesni	51.8*	26.9 $p < 0.001$
severe sore mouth močno vnetje ustne sluznice	12.0	7.0
short of breath on mild exertion se hitro zadiha	58.0	45.0
chest pain on exertion bolečine v prsih ob naporu	23.0	19.0
prickling areas on skin otrpli predeli na koži	23.0	18.0
get tired easily hitro se utrudi	39.0	31.2
taking medicines currently trenutno jemanje zdravil	27.2	22.6
non smokers nekadilci	54.9	65.4
former smokers prenehali kaditi	7.6	7.4
smokers (10 cig./less) kadilci do 10 cig./dan	7.6 13.0*	$p < 0.01$
smokers (20 cig./less) kadilci do 20 cig./dan	17.9	10.5
smokers (21 cig./more) kadilci 21 in več cig./dan	12.0*	3.7 $p < 0.001$

Systemic diseases and conditions were more often reported in the "diseased" group than in the "healthy" group. Bleeding gums was the only statistically significantly more often presented symptom in periodontal patients in comparison with "healthy" subjects ($p < 0.001$).

More than half of persons in the "periodontal" group were non smokers (54.9%), in the "healthy" group there were 65.4% of non smokers. Moderate smokers (up to 20 cigarettes per day) and heavy smokers (more than 20 cigarettes per day) together amounted to 29.9% in the "diseased" group, and only to 14.2 % in the "healthy" one. The difference is statistically significant ($\chi^2 = 12.1$; $p < 0.001$).

Number of missing teeth in both study groups increases with the age (Tab. 3). Periodontal patients in all the age groups have less teeth in comparison with "healthy" persons. The difference is statistically significant in 35- to 65-yr-old groups.

Tab. 3. *Number of missing teeth (x±s.d.) in "diseased" group and in "healthy" group according to the age.*

Tab. 3. *Število manjkajočih zob (x±s.d.) v "parodontalni" skupini in v " zdravi" skupini glede na starost.*

	"Diseased" group "Parodontalna" skupina	"Healthy" group "Zdrava" skupina	
Age/years Starost/leta	x±s.d.	x±s.d.	P
25	2.04±2.44	1.83±2.41	N.S.
35	5.38±3.99	4.0±3.30	*
45	7.72±4.66	3.37±2.79	**
55	10.97±6.21	5.45±3.41	**
65	12.64±4.57	7.45±3.17	**

* $p < 0.05$
** $p < 0.001$
N.S. = non significant
N. S. = nesignifikantno

Discussion

In the present study the most prevalent self-reported diseases were hives, allergy to drugs, cardiovascular diseases and rheumatoid arthritis. Among cardiovascular diseases high blood pressure was most frequently reported. Our findings can be compared to some foreign researches.

Prevalence of hives in periodontal subjects in our study (23.6%) is slightly higher as stated in the study by Grossi and co-workers (2). They found among 1426 inhabitants of Erie County, New York, from 25- to 74-yr-old having 20.6% prevalence of hives.

Periodontal patients in our study had 18.6% prevalence of allergy to drugs, what is slightly less than in the study by Grossi (25.3%) (2), or in the study of Peacock, where 21.7% of prevalence of allergy in subjects with periodontal diseases was reported (12). Rees and Brasher (8) found 16.1% prevalence of allergy to drugs in subjects with periodontal disease.

Allergy to drugs and hives in our study group are conditions which were insignificantly more frequently reported among subjects in the "healthy" group in comparison with the "periodontal" group. In the study by Grossi et al (2) allergies were less frequently present in patients with greater loss of attachment and bone loss. The association may be related to medications taken by individuals with allergies. (2)

Our study reported higher percentage of high blood pressure (22.1) in comparison with the finding in the study by Grossi (16.4%) (2). In their study Peackock and Carson (12) found out in 590 persons with periodontal disease from 18- to 78-yr-old 20.0% prevalence of cardiovascular diseases, and most of them had high blood pressure. Nery and co-workers (13) found in 581 persons with periodontal disease aged from 20- to 90-yr-old 26% prevalence of cardiovascular diseases.

Prevalence of asthma/hay fever in periodontal patients in our study is lower (10.3%) in comparison with the prevalence in study by Grossi et al. (18,4 %) (2).

In our study a higher percentage of diabetes was found out in persons with periodontal disease (8.7%) than in the study by Grossi et al (4.8%) (2), or by Peacock (3.7%) (12). In our study diabetes is significantly more frequently present in periodontal patients than in "healthy" subjects ($p < 0.05$). In the report by Grossi et al (2) it was found that among 17 systemic diseases only diabetes mellitus correlated with the more severe destructive periodontal disease. Diabetics were twice as likely to exhibit attachment loss than non-diabetics (2). In other studies higher prevalence and severity of periodontal disease in individuals with diabetes in comparison with non-diabetics was reported (14, 15).

Bleeding gums was significantly more frequently reported (< 0.001) in the "diseased" group of our population in comparison with "healthy" subjects. The result is in accordance with the clinical findings of the examined subjects.

In many studies smoking was shown to be a strong risk indicator for the destructive periodontal disease (2, 5, 16, 17). Smoking was highly correlated with attachment loss and alveolar bone loss (2, 5). In our study in "periodontal" patients there were significantly more frequent moderate smokers (up to 20 cigarettes a day) and heavy smokers (21 and more cigarettes a day) in comparison with "healthy" persons.

Regarding the number of missing teeth in the group of "periodontal" patients there were more missing teeth in comparison with persons of the "healthy" group, statistically significantly more in 35- to 65-yr-old groups. In one of the previous

reports we found in the randomly selected population of Ljubljana that in patients with advanced periodontal disease, the number of missing teeth is increased (18). Smokers also have less missing teeth in comparison with non smokers (19). The results of our study regarding diabetes, hives, allergy, bleeding gums and smoking habits are in accordance with the findings of other studies (2). Sistemic diseases and smoking have a potential to lower the host's resistance, and thus increase the possibility of the periodontal disease progression (1-3).

High percentage of systemic diseases and conditions in our present population suggests that thorough medical history of patients, especially of those with periodontal disease, is an important task in the everyday dental practice. A periodontist might expect that 40% or more of patients looking for treatment also have some disease which may modify the treatment outcome (8).

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Ključne besede: *parodontalna bolezen/epidemiologija; sistemske bolezni; medicinska anamneza; sladkorna bolezen; kajenje*

Izvleček - Izhodišča. *Sistemske bolezni spadajo med osebnostne dejavnike, ki zmanjšajo odpornost organizma na bakterije v zobnih oblogah in tako vplivajo na začetek in klinični potek parodontalne bolezni. Pacient s parodontalno boleznijo je najpogosteje srednjih ali starejših let. Velikokrat ga spremljajo še druga bolezenska stanja, ki lahko vplivajo na spremenjen odgovor organizma na parodontalno zdravljenje in ga tudi poslabšajo. Ker so ti pacienti pogosto deležni parodontalno kirurških posegov, mora parodontolog poznati pacientovo splošno zdravstveno stanje, da lahko prepreči neljube zaplete, ki bi lahko ogrozili njegovo zdravje.*
Namen naše raziskave je bil ugotoviti ali so sistemske bolezni pogosteje prisotne pri osebah s parodontalno boleznijo v primerjavi z osebami, ki imajo zdrava obzobna tkiva.

Metode. *Obzobna tkiva 1210 naključno izbranih prebivalcev Ljubljane smo pregledali z uveljavljenim Skupnostnim indeksom potreb po parodontalnem zdravljenju.*
Preiskovance, ki so bili stari 25, 35, 45, 55 in 65 let, smo razdelili v dve skupini. V "parodontalno" skupino smo izbrali 195 oseb z znaki parodontalne bolezni. "Zdravo" skupino pa je sestavljalo 186 oseb z relativno zdravimi obzobnimi tkivi glede na starost. Obe skupini sta predstavljali 31.5% delež pregledane populacije. Prisotnost sistemskih bolezni smo ocenili iz odgovorov na vprašalnik o zdravju (Cornell Medical index health questionnaire). Pri vrednotenju rezultatov smo uporabili test c_c in Studentov t-test.

Rezultati. *V primerjavi z "zdravo" skupino so se v "parodontalni" skupini pogosteje pojavljale kardiovaskularne bolezni, med katerimi je bil največkrat prisoten visok krvni pritisk (22.1%), nato revmatično vnetje sklepov (20.0%), astma/seneni nahod (10.3%) in sladkorna bolezen (8.7%). V "zdravi" skupini pa sta bili koprivnica (31.2%) in alergija na zdravila (24.7%), pogosteje prisotni kot v "parodontalni" skupini. Med pacienti s parodontalno boleznijo je bilo statistično značilno več zmernih in hujših kadilcev, imeli pa so tudi statistično značilno manj zob v ustih v primerjavi z zdravimi osebami.*

Zaključki. *Ugotovili smo, da se pri osebah s parodontalno boleznijo pogosteje pojavljajo sistemske bolezni in stanja v primerjavi z osebami, ki imajo relativno zdrava obzobna tkiva. Istočasno pa več kot četrtina parodontalnih pacientov jemlje tudi zdravila. Zato je zaključna misel naše raziskave, da je natančna in skrbna ocena zdravstvenega stanja pacienta, posebno pacienta s parodontalno boleznijo, ena najpomembnejših in odgovornih nalog v zobozdravnikovi ordinaciji pred začetkom zdravljenja.*

Review article/Pregledni prispevek

ORAL PATHOLOGY OF HIV INFECTION AND TREATMENT

PATOLOGIJA USTNE VOTLINE IN ZDRAVLJENJE PACIENTOV OKUŽENIH Z VIRUSOM HIV

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Key words: *oral pathology; HIV; candidosis; hairy leukoplakia; periodontal disease; Kaposi's sarcoma*

Abstract – *Oral lesions are common in patients infected with human immunodeficiency virus (HIV), are often the presenting feature, and may herald deterioration in the general health and a poor prognosis. A wide range of oral lesions can be seen, notably fungal and viral infections. An overall picture of oral disease in the whole spectrum of HIV infection and the acquired immune deficiency syndrome (AIDS) has emerged, with a restricted series of fairly common oral lesions such as candidiasis, viral infections including hairy leukoplakia, gingival and periodontal disease and Kaposi's sarcoma. However, a panoply of less common lesions can also be seen.*

Any treatment regimes used in persons with HIV infection should avoid further immunosuppression, avoid cariogenic agents (such as sucrose-containing medications), avoid agents aggravating symptoms such as xerostomia and must take into account adverse drug reactions and possible drug interactions, as well as the immune defect and bleeding tendency.

Management of the HIV-infected person is a team approach and there should always be close collaboration with the responsible appropriate colleagues, particularly when the oral lesions are not in isolation. Many of the oral lesions seen in HIV infection regress or clear during treatment with anti-HIV agents.

This paper reviews the more common oral lesions and their management.

Introduction

Oral lesions are common in patients infected with human immunodeficiency virus (HIV) are often the presenting feature, and may herald deterioration in the general health and a poor prognosis. A wide range of oral lesions can be seen notably fungal and viral infections. However, a panoply of less common lesions can also be seen.

An overall picture of oral disease in the whole spectrum of HIV infection and the acquired immune deficiency syndrome (AIDS) has emerged, with a restricted series of fairly common lesions such as candidiasis, viral infections including hairy leukoplakia, gingival and periodontal disease and Kaposi's sarcoma (1-15). Recent classifications take this into account (16, 17).

This paper reviews the more common oral lesions (Tab. 1) but space precluders consideration of the less common lesions, and salivary disorders (Tab. 2).

Discussion

Many of the oral lesions seen in HIV infection regress on clear during treatment with anti-HIV agents. Management of the HIV infected person is a team approach, and there should always be close collaboration with the responsible appropriate colleagues, particularly when the oral lesions are not in isolation.

Common dental problems may be seen in HIV-infected person, one Australian study showed that nearly 65% of patients suffered toothache and because of oral problems 17% did not go out. Nearly 30% of HIV-infected individuals have oral discomfort, typically due to candidosis but sometimes caused by other infections ulcers or tumours.

Prevention and the treatment of oral disease, particularly painful conditions and opportunistic infections, is especially important to maintain quality of life and possibly prevent more serious complications. To this end the highest standards of oral hygiene and nutrition must be maintained. Where the HIV-infected person has an immune defect that is severe enough to predispose to clinical infections - or where an invasive procedure is contemplated and may predispose to infection with oral or other bacteria, antimicrobial prophylaxis may also be indicated, though unequivocal evidence for this is not available.

Any treatment regimes used in persons with HIV infection should avoid further immunosuppression, avoid cariogenic agents (such as sucrose-containing medications), avoid agents aggravating symptoms such as xerostomia and must take into account adverse drug reactions and possible drug interactions. For example, acetylsalicylic acid may aggravate the bleeding tendency caused by the immune thrombocytopenia. Dental treatment should also take into account other complicating factors, such as other sexually transmitted diseases, viral hepatitis, or a history of endocarditis found - especially intravenous drug misusers.

Certain basic principles underlie the treatment of infections in HIV-infected persons:

(a) Fungal, viral and parasitic infections are rarely *curable*: long term antimicrobial therapy may be needed.

(b) Most infections are opportunistic, reflect the local prevalence of such infections, and represent little or no threat to

Tab. 1. *Oral disorders in HIV disease.*

Tab. 1. *Spremembe v ustni votlini pri osebah okuženih z virusom HIV.*

More common Pogosto prisotne	Less common Redko prisotne
INFECTIONS INFEKCIJE	INFECTIONS INFEKCIJE
Fungal **Glivične** Candidosis	**Fungal** **Glivične** Aspergillosis Histoplamosis Cryptococcus neoformans Mucomycosis Geotrichosis
Bacterial **Bakterijske** HIV-G HIV-P HIV-like	**Bacterial** **Bakterijske** Tuberculosis Mycobacterium avium-intracellulare Klebsiella pneumoniae Enterobacterium cloacae Escherichia coli Samonella enteritidis Sinusitis Exacerbation of apsial periodontitis Submandibular cellulitis Rochalimea henselae
Viral **Virusne** HSV VZV EBV (including hairy leukoplakia)	**Viral** **Virusne** HPV CMV Molluscum contaglosum
NEOPLASMS NOVOTVORBE	NEOPLASMS NOVOTVORBE
Kaposis sarcoma	non-Hodgkins lymphoma
	NEUROLOGICAL DISTURBANCE NEVROLOŠKE MOTNJE
	Paraesthesia Facial palsy Hyperaesthesia Dysphagia
	MISCELLANEOUS OSTALO
	Recurrent aphthous ulceration Progressive necrotising ulceration Toxic epidermolysis Erythema multiforme Delayed wound healing Thrombocytopenia Xerostomia and Sicca type syndrome HIV-embryopathy Submandibular lymphadenopathy Granulosum annulare Exfoliative cheilitis Lichenoid reactions

Tab. 2. *Salivary disorders in HIV infection.*

Tab. 2. *Motnje v delovanju žlez slinavk pri osebah okuženih z virusom HIV.*

Xerostomia
Sicca-type diseases
Cystic-benign lymphoepithelial lesion
Lymphadenopathy
Lymphoma
Kaposi's sarcoma
Sialadenitis and other infections

other persons. Tuberculosis, hepatitis B and some sexually transmitted diseases however, are exceptions.

(c) Multiple concurrent or consecutive infections with different micro-organisms are common.

(d) Infections are often severe, persistent, and may disseminate - especially where the CD4 count is low.

The management of oral health in persons infected with human immunodeficiency virus has been reviewed elsewhere (1, 6-9, 18-23).

Candidosis

Oral carriage of Candida species is increased, and candidosis is common (Scully et al. 1994). Oral candidosis is related to the immune defect, xerostomia, smoking, antimicrobial use and other factors. Anti-candidal factors such as calprotectin, IgA and lactoferring are reduced in saliva.

(a) Significance

Oral candidosis is often the initial manifestation of symptomatic infection with HIV. It may predict a CD4 count below 200 cells/mm^3 the concurrent presence of oesophageal candidosis and a liability to other opportunistic infections usually within 3 months. The development of oral candidosis without a local cause such as xerostomia or systemic therapy with antimicrobials corticosteroids or other immunosuppressive drugs, is strongly suggestive of HIV infection, and has been used as a marker of disease severity in most classifications of HIV infections.

(b) Clinical types of oral candidosis

Though thrush (pseudomembranous candidosis) is one of the most obvious oral lesions in HIV infection, studies have now shown that other types of candidosis may also be seen, especially erythematous, angular stomatitis (cheilitis) and erythematous candidosis. Indeed, the erythematous form of candidosis may be a common early oral manifestation of HIV infection and presents as pink or red macular lesions typically on the palate and dorsum of tongue.

(c) Candidal species implicated

Candida albicans is responsible for more than 85% of clinical oral candidosis. Up to 35 strains of *C. albicans* have been implicated. The strain of Candida species usually remains constant and may be transmitted between. Non-albicans species may also be implicated, including *C. kruseii, C. tropicalis, C. parapsilosis, C. lambica, C. kefyr, Torulopsis glabrata* and *Saccharomyces ceresisiae*. Up to one quarter of patients have such species in addition to *C. albicans* and the incidence of infection with non-albicans species appears to be increasing with increasing use of chronic antifungal therapy. *C. kruseii* and *I. glabrata* are particularly becoming a problem since they are less likely to respond to fluconazole.

(d) Oral Candida albicans in HIV-infection

C. albicans isolates from HIV-infected persons show increased virulence as shown by aspartyl proteinase secretion, the ability to degrade host defence proteins, and decrease in susceptibility to some antifungal drugs, despite no previous exposure, as well as increased adherence to oral epithelial cells. There are strain differences, with a change from the serotype A predominant biotype to serotype B. Serotype B in particular appears resistant to fluconazole.

(e) Management of oral candidosis

Early treatment is warranted not only because of the discomfort but also because the foci may act as reservoirs spread of disease particularly to the oesophagus. Predisposing factors such as smoking and werostomia should be controlled. Underlying xerostomia should be treated with, for example, bethanecol. Antiretroviral and protease inhibitor treatment may aid resolution. Topical treatment with gentian violet, nystatin, amphotericin or clotrimazole is often successful initially

but relapses are common and these agents are not always palatable, or accepted by children. Chlorhexidine oral rinses may also be of some benefit.
Fluconazole produces remission within about one week and is well tolerated-though nausea or headache have been reported in a few patients. The most usual dose is 50 mg daily or 100 mg daily but a single oral dose of 150 mg may be effective. Once weekly fluconazole 150 mg may be effective prophylaxis.
Fluconazole resistance is now a clinical problem. One US study found that at least one fungus resistant to fluconazole was isolated from 41% of patients with AIDS. Fortunately, there may still be clinical response to fluconazole in fluconazole-resistant candidosis. Itraconazole may remain effective though about 30% of fluconazole-resistant isolates may be resistant to itraconazole.

Viral infections

Oral or peri-oral infections with herpes viruses, especially herpes simplex virus (HSV), cytomegalovirus (CMV) and Epstein-Barr virus (EBV), are fairly common in HIV infection (25).

(a) Herpes simplex

HSV infections are particularly severe and persistent, are particularly seen when CD4 counts fall below 100 to 50 × 10^6/l when 60% of mouth ulcers contain HSV but rarely disseminate.

(b) Herpes varicella-zoster

Zoster may herald a poor prognosis, is more common cranially than in non-HIV persons and is an early and readily detectable manifestation.

Management of herpes simplex and varicella-zoster virus infections

Apart from supportive care, aciclovir in daily oral doses of 1 to 4 grams in divided doses is the most generally accepted therapy though subsequently maintenance of 200 mg orally 2 to 5 times daily may be required. HSV is sometimes resistant to aciclovir, and then foscarnet for at least 10 days may be effective. Recently aciclovir-resistant strains of VZV have occurred in HIV disease warranting foscarnet therapy.

(c) Cytomegalovirus (CMV)

Chronic oral ulceration associated with CMV infection is occasionally seen in AIDS.

Management of cytomegalovirus (CMV) infections

Chronic oral ulceration associated with CMV infection may respond to ganciclovir or foscarnet. Ganciclovir should only be used where there are sight-threatening or life-threatening infections. Occasional strains are ganciclovir-resistant.

(d) Epstein-Barr virus infection

EBV is found in normal oral epithelium and may be associated with hairy leukoplakia, lymphomas and occasionally with oesophageal ulceration. Immunodeficient individuals, especially those HIV disease, showed increased infection with EBV type B, or dual infection. Hairy leukoplakia (HL) is an adherent white patch usually seen on the parakeratinised mucosa on the lateral margins of the tongue, seen in at least one quarter of HIV-infected persons and in some studies, has been the most common lesion. HL is typically identifiable about 20 to 40 months before death with a CD4 count below 200 cell/mm^3 and may be a predictor of poor prognosis. Cigarette smoking appears to increase the risk of HL. The lesions of HL are mostly symptomless and unlike some oral keratoses, have no known pre-malignant potential.
HL was generally regarded as pathognomonic of HIV disease, though it is now clear that similar lesions can be seen in some other severely immunocompromised persons or even in the apparently immunocompetent.
Although HL regresses with antivirals, no treatment is required.

Gingival and periodontal disease

Gingivitis and periodontitis are not uncommon in HIV-infected persons particularly where CD4 counts are falling. A number of different lesions can arise and several classifications have been developed (26).
i. Non specific gingivitis is indistinguishable from plaque-related gingivitis affecting non-HIV infected individuals.
ii. Linear gingival erythema (HIV gingivitis; HIV-G; linear gingivitis, previously termed generalised atypical gingivitis (AZYP)) is seen in about 9%, though some have found higher rates in this, there is gingivitis with erythema and cedema failing to respond to improved oral hygiene. Tobacco use contributes to this lesion.
iii. Necrotising ulcerative gingivitis (NUG) may be seen, essentially indistinguishable from ANUG in non-HIV-infected patients, giving rise to gingival pain, ulceration, pseudomembrane formation, and interdental gingival cratering.
iv. Necrotising ulcerative periodontitis (HIV-associated periodontitis: HIV-P). This is most likely where CD4 counts are low. It is characterised by rapid localised or more generalised periodontal destruction unrelated to plaque levels giving rise to pain, spontaneous bleeding, tooth mobility, and infrabony pocketing. There may be painful extensive soft tissue and bony necrosis with sequestration.
The precise aetiology of these gingival and periodontal lesions is still unclear, but *Candida albicans, Porphyromonas (Bacteroides) gingivalis, Prevotella (Bacteroides) intermedias, Eikenella corrodens, Actinobacillus actinomycetemcomitans, Eusobacterium nucleatum, Campylobacter rectus* and *Peptostreptococcus micros* are frequently present. A directly proportional relationship between the severity of periodontal disease and degree of immunodeficiency in HIV disease has been found in some studies. The microbiota involved in HIV-G differs from that of non-HIV gingivitis and is similar to that of HIV-P and periodontitis in non-HIV persons suggesting HIV-G is a precursor of HIV-P.

Management

Patients with HIV infection should have periodontal assessments at three monthly intervals. Antimicrobial cover is not required before scaling. Local debridement with the application of 10% providone iodine and the possible inclusion of antifungals to treat candidosis has been suggested. Twice daily oral rinses with aqueous chlorhexidine help reduce gingival erythema, bleeding and pocket depths. Metronidazole can also be a useful adjunct.

Oral and periodontal malignant neoplasms

Malignant tumours affecting persons with AIDS not infrequently affect the oral tissues (8, 9, 27-29).
The most common malignant tumours in HIV disease are Kaposi's sarcoma (KS: sometimes termed epidemic KS or EKS), which accounts for 83% of neoplasms, and non-Hodgkin's lymphomas (NHL) which account for 13%.

(a) Kaposi's sarcoma

Epidemic KS is more aggressive than the classical endemic form, or that related to iatrogenic immunosuppression. KS in

western countries is now found mainly in sexually transmitted HIV disease. KS has been regarded as an endothelial cell multicentric malignant neoplasm but there is now a body of opinion that suggests that KS is a multifocal vascular proliferative disorder involving spindle cells (similar to smooth muscle and endothelium). The recent identification of a possible new herpes virus (KSHV) in KS has been confirmed, and KISHV has now been found in oral KS. Oral KS is found mainly in those with CD4 cell counts below 200/mm^3 and is often an early manifestation of severe HIV disease. The prevalence of KS in AIDS has been decreasing during the recent past, and it is now a less common oral manifestation than, for example, oral candidosis and hairy leukoplakia.

The clinical appearance of oral KS is variable, but early lesions typically present in the palate, and usually as a pigmented macule or nodule. Sometimes the tongue, gingiva, or other sites are affected and KS may present, as red, bluish, or purple patches or nodules, sometimes ulcerated. Early lesions then are macular, but they later become nodular. Biopsy may well be indicated not least to differentiate from epithelioid angiomatosis.

Management of KS

The overall prognosis for survival in patients with KS appears to depend on the severity of immune defect and HIV infection. There is more than 80% survival at 2 years in patients without opportunistic infections but less than 20% in patients with opportunistic infections. There is no curative treatment for KS. Systemic management of HIV with zidovudine (azidothymidine) may cause regression of oral KS. Thalidomide has also been effective as may be human chorionic gonadotropin. Neither local or systemic treatment of KS have been shown to alter the ultimate course of the disease but they may reduce the size of lesions and alleviate discomfort. Overall, studies show that single chemotherapy agents may control KS in approximately 30% of patients, while combination chemotherapy produces responses in about 70% of patients. No data show that treatment improves survival. Systemic therapy with vinca alcoloids produces regression of KS in about one third of patients and many other lesions cease growth. Systemic etoposide alone, or in combination with other cytotoxics, is preferred by come. Intramuscular bleomycin can produce regression. Others use vincristine with bleomycin or liposomal doxorubiein. Systemic alpha-interferon produces benefit in up to 30% of patients. Interferon combined with zidovudine or with zidovudine and vinblastine have been tried. Adverse effects can of course be a significant disadvantage.

KS is generally very responsive to radiation therapy not, of course, without significant, adverse effects.

Nearly two thirds of patients with oral KS have symptoms of pain or discomfort, or complain of unsightly aesthetics: thus treatment of oral lesions alone with intralesional vinblastine may sometimes be indicated. Nearly 70% of lesions resolve. Intralesional sodium tetradecyl sulphate or interferon-alpha may also produce regression of oral KS.

Surgery can have a place for isolated oral KS lesions. Carbon dioxide or argon laser or photodynamic therapy or surgical excision are sometimes useful. Cryosurgery has been used but KS often persists beneath the cryo-lesion. Oral lesions can also be directly treated with radiotherapy though unusually severe mucositis and xerostomia tend to result. Supervoltage therapy has been recommended.

(b) Lymphomas

Lymphomas are fairly common in HIV disease - particularly in intravenous drug abusers. High-grade NHL are the most common. Most are of the small non-cleaved cell (SNCC) type, some are immunoblastic lymphomas (IBL) and few have intermediate features. Many SNCC are Burkitts lymphomas which accounts for 25% of all HIV-associated lymphomas.

In one-third of cases, the onset of lymphomas in HIV disease is preceded by persistent generalised lymphadenopathy (PGL). Enlargement of pre-existent palpable lymph nodes or a rapidly enlarging mass in the head and neck in HIV disease is always an indication for a biopsy to exclude malignant lymphoma. Nevertheless, at initial presentation, the lymphoma is typically widely disseminated, with extranodal sites of disease in up to 98% of patients.

Oral lymphomas, almost exclusively NHL, have been recorded mainly in homosexuals/bisexuals. They typically present in the fauces or gingiva as a rapidly growing mass, ulcer, or tooth mobility. Burkitts lymphoma, anaplastic large cell lymphoma and Hodgkins disease have been reported in the mouth.

Lymphomas in HIV disease, especially IBL may be associated with Epstein-Barr virus (EBV).

Management of lymphomas

Staging ideally includes bone marrow biopsy, chest and gastrointestinal radiography, CT scans of thorax, abdomen, pelvis and brain, gallium-67 scanning, ENT examination, and lumbar puncture. Treatment presents several problems. First, patients often have advanced disease and the lymphomas are aggressive and involve the bone marrow and CNS. Second, the immunodeficiency and possibility of opportunistic infections complicate chemotherapy. Thirdly, leukopenia makes the use of conventional multiagent chemotherapy regimens hazardous.

Various chemotherapeutic regimens are used. More than 50% of patients with stage I or II lymphomas have a complete remission whereas only 25% of those with stage III have remission. Low dose chemotherapy regimens with anteretroviral agents and marrow-protective colony stimulating factors show promise. The prognosis is better in the large non-cleaved cell NHL (median survival 7.5 months) compared with small non-cleaved cell NHL (5.5 months) and immunoblastic lymphoma (2.0 months). Most patients with HIV-related lymphomas will not have a long disease-free survival after combination chemotherapy, though a few can be expected to live 1 or 2 years in complete remission. Oral lymphomas are usually managed by radiotherapy unless there is systemic dissemination, when chemotherapy may be warranted.

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Ključne besede: *oralna patologija; HIV; kandidiaza; lasasta leukoplakija; parodontalna bolezen; Kaposijev sarkom*

Izvleček – *Lezije v ustni votlini so pogosto prisotne pri pacientih, okuženih z virusom imunske pomanjkljivosti pri človeku (HIV) in lahko napovedujejo slabšanja sistemskega zdravja in neugodno prognozo. V ustih okuženih pacientov lahko vidimo mnogo različnih lezij, najpogosteje kosti, ki jih povzročajo glivice in virusi. Pri pacientih z AIDS-om se je pokazala pestra slika ustnih bolezni, med katerimi so najpogostejša kandidiaza, virusne infekcije vključno z lasasto leukoplakijo, gingivitis in parodontalna bolezen ter Kaposijev sarkom. Seveda pa je včasih mogoče opaziti tudi mnogo manj običajnih lezij.*

Katerakoli oblika zdravljenja pri okuženih s HIV ne sme povzročiti dodatnega zaviranja imunskega sistema, ne sme vsebovati kariogenih agensov (kot so zdravila, ki vsebujejo saharozo), ne sme pospeševati kserostomije in mora upoštevati stranske učinke zdravil in njihovo medsebojno delovanje.
Zdravljenje pacientov okuženih s HIV mora biti skupinsko v tesnem sodelovanju z drugimi specialisti, posebno takrat, kadar pacient nima lezij samo v ustni votlini. Mnoge lezije v ustni votlini se pri teh pacientih zmanjšajo ali izginejo med sistemskim zdravljenjem.
Članek pregledno opisuje bolj pogoste lezije v ustni votlini okuženih pacientov in njihovo zdravljenje.

Research article/Raziskovalni članek

PREVALENCE OF ORAL PATHOLOGY AND PERIODONTAL DISEASE IN HIV-1 INFECTED PATIENTS IN SLOVENIA

POGOSTNOST BOLEZNI USTNE SLUZNICE IN OBZOBNIH TKIV PRI PACIENTIH, OKUŽENIH S HIV-1, V SLOVENIJI

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Key words: *HIV; candidiasis; hairy leukoplakia; epidemiology*

Abstract – Background. *Prevalence of oral pathology and periodontal disease is frequent in HIV infected patients. In this study we examined the oral mucosa and periodontal tissues in HIV-1 infected patients in Slovenia, and compared the oral findings to the progression of HIV-1 infection.*

Patients and methods. *We examined 35 HIV-1 infected patients (27 males and 8 females; age: 19–56 years; mean: 38.8 years), and 35 gender and age matched seronegative control subjects. Oral mucosa lesions were assessed by an oral clinical examination. Plaque deposits and periodontal tissues were evaluated by the established clinical parameters: Plaque Index (PI), Gingival Index (GI), probing depth (PD) and epithelial attachment loss (EAL). The stage of HIV-1 infection was assessed by the number of CD4⁺ T-lymphocytes and the number of HIV-1 RNA copies/ml in the patients sera. A statistical analysis was performed by the Student's t-test and the Pearson's correlation coefficient.*

Results. *In 10 patients (28.6%) we found clinical manifestations of pseudomembraneous oral candidiasis. Ten patients (28.6%) also showed the manifestation of hairy leukoplakia on the lateral margins of the tongue. No oral mucosa lesions were observed in the control group. HIV-1 infected patients with oral candidiasis and hairy leukoplakia had a significantly lower number ($t = 2.22$, $P < 0.05$; $t = 2.23$, $P < 0.05$, respectively) of CD4⁺ T-lymphocytes, and a non-significantly higher number of HIV-1 RNA copies/ml in the sera comparing to the values found in HIV-1 infected patients without oral candidiasis and hairy leukoplakia. The average value of dental plaque (PI = 1.27±0.67), gingival inflammation (GI = 0.99±0.58), pocket depth (PD = 1.73±0.47), and epithelial attachment loss (EAL = 2.18±1.01) in the infected patients were similar to the values found in non-infected subjects (PI = 1.23±0.65, GI = 1.01±0.53, PD = 1.99±0.73, EAL = 2.52±1.32).*

Conclusions. *We are concluding that HIV-1 infected patients had high prevalence of oral candidiasis and hairy leukoplakia, but a similar level of periodontal disease when compared to HIV-seronegative subjects. The prevalence of oral candidiasis and hairy leukoplakia was significantly higher in patients with the lower number of CD4⁺ T-lymphocytes in the sera.*

Introduction

Oral lesions in HIV infected individuals are frequent, and are among the first symptoms of the infection. All oral lesions have been recently classified into three major groups (1, 2):

1. lesions strongly associated with HIV infection (candidiasis, hairy leukoplakia, Kaposi's sarcoma, non-Hodgkin's lymphoma and periodontal disease: linear gingival erythema, necrotising gingivitis, necrotising periodontitis)
2. lesions less commonly associated with HIV infection (bacterial infections – *Mycobacterium sp.*, melanotic hyperpigmentation, necrotising stomatitis, salivary gland disease, thrombocytopenic purpura, ulceration NOS – not otherwise specified and viral infections – *herpes simplex virus, human papillomavirus, varicella – zoster virus*)
3. lesions seen in HIV infection (bacterial infections – *Actinomyces israelii, E. coli, Klebsiella pneumoniae*, cat-scratch disease, drug reactions, epithelioid angiomatosis, fungal infections other than candidiasis, neurologic disturbances, recurrent aphthous stomatitis and viral infections – *CMV, Molluscum contagiosum*).

As there is no report on oral pathology and periodontal disease in HIV infected patients in Slovenia, we decided to examine the oral mucosa and periodontal tissues of all available HIV-1 infected patients under treatment in Slovenia.

Methods

Patients

The subjects of this study consisted of 35 HIV-1 infected patients, 27 males and 8 females, in the age range from 19 to 56 years (mean: 38.8 years). Oral examination of all the patients was performed at the Department of Infectious Diseases and Febrile Illness, University Medical Center, Ljubljana, Slovenia, between December 1996 and February 1997. A way of getting the infection, clinical category of the disease, and treatment modality of patients are described in the companion paper (3).
The control group consisted of 35 seronegative, age and gender matched blood donors, without any systemic diseases or disorders.

Clinical oral examination

The diagnosis of oral lesions or diseases was based on clinical diagnostic criteria as previously established (1, 2).
Clinical evaluation of periodontal tissues included established parameters in clinical periodontology. The amount of dental plaque deposits was evaluated by Plaque Index (PI) (4) and degree of gingival inflammation estimated by Gingival Index (GI) (5). The level of probing depth (PD) in mm and the presence of gingival recession in mm were measured by a William's periodontal probe. Epithelial attachment loss (EAL) in mm was calculated from the values of probing depth and gingival recessions. Clinical parameters were recorded mesiobuccally, buccally, distobuccally and orally around all the teeth in the oral cavity except for the third molars. The average values of PI, GI, PD and EAL were calculated for each patient.

Statistical analysis

Statistical evaluation of the results was performed by the use of the unpaired Student's *t*-test and the Pearson's correlation coefficient.

Results

Oral lesions and diseases

In the examined population we found 10 patients (28.6%) with clinical manifestations of pseudomembraneous oral candidiasis. Hairy leukoplakia on the lateral margins of the tongue was observed in 10 patients (28.6%), 5 of these patients also showed signs of oral candidiasis. Ulceration NOS (not otherwise specified) of buckle mucosa was found in one patient (2.9%) and verruca vulgaris also in one patient (2.9%).
Statistical analysis demonstrated that patients with oral candidiasis and hairy leukoplakia had a significantly lower number of $CD4^+$ T-lymphocytes (Fig. 1, 2). The number of the HIV-1 RNA copies/ml (N) in the sera of patients varied between 2000 and 1276000. The average number of HIV-1 RNA copies/ml in the sera of patients with oral candidiasis (N = 176240±174903), and of patients with hairy leukoplakia (N = 196920±257157) was higher than the number of HIV-1 RNA copies/ml in the sera of patients without oral candidiasis (N = 154325±336046), and of patients without hairy leukoplakia (N = 146053±314600), but the differences between both groups did not reach the significance.
No oral mucosa lesions or diseases were observed in the control group.

Periodontal disease

The average values of PI, GI, PD and EAL in HIV infected patients and in control subjects are presented in Tab. 1.

Tab. 1. *Periodontal parameters in HIV-1 infected patients and control subjects.*

Tab. 1. *Parodontalni parametri pri pacientih, okuženih s HIV-1, in kontrolnih osebah.*

Clinical parameters	HIV infected patients	Control subjects
average PI	1.27±0.67	1.23±0.65
average GI	0.99±0.58	1.01±0.53
average PD	1.73±0.47	1.99±0.73
average EAL	2.19±0.69	2.52±1.24

Statistical analysis of the results shows no statistical differences in any of the periodontal parameters between both groups (Tab.1.).
The correlation between periodontal parameters, the number of $CD4^+$ T-lymphocytes, and HIV-1 RNA copies/ml in the sera of HIV-1 infected patients shows no significant correlation. However, the average periodontal pocket depth and epithelial attachment loss show a tendency to be greater in patients with a lower number of $CD4^+$ T-lymphocytes, and with a higher number of HIV-1 RNA copies/ml in the sera (Fig. 3).

Discussion

It is not surprising that early indicators of immunodeficiency occur in the oral cavity, because concurrent immune suppression allows normally non-pathogenic microbes to proliferate, resulting in characteristic oral lesions such as hairy leukoplakia or pseudomembraneous candidiasis. Those two lesions indicate a strong likelihood that HIV infection is progressing towards AIDS (6). According to the number of clinical studies the high prevalence of oral candidiasis has been recorded, and the number varies between 12.1–94.0% (7–9). The relatively low percentage (28.6%) of patients with oral candidiasis in our study could be explained by the fact, that all the patients have been advised to take antifungal agent fluconazole (Diflucan), 100 mg daily, in the case of the oral candidiasis appearance. Hairy leukoplakia as a common early indicator of HIV-1 infection was present in 10 patients (28.6%), which is consistent with other studies where a percentage of 5–43% has been reported (10–12).
Our findings that oral candidiasis and hairy leukoplakia were present in patients with a significantly reduced number of $CD4^+$ T-lymphocytes in the sera were in accordance with previous reports, which had also demonstrated higher prevalence of both manifestations in full blown AIDS (8).
The prevalence of periodontal disease in HIV infected persons is unresolved. Numerous reports have been published, but the data are conflicting in part due to different populations studied, and lack of consensus criteria for the disease (13). The level of plaque accumulation, gingival inflammation, probing depth, and attachment loss in our HIV-1 infected patients were similar to the level of these parameters in control subjects. This finding is in accordance with other authors who also didn't find any unique or pathognomonic characteristics that could set HIV-periodontal disease apart from the periodontal disease in HIV-negative population (14, 15). However, in one study, HIV-seropositive subjects experienced more severe attachment loss in the lower incisor region (15). We also observed a tendency of deeper pocket depth and greater attachment loss in patients with a more advanced stage of HIV-1 infection, as reflected by a low number of $CD4^+$ T-lymphocytes, and a high number of HIV-1 RNA copies/ml in the sera. Further longitudinal studies are needed to evaluate if periodontal inflammation and the destruction are present in HIV infected patients as reported by the pioneer investigators (16).

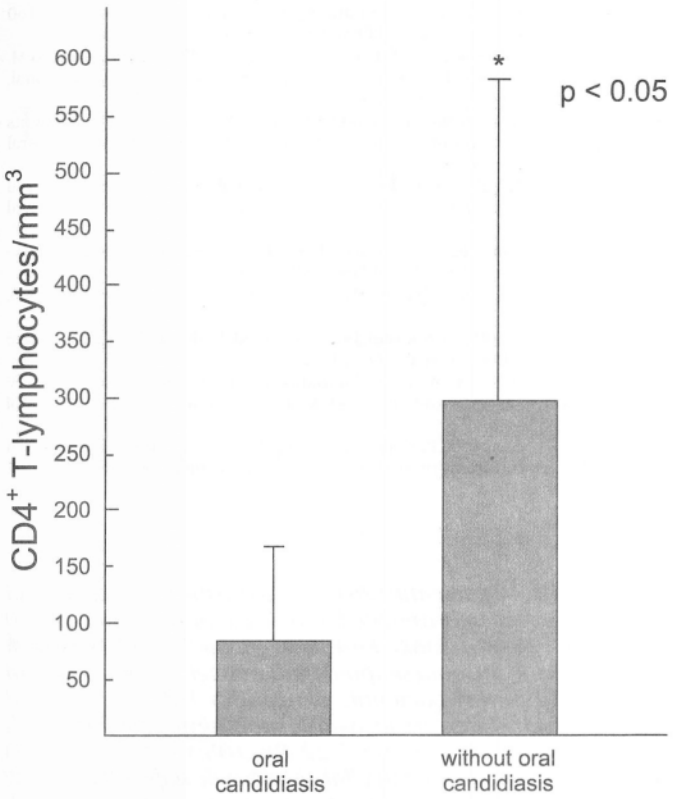

Fig. 1. *The average number of CD4⁺ T-lymphocytes in the sera of HIV-1 infected patients with (N = 10) and without (N = 25) presence of oral candidiasis. Significant differences (t = 2.22; P < 0.05) exist between both groups.*

Sl. 1. *Povprečno število T-limfocitov CD4⁺ v serumu pacientov, okuženih s HIV-1, ki so (N = 10) oz. niso imeli ustne kandidiaze. Med obema skupinama obstaja statistično značilna razlika (t = 2,22; P < 0,05).*

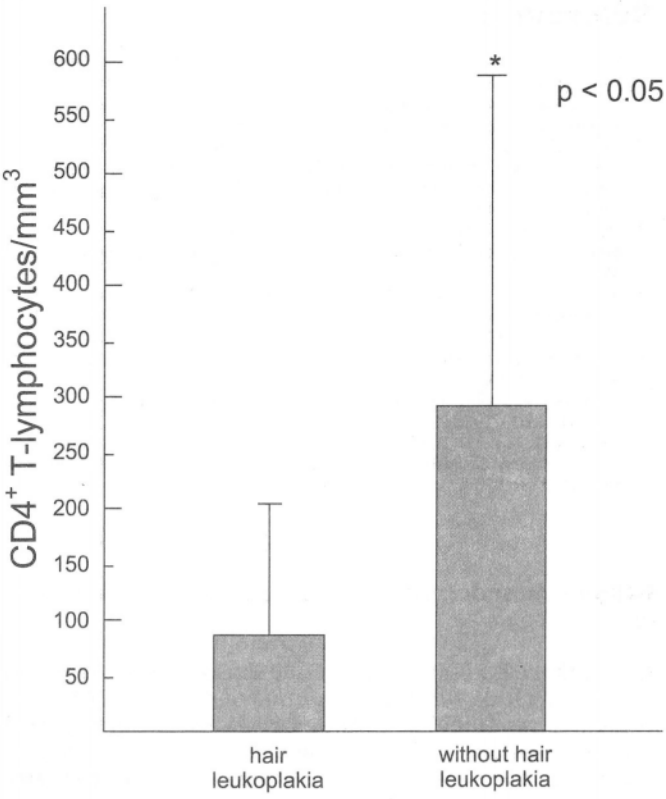

Fig. 2. *The average number of CD4⁺ T-lymphocytes in the sera of HIV-1 infected patients with (N = 10) and without (N = 25) presence of hairy leukoplakia on the lateral margins of the tongue. Significant differences (t = 2.21; P < 0.05) exist between both groups.*

Sl. 2. Povprečno število T-limfocitov CD4⁺ v serumu pacientov, okuženih s HIV-1, ki so (N = 10) oz. niso (N = 25) imeli lasaste levkoplakije na lateralnih robovih jezika. Med obema skupinama obstaja statistično značilna razlika (t = 21; P < 0,05).

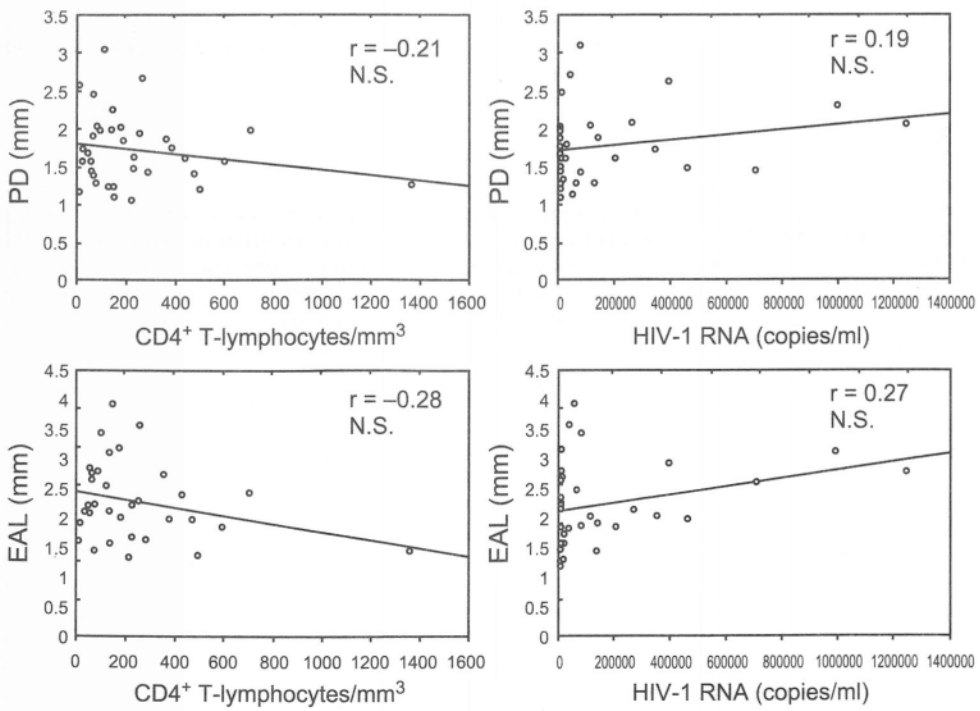

Fig. 3. *Correlation between clinical parameters (average probing depth – PD in mm and epithelial attachment loss – EAL in mm) and a degree of HIV-1 infection as measured by the number of CD4⁺ T-lymphocytes and HIV-1 RNA copies/ml in the sera of 35 patients.*

Sl. 3. *Razmerje med kliničnimi parametri (povprečno globino sondiranja – PD v mm in izgubo epitelijskega prirastišča – EAL v mm) in stopnjo okužbe s HIV-1, ki smo jo določili s številom T-limfocitov CD4⁺ in številom kopij HIV-1 RNK na ml seruma pri 35 pacientih.*

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Ključne besede: *HIV; kandidiaza; lasasta levkoplakija; epidemiologija*

Izvleček – Izhodišča. *Bolezni ustne sluznice in obzobnih tkiv so pogosto prisotne pri pacientih, okuženih s HIV. Med raziskavo smo pregledali stanje ustne sluznice in obzobnih tkiv pri pacientih v Sloveniji, okuženih s HIV-1, in stanje v ustni votlini primerjali s stopnjo napredovanja okužbe s HIV-1.*

Pacienti in metode. *Pregledali smo 35 pacientov, okuženih s HIV-1 (27 moških in 8 žensk, starih 19–56 let in s povprečno starostjo 38,8 let) ter 35 po spolu in starosti primerljivih seronegativnih kontrolnih oseb. Spremembe na ustni sluznici smo ocenili s kliničnim pregledom. Količino zobnih oblog in stanje obzobnih tkiv smo določali z ustaljenimi kliničnimi parametri kot so: indeks plaka (PI), gingivalni indeks (GI), globina sondiranja in izguba epitelijskega prirastišča. Stopnjo razvoja okužbe s HIV-1 smo pri pacientih ocenili s številom CD4⁺ T-limfocitov in številom kopij HIV-1 RNA/ml v serumu. Rezultate smo statistično ovrednotili s Studentovim t-testom in Pearsonovim korelacijskim koeficientom.*

Rezultati. *Pri 10 pacientih (28,6%) smo v ustni votlini ugotovili znake psevdomembranozne kandidiaze in prav tako pri 10 pacientih (28,6%) znake lasaste levkoplakije na lateralnem robu jezika. Kontrolna skupina ljudi ni imela znakov bolezni ustne sluznice. Pri pacientih, okuženih s HIV-1, ki so imeli oralno kandidiazo in lasasto levkoplakijo, smo izmerili značilno nižje vrednosti ($t = 2,22$, $P < 0,05$; $t = 2,21$, $P < 0,05$) CD4⁺ T-limfocitov in neznačilno višje število kopij HIV-1 RNA/ml v serumu v primerjavi z vrednostmi pri pacientih, okuženih s HIV-1 brez oralne kandidiaze in lasaste levkoplakije. Prisotnost zobnih oblog (PI = 1,27±0,67), vnetje dlesni (GI = 0,99±0,58), globina obzobnih žepov (PD = 1,73±0,47) in izguba prirastišča (EAL = 2,18±1,01) je bila pri okuženih pacientih podobna kot pri zdravih preiskovancih (PI = 1,23±0,65, GI = 1,01±0,53, PD = 1,99±0,73, EAL = 2,52±1,32).*

Zaključki. *Rezultati raziskave so pokazali, da imajo pacienti, okuženi s HIV-1, visoko stopnjo pojavnosti oralne kandidiaze in lasaste levkoplakije ter podobno stopnjo parodontalne bolezni, če jih primerjamo s seronegativnimi osebami. Pogostnost pojavljanja oralne kandidiaze in lasaste levkoplakije je statistično značilno višja pri pacientih z razvitejšo stopnjo okužbe s HIV-1, ocenjeni z nižjim številom CD4⁺ T-limfocitov v serumu.*

Professional article/Strokovni prispevek

DETECTION OF ANTI HIV-1/2 ANTIBODIES IN SALIVA OF HIV-SEROPOSITIVE PERSONS IN SLOVENIA

PRISOTNOST PROTITELES PROTI HIV-1/2 V SLINI OSEB, OKUŽENIH S HIV V SLOVENIJI

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Key words: *HIV-1 infection; epidemiologic surveillance; saliva anti HIV-1/2*

Abstract – Background. *To evaluate the sensitivity and specificity of saliva-based anti HIV antibody testing in the Slovenian population due to heterogeneity of HIV.*

Patients and methods. *Serum and saliva samples from 35 persons previously determined as HIV-1 seropositive, and 50 persons of unknown HIV status with no risk factors for HIV infection were evaluated in a prospective, blinded fashion for anti HIV-1/2 antibodies. The clinical classification of HIV infection, serum HIV-1 RNA viral load and antiretroviral drug use were well documented in all HIV-seropositive persons. Saliva was gathered with the Omni-Sal collection device designed to concentrate crevicular fluid rich saliva, and analysed for the presence of anti HIV-1/2 antibodies using the commercial enzyme immuno assay Wellcozyme HIV 1+2 GACELISA, and confirmatory Western blot test. Independently, serum samples were analysed for anti HIV-1/2 antibodies using two different commercial screening enzyme immuno assays (Wellcozyme HIV 1+2, Enzygnost Anti-HIV 1+2), and three different confirmatory Western blot tests.*

Results. *All 35 persons previously determined as HIV-1 seropositive, expressing most various stages of HIV disease with a wide range of serum HIV-1 RNA load, and different variations in antiretroviral therapy, had detectable anti HIV-1 antibodies in saliva by both screening and confirmatory test. None of the 50 controls were positive by testing of serum or saliva specimens for the presence of anti HIV-1/2 antibodies. According to our study both the sensitivity and specificity of Wellcozyme HIV 1+2 GACELISA were 100%.*

Conclusions. *Our study confirmed that testing of saliva appears to be a simple, safe, sensitive and specific method for detecting anti HIV antibodies, used mostly for surveillance purposes.*

Introduction

As the human immuno deficiency virus (HIV) continues to infect more than 22 million people throughout the world, simplified testing methods are needed to identify asymptomatic HIV carriers for an early treatment and prevention of transmission (1). Serologic tests for anti HIV antibodies have been available since the mid-1980s, soon after the HIV virus was identified (2). The presence of the anti HIV antibody in saliva was first described by Archibald et al in 1986 (3). Soon its specificity has been demonstrated by radioimmunospecification assays and Western blot (4, 5). Saliva offers an attractive alternative to blood for diagnostic purposes because technically it provides a non-invasive, user-friendly and safe sample for the antibody status assessment. However, its disadvantage is that it contains only a small amount of antibody compared to serum or plasma, and thus requires sensitive detection systems (6). Recently, commercial collection devices as well as commercial assays for anti HIV-1/2 antibody detection have been introduced to the market (7). In general the specificities attained have been excellent, but many have demonstrated suboptimal sensitivity (8–13). Failures to detect salivary anti-HIV antibodies in the known seropositive subjects have been noted in all systems, especially in seroconverting patients (6, 14). Use of commercial testing devices tested only in certain countries could lead also to failures due to HIV heterogeneity in different populations.

In the population of 2 million of the Slovenians the incidence of HIV infection has been increasing very slowly but steadily (15–18). From 1986 till February 1997 there were 120 HIV-seropositive persons registered. During the first nine years the annual incidence rate of the acquired immuno deficiency syndrome (aids) varied between 0,5 to 3,5 per million of population. Homosexual men still represent the majority of reported cases. Prior to more extended use of anti HIV salivary tests a pilot study was introduced to assess the sensitivity and specificity of the tests in the Slovenian population.

Patients and methods

Serum and saliva specimens from 35 persons previously determined as HIV-1 seropositive and 50 persons of unknown HIV status were evaluated in a prospective, blinded fashion for anti HIV-1/2 antibodies. They were all advised not to eat or practice oral hygiene for two hours prior to the collection procedure.

Patients

HIV-1 seropositive patients were recruited from the Clinic of Infectious Diseases, University Medical Centre Ljubljana, Slovenia, where all the HIV-seropositive patients in Slovenia are hospitalised and/or followed up. We enrolled all the HIV-1 seropositive patients that attended the Clinic between October 1996 and February 1997. A history of risk behaviours for HIV infection, clinical classification (according to the 1993 revised Centre for Disease Control and Prevention HIV classification system), serum HIV RNA viral load and antiretroviral medication use, were noted (19).
Fifty randomly chosen patients, who were hospitalised at the same Clinic in the same period of time, and whose HIV antibody status was unknown, served as a control group. They had no known risk factors for HIV infection, and matched the HIV-seropositive group according to age and gender.

Sample collection

Vials of blood and saliva were collected from each individual, using the Vacutaner system and the Omni-Sal saliva collection device [Saliva Diagnostic Systems, Inc. (SDS), Vancouver, Washington, USA], respectively (7). The latter consists of a cotton pad and a tube containing a transport medium. Antimicrobial and antiproteolytic agents in the transport medium stabilise the specimen during the period between the collection and testing. The collection pad is designed to hold 1 ml fluid when fully saturated, to fit into the transport tube, and to allow contact with the transport medium. Extraction of fluid from the pad typically yields 1-1,5 ml of cell-free fluid.
The laboratory personnel were completely blind to the linkage between serum and saliva specimens.

Detection of anti HIV-1/2 antibodies

Serum samples were tested for the presence of anti HIV-1/2 antibodies using two different screening enzyme immuno assays (Wellcozyme HIV 1+2 Test, Murex Diagnostics, Dartford, Great Britain and Enzygnost Anti-HIV 1+2 Test, Behring AG, Marburg, Germany) in the routine manner established in our laboratory (20). All repeatedly reactive sera were supplementary tested with three different Western blot tests (Novopath HIV-1 Immunoblot Test, Bio-Rad GmbH, Vienna, Austria; HIV-1 Western blot kit, Cambridge Biotech Corporation, Worcester, MA, USA; New Lav-Blot II, Sanofi Diagnostics Pasteur, Marnes-La-Coquette, France). The Western blot results were interpreted according to CDC/ASTPHLD standards (21). A specimen was considered anti HIV-1 positive when any two of the following bands were present: p24, gp41, and either gp120 or gp160. Assay results were considered to be indeterminate for HIV-1 if the Western blot showed at least one band characteristic of HIV-1 (p17, p24, p31, gp41, p51, p55, p66, gp120, or gp 160), but did not meet the criteria for a positive result. In the absence of all bands a specimen was considered anti HIV-1 negative (21).
Saliva specimens were tested for the presence of anti HIV-1/2 antibodies using commercial enzyme immuno assay Wellcozyme HIV 1+2 GACELISA (Murex Diagnostics, Dartford, Great Britain), following manufacturer's instructions, as described previously (22). All repeatedly reactive saliva specimens were supplementary tested with HIV-1 Western blot kit (Cambridge Biotech Corporation, Worcester, MA, USA), following manufacturer's instructions except for the sample volume (200 ml of saliva specimen was used instead of 20ml of serum). The Western blot results were interpreted as described above.

Results

Out of 35 persons previously determined as HIV-1 seropositive 27 were male and 8 female with the age range from 19 to 56 years (mean 38,8 years). Eleven were homosexual, 5 bisexual men, 5 got infected through blood products as haemophiliacs, 3 were intravenous drug abusers and 11 reported risk heterosexual contacts. Twelve were asymptotic (clinical categories A1, A2, A3), 8 belong to the clinical category B (6 of them into B3) and 15 were ranged into C category, 14 of them having less than 200 × 10^6/L CD4+ T lymphocytes. The serum viral load ranged from less than 2000 up to 1.276.000 HIV-1 RNA copies/ml. 22 patients had been treated by antiretroviral drugs for at least 6 months, 3 of them with zidovudine only, 5 with the combination of two reverse transcriptase inhibitors, and 14 with the combination therapy including proteinase inhibitors. 13 patients had never been treated by any antiretroviral drug.
All were reactive for anti HIV-1 antibodies by Wellcozyme HIV 1+2 GACELISA in saliva specimens. On confirmatory Western blot testing all the 35 GACELISA reactive salivary samples gave positive results.
Among 50 persons of the control group there were 38 male and 12 female with the average age of 37,8 years (range from 19 to 54 years). None of the controls was positive by testing of serum or saliva specimens for the presence of anti HIV-1/2 antibodies (Tab. 1).

Tab. 1. *Results of anti HIV-1/2 testing in saliva and serum samples.*

Tab. 1. *Rezultati testiranja vzorcev sline in krvi na prisotnost protiteles proti HIV-1/2.*

	Serum anti HIV-1	
	+	-
Saliva anti HIV-1		
+	35	0
-	0	50

According to our results the sensitivity, specificity, and positive and negative predictive values for the Wellcozyme HIV 1+2 GACELISA of saliva samples were each 100 %.

Discussion

To the best of our knowledge the present study represents the first evaluation of reliability of anti HIV-1 testing in saliva specimens in the Slovenian population. Our results, obtained from a group of previously determined HIV-1 seropositive persons and controls assessed in a prospective, blinded manner, demonstrated 100% sensitivity and 100% specificity of assays performed in saliva. Several factors probably contributed to these results. In all the patients included in the study the present clinical status was well documented, their immune status was known, the quantity of viral genome in serum was established, all expressing most various stages of HIV disease. However, they had no influence on detection of anti HIV-1 antibodies in saliva.
The number of HIV-1 seropositive persons included into the present study was relatively small. However, we included all the available HIV-1 seropositive persons in Slovenia that regularly attended the Clinic. Taking a larger group of HIV-seropositive persons one can not exclude the possibility that the result of sensitivity would probably have changed. Failures might also happen in seroconverting persons, but the possibility of randomly chosen such a person is likely to be very small due to low prevalence of HIV infection in Slovenia (15).

The collection device and the assays used in the present study were both commercially available. The disadvantages of serodiagnostics with saliva are few and are becoming mostly from minute amounts of immunoglobulins present (11, 22, 23). The transudatory component of saliva, especially the gingival crevicular fluid, has a similar composition to plasma, being rich in immunoglobulins of all classes including IgG and IgM (7). In condition of chronic gingivitis/periodontitis, which is common in HIV-seropositive persons the gingival crevicular fluid is an exudate and has an increased immunoglobulin concentration (23, 24). So the desirable sample for diagnostic purposes would be crevicular fluid-rich (CFR) saliva (7). The collection device Omni-Sal, used in our study, is designed to collect an adequate volume of homogenous, non-viscous, CFR saliva. Because of the minute amounts of immunoglobulins present, the assays must be very sensitive. The earliest commercial assays had only 57–70% sensitivity for detecting anti HIV-1/2 antibodies in saliva of seropositive patients (3, 25). Recently the sensitivities of saliva EIA have been as high as 97.2%–99.4%, most with 100% specificity (6, 10, 19, 26, 27).

Saliva offers several potential advantages over blood in diagnostics of HIV infection (22). It is easier to collect, there is no need for trained technicians, it is less infectious than blood and safer to manipulate, has better subject compliance because it causes no needle injury and no religious or ethnic objections (7, 22, 28, 29). Many oral samples could be obtained simultaneously. It is very convenient for transportation and storage, since it can be stored for longer periods at the room temperature, or even at tropical temperatures, and so applicable under field conditions (29).

Therefore, saliva is particularly attractive for epidemiologic studies in populations, where blood sampling is difficult to perform (injecting drug abusers, street prostitutes, etc.), and in regions of developing countries lacking trained personnel for blood collection (28, 30–33).

The aids epidemic in Slovenia is still in its early stage (15). However in the future we may expect most intensive spread among injecting drug users. This represents a crucial component of HIV surveillance needed for planning, implementing and monitoring prevention and control efforts. The present pilot study showed a perfect correlation between the blood and saliva samples tested for anti HIV-1/2 antibodies. According to our results, saliva can be recommended as a safe and effective alternative to serum for the surveillance programmes in Slovenia. In some cases it could also be applied to persons who are either unwilling or unable to donate blood specimen (i.e. intravenous drug abusers).

Surveillance programmes are usually performed by simple and cheap methods.

Recently, some reports state that oral fluids may be less expensive to collect and process than serum. However, there are no controlled studies to document this fact (8, 10, 12, 13, 26, 34). Although the aim of the present pilot study was not to determine such a cost-effectiveness, our rough evaluation proposes that the cost of anti HIV-1/2 antibody testing in saliva is five times higher than the one performed in blood.

Preferences

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Ključne besede: *okužba s HIV-1; epidemiološki nadzor; proti HIV-1/2 protitelesa v slini*

Izvleček – Izhodišča. *Zaradi virusne heterogenosti smo želeli na slovenski populaciji oceniti senzitivnost in specifičnost standardnih testnih metod za določanje protiteles proti HIV-1/2 v slini.*

Bolniki in metode. *Študija je bila namenjena pregledu, slepa. Slino in serum 35 HIV-1 seropozitivnih oseb, ki so vse pregledane na Kliniki za infekcijske bolezni Kliničnega centra v Ljubljani, ter 50 naključno izbranih oseb, ki so bile zdravljene v isti ustanovi zaradi drugih bolezni, za katere pa nismo vedeli, če so okuženi s HIV in niso navajali nobenega dejavnika tveganja za okužbo s HIV, smo testirali glede prisotnosti protiteles proti HIV-1/2. HIV-1 seropozitivne osebe so se med seboj razlikovale glede stopnje bolezni, imunskega stanja, količine HIV-1 RNA v serumu in glede prejemanja antiretrovirusnih zdravil. Vzorec sline smo odvzeli s posebnim pripomočkom Omni-Sal, ki je prirejen za zbiranje sline, obogatene z gingivalno tekočino. S standardnim encimsko imunskim testom Wellcozyme HIV 1+2 GACELISA smo vzorce sline testirali na prisotnost protiteles proti HIV-1/2 in pozitivne rezultate potrdili še z Western blot testom. Povsem ločeno smo testirali serum na prisotnost protiteles proti HIV-1/2 z dvema različnima standardnima encimsko imunskima presejalnima testoma (Wecozyme HIV 1+2 in Enzygnost Anti-HIV 1+2) ter pozitivne rezultate potrdili s tremi različnimi potrditvenimi Western blot testi.*

Rezultati. *Vseh 35 HIV-1 seropozitivnih oseb je imelo v slini prisotna protitelesa proti HIV-1, kar smo prikazali tako s presejalnim kot tudi s potrditvenim testom. Nihče v kontrolni skupini ni imel prisotnih protiteles proti HIV-1/2, niti v serumu niti v slini. Senzitivnost in specifičnost Wellcozyme HIV 1+2 GACELISA testa za ugotavljenje protiteles proti HIV-1/2 v slini je bila 100%.*

Zaključki. *Študija predstavlja prvo tovrstno raziskavo za oceno zanesljivosti metode določanja protiteles proti HIV-1/2 v slini v Sloveniji. Potrdili smo rezultate drugih, že objavljenih študij o visoki senzitivnosti in specifičnosti take metode. Naš vzorec je bil relativno majhen, vendar so bili zajeti vsi možni bolniki, ki redno obiskujejo kliniko. Rezultat senzitivnosti bi bil verjetno nekoliko drugačen, če bi bil vzorec večji. Napake so možne tudi pri HIV-seropozitivnih osebah v obdobju serokonverzije, vendar je verjetnost za naključen izbor take osebe v Sloveniji zelo majhna, saj je v dvo milijonski populaciji zaenkrat znanih le 120 HIV-seropozitivnih oseb. Testi za ugotavljanje protiteles proti HIV-1/2 v slini so torej enostavni, varni, dovolj senzitivni in specifični. V Sloveniji bi bila njihova uporaba najprimernejša v široko zastavljenih programih epidemiološkega nadzora HIV okužbe.*

Research article/Raziskovalni prispevek

SUPEROXIDE RELEASE AND CYTOPATHOGENIC EFFECTS IN GINGIVAL FIBROBLASTS EXPOSED TO HUMAN IMMUNODEFICIENCY VIRUS (HIV) AND HERPES SIMPLEX VIRUS (HSV)

VPLIV VIRUSA IMUNSKE POMANJKLJIVOSTI (HIV) IN HERPES SIMPLEKS VIRUSA (HSV) NA SPROŠČANJE SUPEROKSIDA IN CITOPATOGENE UČINKE PRI FIBROBLASTIH DLESNE

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Key words: HIV; HSV; fibroblasts; superoxide; cytopathogenic effects

Abstract – Background. *Clinical signs of periodontal disease and herpes simplex virus (HSV) infections are recognized complications in HIV infected patients. The aim of this study was to evaluate whether viral infections such as HIV and HSV induce the release of superoxide and contribute to cytopathogenic effects and cell death of gingival fibroblasts.*

Materials and methods. *Primary cultures of gingival fibroblasts were prepared by the method of explant culture. Fibroblasts were exposed to HIV-1 and HSV-1 and the release of superoxide measured spectrophotometrically by the reduction of ferricytochrome C for the period of 5 hours. Cytopathogenic effects and cell death of fibroblasts exposed to HIV-1 and HSV-1 were observed by light microscopy and the release of ^{14}C-labeled adenine during the 4 days observation period.*

Results. *HIV-1 induced up to 0.5 nmol and HSV-1 up to 1.2 nmol of superoxide from gingival fibroblasts in the 5 hours observation period. The release of O_2^- was only partially suppressed by the addition of superoxide dismutase.*
HIV-1 induced only minor cytopathogenic effects and cell death of gingival fibroblasts in the 4 days observation period. HSV-1, however, induced pronounced cytopathogenic effects and complete lysis of gingival fibroblasts after 4 days of the observation period. The cytolysis of fibroblasts exposed to HSV-1 was not prevented by the addition of superoxide dismutase.

Conclusions. *Human gingival fibroblasts after an exposure to HIV-1 released only a low amount of superoxide and showed only minor morphological changes and cell death. HSV-1 induced a relatively high level of superoxide release in gingival fibroblasts, which might contribute to pronounced cytopathogenic effects and cytolysis of these cells during the 4 days observation period. We are concluding that superoxide released from HSV exposed gingival fibroblasts could play a role in pathogenesis of oral herpes simplex virus infections.*

Introduction

Clinical signs of gingivitis and periodontitis and oral manifestations of herpes simplex virus infections are recognized complications in patients with acquired immunodeficiency syndrome (AIDS) (1). The release of reactive oxygen intermediates (ROI) by leukocytes upon stimulation is well documented as an important defense mechanism against infectious agents (2). Besides this protective role, the ROI are also important in the pathogenesis of cell and tissue injury (3).
More recently, it was recognized that besides leukocytes a number of other cell types such as endothelial cells (4), epithelial cells (5), smooth muscle cells (6), platelets (7) and fibroblasts (8) are also able to release the superoxide anion radical upon stimulation. It appears possible that these cells may contribute to inflammatory reactions by the release of superoxide. These studies were initiated to determine whether viral infections such as human immunodeficiency virus (HIV) and herpes simplex virus (HSV) induced the release of superoxide and cytopathogenic effects in gingival fibroblasts.

Materials and methods

Gingival fibroblasts culture

Primary cultures of gingival fibroblasts were prepared by the method of the explant culture. Pieces of healthy gingiva from patients undergoing surgical removal of the third molars were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco Co., NY) supplemented by 10% Fetal Calf Serum (FCS, Gibco); 100 U/ml Penicillin; 100 µg/ml Streptomycin; 0.25 µg/ml Fungizone; 2 mM Glutamin and 5 µg/ml Gentamycin. The cultures were incubated in humidified atmosphere of 5% CO_2 in air, at 37°C. The media was changed twice weekly. For the subculture the cells were washed twice with PBS, treated with trypsin/EDTA for 5 min, and transferred to 75 cm^2 (Costar,

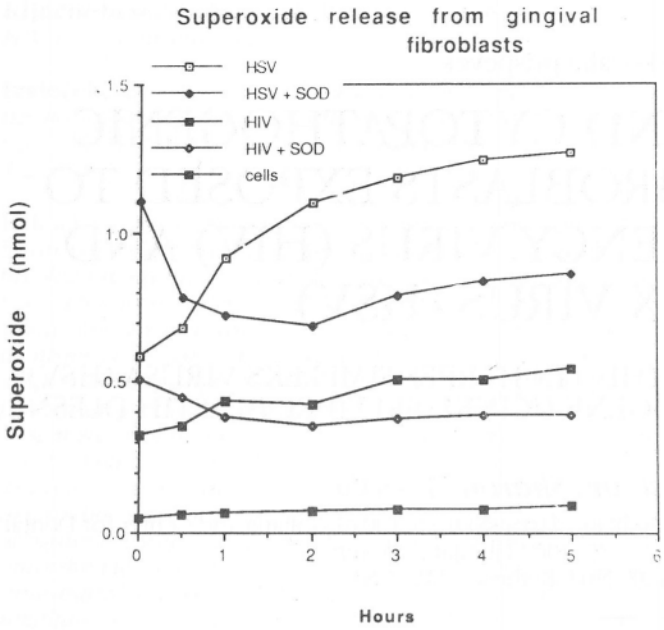

Fig. 1. *Superoxide release (nmol) from gingival fibroblasts exposed to HSV and HIV in 5 hours of experiment.*

Cambridge, MA) culture flasks. After reaching a confluence the cells were trypsinized, counted and plated at 4×10^4 cell/well in 96 well plates (Costar, Cambridge, MA) in serum free media for 48 hours.

Superoxide anion (O2-) assay

HIV-1 strain Ba-L, a macrophage-derived isolate obtained from primary lung cultures of a patient who died of the acquired immunodeficiency syndrome (9) and HSV-1 strain HF, obtained from the American-type collection (Bethesda) were used for the stimulation of the superoxide release from fibroblast cells.

The production of O_2^- by gingival fibroblasts was measured by the reduction of ferricytochrome C (Sigma, St.Louis, MO). Ferricytochrome C (100 µl at 2 mg/ml in Earle's balanced solution with 1 mM $CaCl_2$) was added to the cells which were then stimulated by HIV and HSV in the ratio of 10 virus particles/cell. Control cells received 10 µl of PBS or 10 µl of superoxide dismutase (SOD) (100 µg/ml) (Sigma). The production of O^-_2 was monitored spectrophotometrically at 550 nm (MR 600 Microplate Reader, Dynatec, Alexandria, VA) for up to 5 hours. The amount of O_2^- per well was calculated by the formula: nanomoles O_2^- = (mean absorbance at 550 × 100)/6.3 which is based on the extinction coefficient for the absorption of reduced ferricytochrome C minus oxidized ferricytochrome C corrected for the 3 mm vertical light path through the well (10).

Cytopathogenic effects and cell death

Fibroblast cultures were examined for cytopathogenic effects (CPE) by the light microscopy for 4 days. Cell death was observed by a technique for labeling cellular nucleotide pools of fibroblasts and followed by the release ^{14}C-labeled adenine into the medium as a result of the cell injury by viruses (11).

Results

Figure 1 shows the release of O_2^- from gingival fibroblasts exposed to HIV and HSV, and from control cells. The results represent the average values for 5 measurements of the O_2^- release. We can observe the minimal release of O_2^- from control cells, but the increased superoxide release (up to 0.5 nmol) from gingival fibroblasts exposed to HIV virus and higher values (up to 1.2 nmol) of the O_2^- release after the exposure of cells to HSV in the 5 hours observation period. An addition of SOD suppressed the release of O_2^- for about 20% and 35% in cells exposed to HIV and HSV, respectively.

Gingival fibroblasts were also examined as to their response to HIV and HSV infection by the appearance of cytopathogenic effects in the absence or presence of SOD (100 µg/ml). The figure 2 presents the control fibroblasts after four days in culture without (Fig. 2a) and with the presence of SOD (Fig. 2b). We can observe that cells exposed to HIV (Fig. 3a and 3b) have similar morphology as control cells. On the other side, the HSV induced a pronounced cytopathogenic effect. The first microscopic damage to the fibroblasts was noticed 48 hours after the infection. The cells became rounder and lost their typical elongated form. After four days the cell monolayer was almost completely destroyed (Fig. 4a). The addition of SOD did not prevent a cytolysis of the fibroblasts exposed to HSV (Fig. 4b).

To evaluate if cytopathogenic effects in gingival fibroblasts observed microscopically correlate with the cell death we monitored the ^{14}C-adenine release from fibroblasts exposed to both viruses for up to 4 days (Fig. 5). We can observe that

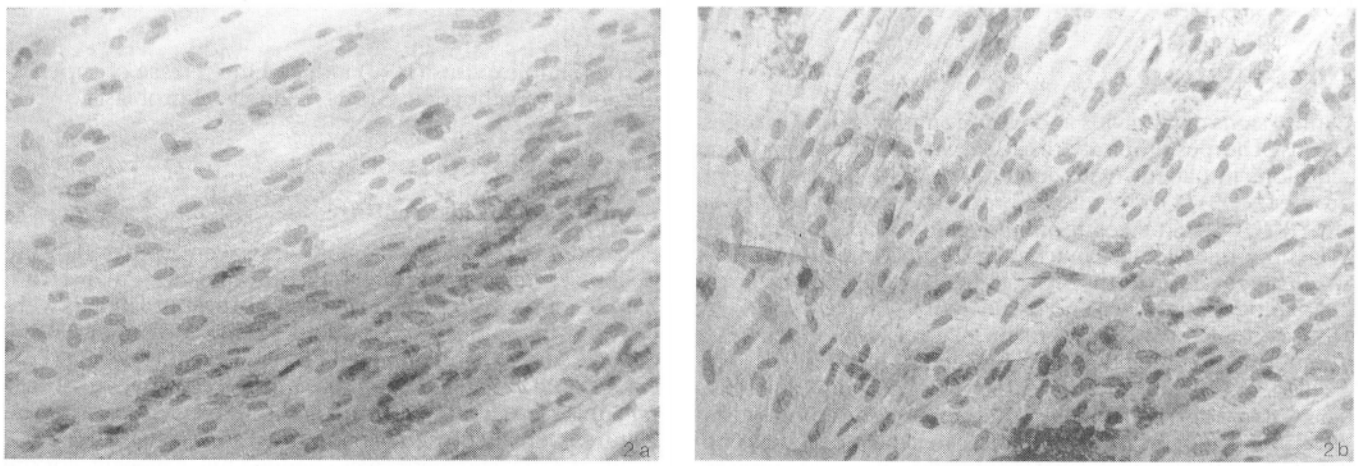

Fig. 2. *Normal gingival fibroblasts in culture after 96 hours in the absence (a) and in the presence (b) of SOD (100* mg/ml*) (HE-staining, magnification × 100).*

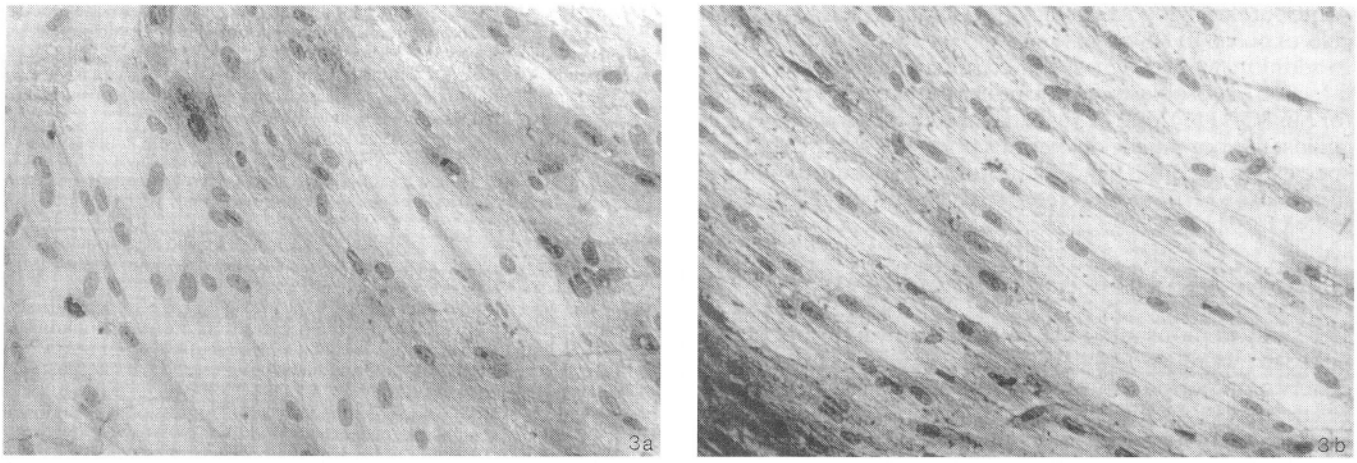

Fig. 3. *Gingival fibroblasts 96 hours after the infection with HIV in the absence (a) and in the presence (b) of SOD (100* mg/ml*) (HE-staining, magnification × 100).*

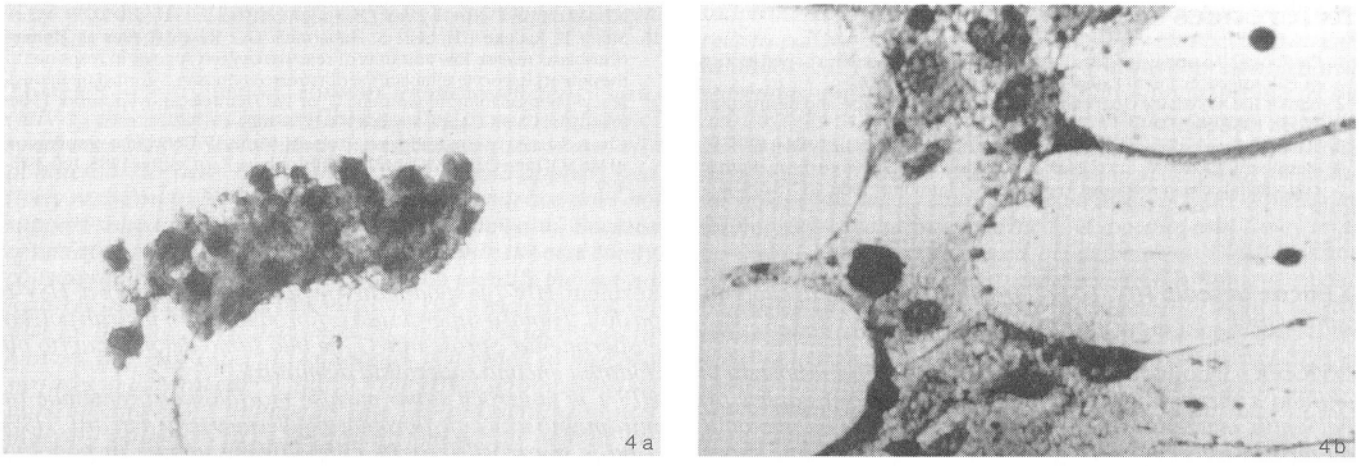

Fig. 4. *Gingival fibroblasts 96 hours after the infection with HSV in the absence (a) and in the presence (b) of SOD (100* mg/ml*) (HE-staining, magnification × 100).*

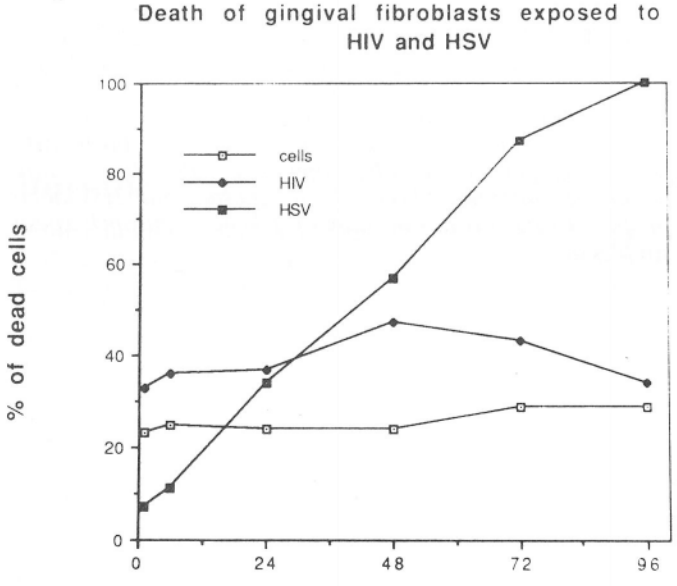

Fig. 5. *Percentage of dead gingival fibroblasts exposed to HIV and HSV in 96 hours of experiment.*

the percentage of dead fibroblasts exposed to HIV is only about 20% higher than in the control cells during the 4 days observation period. The number of dead fibroblasts exposed to HSV, however, constantly increased. After 48 hours half of the cells died and after 4 days all fibroblasts exposed to HSV were killed.

Discussion

Gingivitis and periodontitis are recognized clinical complications in HIV infection and the occurrence of severe forms of periodontal destruction appears to be associated with the pronounced immune suppression (12). Recently, the HIV and HSV were detected in the pockets of periodontal patients (13). As it has been demonstrated that HIV can also infect CD4-negative human fibroblastoid cells (14), and that fibroblasts are sensitive to HSV infection (15), we have been interested if gingival fibroblasts release superoxide and show cytopathogenic effects after an exposure to HIV and HSV. It has already been demonstrated that human fibroblasts release low amount of ROI in response to potent phagocyte stimulants (16). HIV also induced the low levels of the O_2^- release from gingival fibroblasts, but HSV induced the 2.5 times higher levels of O_2^- (Fig. 1). The release of O_2^- by both viruses in fibroblasts was only partially inhibited by SOD. A constant release of relatively high levels of superoxide from gingival fibroblasts could

contribute to cytopathogenic effects and probably death of cells exposed to HSV (Fig. 4 and 5).
In addition, we also demonstrated (data not shown) that HSV infection of fibroblasts did not induce the expression of mRNA for Mn SOD (17), one of vital enzymes in protecting the cells against the potentially deleterious effects of reactive oxygen species. On the other hand, a continuous exposure of gingival fibroblasts to HIV induced cells to express elevated levels of mRNA for Mn SOD.

Conclusions

The study demonstrated that human gingival fibroblasts exposed to HIV released the low amount of O_2^- in 5 hours of the observation period. Only minor morphological changes and cell death was observed in gingival fibroblasts exposed to HIV for 4 days. The exposure of gingival fibroblasts to HSV for 5 hours, however, induced relatively high level of the O_2^- release which might contribute to pronounced cytopathogenic effects and cell death in the 4 days observation period.

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Ključne besede: *HIV; HSV; fibroblasti; superoksid; citopatogeni učinki*

Izvleček – Izhodišča. *Klinični znaki parodontalne bolezni in okužba z virusom herpesa simpleksa so znani zapleti pri pacientih, okuženih s HIV. Namen raziskave je bil ugotoviti, ali virusni infekciji s HIV in HSV sprožita sproščanje superoksidnega radikala iz fibroblastov dlesne in prispevata k citopatogenim učinkom ter uničenju teh celic.*

Materiali in metode. *Primarne kulture fibroblastov dlesne smo pripravili iz vzorcev zdrave dlesne, odvzetih med kirurškim odstranjevanjem impaktiranega modrostnika. Fibroblaste smo izpostavili HIV-1 in HSV-1 in spektrofotometrično merili sproščanje superoksida z metodo reduciranega fericitokroma C v razponu 5 ur. Citopatogene učinke in uničenje fibroblastov, izpostavljenih HIV-1 in HSV-1 v obdobju 4 dni, smo opazovali s svetlobnim mikroskopom in z merjenjem količine sproščenega ^{14}C adenina v celični medij.*

Rezultati. *HIV-1 je spodbudil sproščanje do 0,5 nmol-a, HSV-1 pa do 1,2 nmol-a superoksida iz fibroblastov v 5-ih urah poteka eksperimenta. Sproščanje O_2^- je bilo samo deloma zavrto ob dodatku encima superoksid dismutaze.*
HIV-1 je povzročil samo manjše morfološke spremembe in minimalno uničenje fibroblastov dlesne v teku 4-ih dni, HSV-1 pa je povzročil izrazite citopatogene učinke in popolno razgradnjo fibroblastov dlesne v enakem opazovalnem obdobju. Dodatek encima superoksid dismutaze ni preprečil uničenja celic.

Zaključki. *HIV-1 sproži samo nizko količino superoksida in povzroči majhne morfološke spremembe na fibroblastih človeške dlesne. HSV-1 pa nasprotno sproži v fibroblastih relativno veliko količino superoksida in povzroči obsežne citopatološke učinke in razgradnjo celic med 4-dnevnim potekom eksperimenta. Zaključujemo, da superoksidni radikal, ki se sprosti iz fibroblastov dlesne, izpostavljenih HSV lahko prispeva k razvoju oralnih znakov infekcije z virusom herpesa simpleksa.*

Professional article/Strokovni prispevek

MUCOSAL INHIBITORS OF HIV-1

SLUZNIČNI ZAVIRALCI VIRUSA IMUNSKE POMANJKLJIVOSTI PRI ČLOVEKU (HIV-1)

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Key words: *HIV-1; oral mucosa; saliva; secretory leukocyte protease inhibitor*

Abstract – Introduction. *Human immunodeficiency virus (HIV-1), the causative agent of the acquired immunodeficiency syndrome (AIDS), is commonly transmitted by blood and also at mucosal surfaces. However, the oral mucosa appears relatively resistant to HIV-1 transmission and evidence does not support salivary fluids as a vector for transmission. Because of this apparent protected environment within the oral cavity, considerable effort has been expended to identify the unique features associated with this mucosal site which are responsible for limiting HIV-1 transmission. Among the identified inhibitors are HIV-1-specific antibodies generated at mucosal surfaces in the oral cavity. Saliva from HIV-1 seronegative donors, lacking HIV-1 antibodies, also showed significant neutralizing activity to HIV-l, suggesting that innate defense factors may also contribute to viral resistance. Through isolation and screening of a battery of salivary components for their ability to inhibit HIV-1 infection in vitro assays, the high molecular weight mucins, large glycoproteins secreted by the acinar cells of the submandibular glands, were found to aggregate and entrap viral particles. Another molecule isolated from saliva that was found to block HIV-1 infection of mononuclear cells in culture was identified as secretory leukocyte protease inhibitor (SLPI). At concentrations routinely present in oral secretions, this 12kD single chain polypeptide, produced by acinar cells in parotid and submandibular glands, inhibits HIV-1 infection. The mechanism whereby SLPI inhibits infection by HIV-1 appears to involve an interaction with the cellular hosts of the virus and not the virus itself. SLPI binding to monocytes, a key target of HIV-1, is specific, and time-, temperature-, concentration- and pH-dependent. Binding data reveal the presence of approximately 7000 binding sites per monocyte. In vitro, a single transient exposure of mononuclear cells to physiological conceptions of SLPI cause prolonged resistance to infection. However, once the cell is infected, SLPI is unable to influence viral replication. Based on these analyses, SLPI appears to serve a barrier function, blocking new rounds of infection.*

Conclusions. *Recognition of the ability of this naturally occurring polypeptide to block HIV-1 infection provides a basis for the antiretroviral activity of saliva and the infrequent oral transmission of HIV.*

Introduction

Human immunodeficiency virus (HIV-1), the causative agent of the acquired immunodeficiency syndrome (AIDS), is commonly transmitted by blood and also at mucosal surfaces. Nonetheless, the oral mucosa appears relatively resistant to HIV-1 transmission and evidence does not support salivary fluids as a vector for transmission. Because of this apparent protected environment within the oral cavity, considerable effort has been expended to identify the unique features associated with this mucosal site which are responsible for inhibiting transmission. Although initially suggested that the failure to transmit HIV-1 by the oral route might simply be due to the absence of HIV-1 in the oral cavity, a number of studies have identified HIV-1, some of it infectious, and proviral sequences in the oral fluids and tissues (reviewed in 1). That HIV-1 could be identified in the oral cavity, yet was not readily transmitted by saliva, heightened the interest in identifying potential endogenous antiviral inhibitors.

Antiretroviral agents in saliva

Numerous components of the oral milieu have been suggested as antiretroviral candidates and evaluated for their contributing roles since the initial findings that whole saliva inhibits HIV-1 infection (2). For example, HIV-1-specific antibodies generated at mucosal surfaces in infected individuals would be envisaged as effective inhibitors of HIV-1 in the oral cavity. In this regard, HIV-1 specific IgG, IgA and IgM have been quantified in saliva from HIV-1 seropositive individuals (3) (Tab. 1). However, saliva from HIV-1 seronegative donors, lacking HIV-1 antibodies, also showed significant neutralizing activity to HIV-l, suggesting that innate defense factors may also contribute to viral resistance. Saliva is comprised of a plethora of additional proteins with the potential for interfering with viral transmission including mucins, lysozyme, peroxidase, amylase, statherin and proline-rich proteins. Among the first of these shown to be aligned with antiretroviral activity were the high molecular weight mucins. These large glycoproteins secreted by the acinar cells of the submandibular glands aggregate and entrap viral particles, thereby obstructing viral

transmission (4, 5). While this may be an important deterrent, parotid saliva, which lacks mucins also possesses antiretroviral activity (6), thereby implicating additional factors in viral defense. Through isolation and screening of a battery of salivary components for their ability to inhibit HIV-1 infection in vitro assays, lysozyme, lactoferrin and cystatin were independently shown to have antiviral activity, but at concentrations exceeding those present in saliva (7). Whether or not these and other molecules work in tandem to reduce infectivity has not been established. Other potential HIV-1 inhibitors under consideration were the CD8 T lymphocyte-derived chemokines (8), natural ligands for the HIV-1 coreceptors which are members of the chemokine receptor family (9, 10). The levels of the chemokines, RANTES and MIPla, present in saliva and other mucosal fluids were inconsistent with the presence or absence of antiviral activity in these fluids (Tab. 1) (3).

Tab. 1. *Levels of Potential Antiretroviral Molecules in Saliva.*

Tab. 1. *Koncentracije potencialnih antiretrovirusnih molekul v slini.*

Molecule Molekula	Saliva Levels† Koncentracija v slini
SLPI	727+193 ng/ml
RANTES	8.5+1.3 pg/ml
MIPla	3.8+0.9 pg/ml
HIV Specific Antibodies* HIV specifična protitelesa*	
IgG	1,008
IgA	32
IgM	20

† Modified from Janoff et al (3)
Prirejeno po Janoff in sod. (3)
* Reciprocal geometric mean titers of HIV-1 infected subjects.
Recipročne geometrijske srednje vrednosti titrov pri HIV-1 okuženih osebah.
Levels were not detectable in HIV-1-negative subjects.
Koncentracije antiretrovirusnih molekul v slini niso bile zaznavne pri HIV-1 negativnih osebah.

Secretory leukocyte protease inhibitor (SLPI)

One molecule isolated from saliva was consistently found to block HIV-1 infection of mononuclear cells in culture at concentrations routinely present in oral secretions (7, 11, 12). This 12kD single chain polypeptide, identified as secretory leukocyte protease inhibitor (SLPI), is produced by acinar cells in parotid and submandibular glands (6, 13), consistent with the ability of secretions from either of these glands to impede HIV-1 infection (6). This molecule, initially recognized and purified from parotid saliva based on its potent antiprotease activity, inhibits the serine proteases, neutrophil elastase and cathepsin G (14), suggestive of an important role for SLPI in the host protection from inflammatory excesses. However, the antiprotease and antiretroviral activities of SLPI may be dissociable. Composed of two highly homologous domains, the COOH domain (domain 2) of SLPI contains the proteinase inhibitory site (15), whereas both domains are required for its antiretroviral action (11). Moreover, SLPI-derived mutant proteins that contain single amino acid substitutions and exhibit reduced antiprotease activity retained their antiretroviral activity (11).

Recognition of the surprising activity of this naturally occurring polypeptide to block HIV-1 infection provided a basis for the antiretroviral activity of saliva and the infrequent oral transmission of HIV. SLPI from parotid, submandibular, sublingual and minor salivary glands accumulates in the oral cavity and depletion of SLPI from saliva results in reduction of the majority of saliva's antiretroviral activity (7). SLPI protein levels, quantified by enzyme linked immunosorbent assay (ELISA), were variable, but on average, were higher in parotid secretions than in whole saliva (Fig.1) (17), likely reflecting degradation of SLPI in the oral cavity and formation of complexes with mucins and other salivary molecules. SLPI can also be found in glandular epithelia at other mucosal surfaces exposed to the external environment, including the lung (6, 16).

The mechanism whereby SLPI inhibits infection by HIV-1 appears to involve an interaction with the cellular hosts of the virus and not the virus itself (1, 7, 11). SLPI does not bind to the virus, nor to isolated gp120, gp160, HIV protease or p24, but binds to the surface of monocytes and lymphocytes which express the CD4 receptor for HIV-1 and its co-receptors, CCR5 and fusin (9–11). SLPI binding to monocytes, a key target of HIV-1 (18), is specific and time-, temperature-, concentration- and pH-dependent. Binding data reveal the presence of approximately 7000 binding sites per monocyte (11). In vitro, a single transient exposure of mononuclear cells to physiological concentrations of SLPI causes prolonged resistance to infection. However, once a cell is infected, SLPI is unable to influence viral replication. Based on these analyses, SLPI appears to serve a barrier function, blocking new rounds of infection (Tab. 2)

Tab. 2. *Role of SLPI in host defense**

Tab. 2. *Vloga SLIP-a v obrambni reakciji.*

- Found at mucosal sites open to external environment
- Nahaja se na površini sluznic, ki so odprte zunanjemu okolju

- Antiprotease activity against PMN elastase and cathepsin G
- Zavira proteazno delovanje elastaze in katepsina G

- Antibacterial activity
- Protibakterijsko delovanje

- Inhibits bacterial LPS-induced signaling and secretion
- Zavira signalizacijo in sekrecijo povzročeno z bakterijskimi LPS

- Serves a barrier function, inhibiting cell internalization of HIV-1
- Deluje kot ovira, zavira vdor in razporeditev HIV-1 v celicah

- Does not inhibit HIV-1 once the cell has been infected
- Ne zavira HIV-1, ko je celica že okužena

Inhibition of infection of HIV-1 in CD4+ monocytes and T cells is evident by SLPI-induced reduction in reverse transcriptase activity, viral p24 capsid antigen levels and by ultrastructural documentation of reduced or absent cell-associated virus. Inhibition of infection occurs with both lab and clinical virus isolates. The virus inhibitory effects of SLPI appear to be specific for HIV in that it blocks HIV-1 and also HIV-2 (7, 11, 12). However, SLPI does not inhibit cytomegalovirus (CMV) infection in vitro, nor block cell-specific infection by herpes virus (HSV) (1). Using a PCR-based assay which assesses nascent viral DNA synthesis, SLPI has been shown to act prior to viral reverse transcription (11). Collectively, the data indicate that SLPI prevents HIV-1 infection early in the virus life cycle, most likely in conjunction with the process of virus internalization (11). Although SLPI inhibits infection by cell-free virus, it does not inhibit cell-cell fusiozi, reflecting a similar specificity of action of intact saliva. That SLPI impairs initial events in the infection process emphasizes its potential as an agent of natural defense against retroviral infection.

The presence of appreciable amounts of SLPI in saliva from both HIV-1-infected and seronegative persons (6) suggests that SLPI may serve as an important innate mechanism of defense against oral HIV-1 transmission by the infected patient and against primary infection of the susceptible host. The potential HIV-1 infectivity of saliva is significantly less than serum and in fact, serum levels of SLPI are far below levels required for antiviral activity (Fig. 1). In this regard, comparison of serum and whole saliva from HIV-1 infected individu-

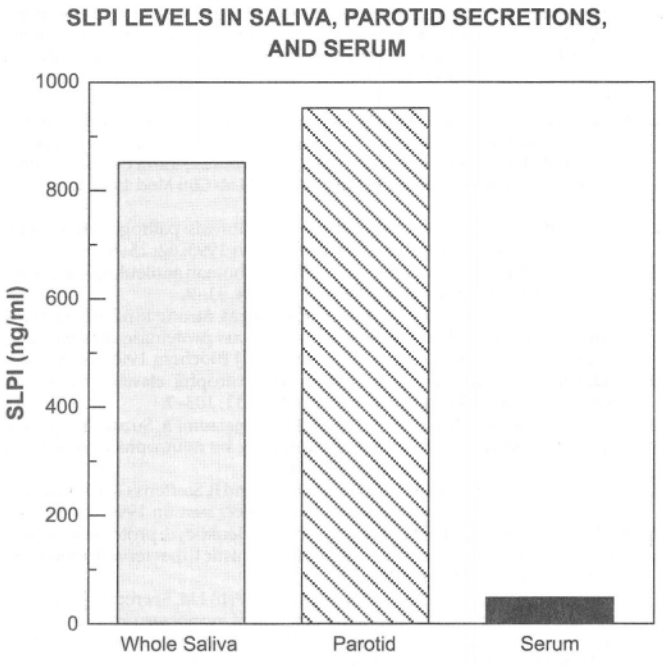

Fig. 1. *Comparison of SLPI levels in saliva, parotid secretions and serum. Whole saliva, parotid secretions or serum were collected from HIV-1 seronegative subjects and SLPI protein levels were quantified by ELISA. Data represent the mean levels (ng/ml) of 6 subjects for saliva and parotid secretions and of 6 serum samples.*

Sl. 1. *Primerjava koncentracij SLPI-a v slini, izločku glandule parotis in serumu. Celotna slina, parotidni izloček ali serum so bili odvzeti HIV-1 seronegativnim osebam. Koncentracije SLPI so bile določene z metodo ELISA. Podatki predstavljajo srednje vrednosti koncentracij SLPI (ng/ml) v slini in parotidnem izločku pri šestih osebah, ter v šestih serumskih vzorcih.*

als for infectious virus revealed that infectious virus could be identified in 38% of the tested sera, but only 1% of the saliva specimens (19). Even with severe periodontal lesions and potential bleeding into the oral cavity, only low concentrations or no infectious virus could be detected in saliva obtained from HIV-1 infected patients (20). Observations such as these suggest that even HIV-1 in blood may be reduced in infectivity by agent(s) in saliva. In recent studies, we have also compared SLPI levels in saliva, distinguished by imperceptible rates of person-to-person HIV-1 transmission, with those in another mucosal fluid, breast milk, through which maternal-child transmission of HIV-1 occurs more frequently (3). SLPI levels in saliva exceeded those in breast milk by 10–100 fold. Thus, there appears to be an inverse correlation between fluid levels of SLPI and HIV-1 transmissibility.

The exact physiological role of SLPI remains enigmatic. As a serine protease inhibitor, an important role appears to be protection of the mucosal epithelium against proteolytic (elastase and cathepsin G) attack (14). In this regard, SLPI has been explored as a therapeutic intervention for inflammatory disorders characterized by overabundance of destructive proteolytic enzymes, including emphysema and cystic fibrosis (21–23). A complicating factor in such an approach is the attenuation of SLPI's anti-elastase properties when it complexes with mucins (24, 25). However, it is unclear whether mucin-SLPI complexes are more or less antiretroviral than either of these molecules alone. In saliva, native SLPI is also degraded, being cleaved at 4 different sites (26), and it remains unknown whether the antiretroviral activity is retained in these fragments. Based on the evidence that both domain 1 and 2 are essential for viral inhibition, the cleaved protein may also lose its activity against HIV-1.

Production of SLPI by submucosal glands at mucosal sites open to the external environment and its secretion into saliva, bronchial mucus, tears, cervical mucus and seminal plasma point toward a role(s) for SLPI in host defense towards environmental pathogens (Tab. 2). The concept that SLPI represents an important mechanism of mucosal defense against environmental pathogens is supported by its presence in saliva from neonates (3). Although the classic mechanism of mucosal defense, secretory IgA, is not produced normally until later in development, levels of SLPI in saliva from young infants were equal or higher than those from adults. Despite the immaturity of the neonatal salivary secretory system, immunohistochemical staining for SLPI protein was clearly evident in newborn salivary gland tissues. Although the levels produced are likely sufficient to facilitate local protection against mucosal pathogens, the extremely low volumes of saliva present in newborns, compared to the large volumes of fluid consumed, may negate any protective effect against maternal HIV-1 (3). Beyond its newly described antiretroviral activity, recent evidence also demonstrates that SLPI is bactericidal (27). SLPI not only kills bacteria, but also inhibits bacterial endotoxin-induced production of inflammatory mediators (Tab. 2) (28, 29).

Conclusions

Possession of both antibacterial and antiviral activity is consonant with an anti-infectious role for this endogenous peptide. Thus, SLPI may be a key component of the innate defense system. Such agents of natural immunity are produced prior to antigenic exposure, and likely contribute to defense against primary infection of a range of organisms. Together with other components of mucosal defense found in saliva, SLPI may contribute to the relatively infrequent transmission of HIV-1 by the oral route. Of considerable current interest is whether the levels of this endogenously generated molecule can be augmented to promote host defense. Exogenous administration, gene transfer and/or stimulation of secretion may provide locally elevated SLPI levels to bolster host defense to HIV-1 and to other vulnerable infectious pathogens entering the host at mucosal surfaces.

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Ključne besede: *HIV-1; ustna sluznica; slina; sekretorni levkocitni proteinazni inhibitor*

Izvleček – Izhodišča. *Virus človeške imunske pomanjkljivosti (HIV-1), ki povzroča AIDS, se prenaša preko krvi in površin sluznic. Različne raziskave so pokazale, da je ustna sluznica relativno odporna proti HIV-1 in da je slina zelo redko tekočina, preko katere bi se prenašala okužba s HIV-1.*
Slina vsebuje mnogo proteinov, ki lahko vplivajo na prenos okužbe z virusi. Avtorji podrobno opisujejo sekretorni levkocitni proteinazni inhibitor (SLPI), ki je v slini vedno v taki količini, ki uspešno zavre infekcijo monocitov z virusom HIV-1. SLPI so našli, razen v slinavkah in slini, tudi v epiteliju drugih žlez in na površini sluznic, ki so odprte zunanjemu okolju. SLPI se ne veže na virus ali njegove sestavne dele, temveč na površino monocitov in limfocitov, ki izražajo CD4 receptorje in koreceptorje: SLPI tako v fizioloških koncentracijah povzroči odpornost proti virusni okužbi, vendar ne more preprečiti razmnoževanja virusa v že okuženih celicah. SLPI so našli v slini HIV okuženih in neokuženih oseb. SLPI je v serumu v taki koncentraciji, ki ne omogoča protivirusnega delovanja, zato ne preseneča, da so našli virus v 38% v serumu in samo v 1% v slini okuženih oseb.
Natančna fiziološka vloga SLPI-ja ni poznana. Deluje kot inhibitor serumskih proteinaz, ima protivirusno, pa tudi protibakterijsko delovanje. Izgleda, da je pomemben pri imunološki obrambi sluznic, saj ga najdemo v normalni koncentraciji že v slini novorojenčkov.

Zaključki. *Avtorji kažejo na možnost, da bi z lokalnim ali spodbujanjem izločanja SLPI-ja zvišali imunološko obrambo proti virusom in bakterijami, ki ogrožajo sluznice.*

NAVODILA SODELAVCEM ZDRAVNIŠKEGA VESTNIKA

Zdravniški vestnik (ZV) je glasilo Slovenskega zdravniškega društva. Naslov uredništva je: Zdravniški vestnik, Komenskega 4, 1000 Ljubljana, telefon (061) 317 868, telefaks (061) 301 955.

Splošna načela

ZV objavlja le izvirna, še ne objavljena dela. Avtor je odgovoren za vse trditve, ki jih v prispevku navaja. Če je članek pisalo več soavtorjev, je treba navesti natančen naslov (s telefonsko številko) tistega avtorja, s katerim bo uredništvo sodelovalo pri urejanju teksta za objavo ter mu pošiljalo prošnje za odtis.

Če prispevek obravnava raziskave na ljudeh, mora biti iz besedila razvidno, da so bile raziskave opravljene skladno z načeli Kodeksa medicinske deontologije in Deklaracije iz Helsinkov/Tokija.

Če delo obravnava poizkuse na živalih, mora biti razvidno, da je bilo opravljeno skladno z etičnimi načeli.

Prispevki bodo razvrščeni v eno od naslednjih rubrik: uvodnik, raziskovalni prispevek, strokovni prispevek, pregledni članek, kakovost v zdravstvu, pisma uredništvu in razgledi.

Raziskovalna poročila morajo biti napisana v angleščini. Dolga naj bodo do 8 tipkanih strani. Slovenski izvleček mora biti razširjen in naj bo dolg do tri tipkane strani. Angleški ne sme biti daljši od 250 besed.

Če besedilo zahteva aktivnejše posege angleškega lektorja, nosi stroške avtor.

Ostali prispevki za objavo morajo biti napisani v slovenščini jedrnato ter strokovno in slogovno neoporečno. Pri raziskovalnih in strokovnih prispevkih morajo biti naslov, izvleček, ključne besede, tabele in podpisi k tabelam in slikam prevedeni v angleščino.

Članki so lahko dolgi največ 12 tipkanih strani (po 30 vrstic) s tabelami in literaturo vred.

V besedilu se uporabljajo le enote SI in tiste, ki jih dovoljuje Zakon o merskih enotah in merilih.

Spremni dopis

Spremno pismo mora vsebovati: 1. izjavo, da poslano besedilo ali katerikoli del besedila (razen abstrakta) ni bilo poslano v objavo nikomur drugemu; 2. da so vsi soavtorji besedilo prebrali in se strinjajo z njegovo vsebino in navedbami; 3. kdaj je raziskavo odobrila Etična komisija; 4. da so preiskovanci dali pisno soglasje k sodelovanju pri raziskavi; 5. pisno dovoljenje za objavo slik, na katerih bi se morebiti lahko prepoznala identiteta pacienta; 6. pisno dovoljenje založbe, ki ima avtorske pravice, za ponatis slik, shem ali tabel.

Tipkopis

Prispevki morajo biti poslani v trojniku, tipkani na eni strani boljšega belega pisarniškega papirja formata A4. Med vrsticami mora biti dvojni razmik (po 30 vrstic na stran), na vseh straneh pa mora biti rob širok najmanj 30 mm. Avtorji, ki pišejo besedila s pomočjo PC kompatibilnega računalnika, jih lahko pošljejo uredništvu v enem izpisu in na 5.25 ali 3.5 inčni disketi, formatirani na 360 KB ali 1,2 MB, kar bo olajšalo uredniški postopek. Ko je le-ta končan, uredništvo disketo vrne. Besedila naj bodo napisana z urejevalnikom Word for Windows ali z drugim besedilnikom, ki hrani zapise v ASCII kodi.

V besedilu so dovoljene kratice, ki pa jih je treba pri prvi navedbi razložiti. Že uveljavljenih okrajšav ni treba razlagati (npr. L za liter, mg za miligram itd.).

Naslovna stran članka naj vsebuje slovenski naslov dela, angleški naslov dela, ime in priimek avtorja z natančnim strokovnim in akademskim naslovom, popoln naslov ustanove, kjer je bilo delo opravljeno (če je delo skupinsko, naj bodo navedeni ustrezni podatki za soavtorje). Naslov dela naj jedrnato zajame bistvo vsebine članka. Če je naslov z avtorjevim imenom in priimkom daljši od 90 znakov, je potrebno navesti še skrajšano verzijo naslova za tekoči naslov. Na naslovni strani naj bo navedenih tudi po pet ključnih besed (uporabljene naj bodo besede, ki natančneje opredeljujejo vsebino prispevka in ne nastopajo v naslovu; v slovenščini in angleščini) ter ev. financerji raziskave (s številko pogodbe).

Druga stran naj vsebuje slovenski izvleček, ki mora biti strukturiran in naj vsebuje naslednje razdelke in podatke:

Izhodišča (Background): Navesti je treba glavni problem in namen raziskave in glavno hipotezo, ki se preverja.

Metode (Methods): Opisati je treba glavne značilnosti izvedbe raziskave, opisati vzorec, ki se preučuje (npr. randomizacija, dvojno slepi poizkus, navzkrižno testiranje, testiranje s placebom itd.), standardne vrednosti za teste, časovni odnos (prospektivna, retrospektivna študija).

Navesti je treba način izbora preiskovancev, kriterije vključitve, kriterije izključitve, število preiskovancev, vključenih v raziskavo in koliko jih je vključenih v analizo. Opisati je treba posege, metode, trajanje jemanja posameznega zdravila, kateri preparati se med seboj primerjajo (navesti je treba generično ime preparata in ne tovarniško) itd.

Rezultati (Results): Opisati je treba glavne rezultate študije. Pomembne meritve, ki niso vključene v rezultate študije, je treba omeniti. Pri navedbi rezultatov je treba vedno navesti interval zaupanja in natančno raven statistične značilnosti. Pri primerjalnih študijah se mora interval zaupanja nanašati na razlike med skupinami. Navedene morajo biti absolutne številke.

Zaključki (Conclusions): Navesti je treba le tiste zaključke, ki izhajajo iz podatkov, dobljenih pri raziskavi; treba je navesti ev. klinično uporabnost ugotovitkov. Navesti je treba, kakšne dodatne študije so še potrebne, preden bi se zaključki raziskave klinično uporabili. Enakovredno je treba navesti tako pozitivne kot negativne ugotovitke.

Ker nekateri prispevki (npr. pregledni članki) nimajo niti običajne strukture članka, naj bo pri teh strukturiranost izvlečka ustrezno prilagojena. Dolg naj bo od 50 do 200 besed; na tretji strani naj bodo: angleški naslov članka, ključne besede v angleščini in angleški prevod izvlečka.

Na naslednjih straneh naj sledi besedilo članka, ki naj bo smiselno razdeljeno v poglavja in podpoglavja, kar naj bo razvidno iz načina podčrtavanja naslova oz. podnaslova, morebitna zahvala in literatura. Odstavki morajo biti označeni s spuščeno vrstico. Tabele, podpisi k slikam in razlaga v tekstu uporabljenih kratic morajo biti napisani na posebnih listih.

Tabele

Natipkane naj bodo na posebnem listu. Vsaka tabela mora biti oštevilčena z zaporedno številko. Tabela mora imeti najmanj dva stolpca. Vsebovati mora: naslov (biti mora dovolj poveden, da razloži, kaj tabela prikazuje, ne da bi bilo treba brati članek; če so v tabeli podatki v odstotkih, je treba v naslovu navesti bazo za računanje odstotka; navesti je treba od kod so podatki iz tabele, ev. mere, če veljajo za celotno tabelo, razložiti podrobnosti glede vsebine v glavi ali čelu tabele), čelo, glavo, morebitni zbirni stolpec in zbirno vrstico ter opombe ali pa legendo uporabljenih kratic v tabeli. Vsa polja tabele morajo biti izpolnjena in mora biti jasno označeno, če morebiti podatki manjkajo.

V besedilu prispevka je treba označiti, kam spada posamična tabela.

Slike

Risbe morajo biti risane s črnim tušem na bel trd papir. Pri velikosti je treba upoštevati, da bodo v ZV pomanjšane na širino stolpca (81 mm) ali kvečjemu na dva stolpca (168 mm). Morebitno besedilo na sliki mora biti izpisano z laserskim tiskalnikom. Pri velikosti črk je treba upoštevati, da pri pomanjšanju slike za tisk velikost črke ne sme biti manjša od 2 mm. Grafikoni, diagrami in sheme naj bodo uokvirjeni.

Na hrbtni strani vsake slike naj bo s svinčnikom napisano ime in priimek avtorja, naslov članka in zaporedna številka slike. Če je treba, naj bo označeno kaj je zgoraj in kaj spodaj.

V besedilu prispevka je treba označiti, kam spada posamična slika.

Literatura

Vsako trditev, dognanje ali misel drugih je treba potrditi z referenco. Neobjavljeni podatki ali osebno sporočilo ne spada v seznam literature. Navedke v besedilu je treba oštevilčiti po vrstnem redu, v katerem se prvič pojavijo, z arabskimi številkami v oklepaju. Če se pozneje v besedilu znova sklicujemo na že uporabljeni navedek, navedemo številko, ki jo je navedek dobil pri prvi omembi. Navedki, uporabljeni v tabelah in slikah, naj bodo oštevilčeni po vrstnem redu, kakor sodijo tabele in slike v besedilo. Pri citiranju več del istega avtorja dobi vsak navedek svojo številko, starejša dela je treba navesti prej. Vsi navedki iz besedila morajo biti vsebovani v seznamu literature.

Literatura naj bo zbrana na koncu članka po zaporednih številkah navedkov. Če je citirani članek napisalo 6 avtorjev ali manj, jih navedite vse; pri 7 ali več je treba navesti prve tri in dodati et al. Če pisec prispevka v originalni objavi ni imenovan, se namesto njega napiše Anon. Naslove revij, iz katerih je navedek, je treba krajšati kot določa Index Medicus.

Primeri citiranja

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– primer za članek iz revije, kjer je avtor organizacija:

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– primer za članek iz suplementa revije:

6. Miller GJ. Antithrombotic therapy in the primary prevention of acute myocardial infarction. Am J Cardiol 1989; 64: Suppl 4: 29B–32B.

– primer za članek iz zbornika referatov:

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Sodelovanje avtorjev z uredništvom

Prispevke oddajte ali pošljite le na naslov: Uredništvo Zdravniškega vestnika, Komenskega 4, 1000 Ljubljana. Za prejete prispevke izda uredništvo potrdilo. V primeru nejasnosti so uredniki na voljo za posvet, najbolje po poprejšnjem telefonskem dogovoru (tel. 061 / 317 868).

Vsak članek daje uredništvo v strokovno recenzijo in jezikovno lekturo. Po končanem redakcijskem postopku, strokovni recenziji in lektoriranju vrnemo prispevek avtorju, da popravke odobri, jih upošteva in oskrbi čistopis, ki ga vrne s popravljenim prvotnim izvirnikom. Med redakcijskim postopkom je zagotovljena tajnost vsebine članka.

Avtor dobi v korekturo prvi krtačni odtis s prošnjo, da na njem označi vse tiskovne napake. Spreminjanja besedila ob tej priliki uredništvo ne bo upoštevalo. Korekture je treba vrniti v treh dneh, sicer uredništvo meni, da avtor nima pripomb.

Rokopisov in slikovnega materiala uredništvo ne vrača.

Dovoljenje za ponatis slik, objavljenih v ZV, je treba zaprositi na Uredništvo Zdravniškega vestnika, Komenskega 4, 1000 Ljubljana.

Navodila za delo recenzentov

Če zaprošeni recenzent prispevka ne more sprejeti v oceno, naj rokopis vrne. Hvaležni bomo, če v tem primeru predlaga drugega primernega recenzenta. Če meni, naj bi uredništvo poleg njega prosilo za oceno prispevka še enega recenzenta (multidisciplinarna ali mejna tema), naj to navede v svoji oceni in predlaga ustreznega strokovnjaka.

Recenzentovo delo je zelo odgovorno in zahtevno, ker njegovo mnenje največkrat vodi odločitev uredništva o usodi prispevka. S svojimi ocenami in sugestijami recenzenti prispevajo k izboljšanju kakovosti našega časopisa. Po ustaljeni praksi ostane recenzent avtorju neznan in obratno.

Če recenzent meni, da delo ni vredno objave v ZV, prosimo, da navede vse razloge, zaradi katerih delo zasluži negativno oceno. Negativno ocenjen članek po ustaljenem postopku skupaj z recenzijo (seveda anonimno) uredništvo pošlje še enemu recenzentu, kar se ne sme razumeti kot izraz nezaupanja prvemu recenzentu.

Prispevke pošiljajo tudi mladi avtorji, ki žele svoja zapažanja in izdelke prvič objaviti v ZV; tem je treba pomagati z nasveti, če prispevek le formalno ne ustreza, vsebuje pa pomembna zapažanja in sporočila.

Od recenzenta uredništvo pričakuje, da bo odgovoril na vprašanja na obrazcu ter bo ugotovil, če je avtor upošteval navodila sodelavcem, ki so objavljena v vsaki številki ZV, in da bo preveril, če so podane trditve in misli verodostojne. Recenzent mora oceniti metodologijo in dokumentacijo ter opozoriti uredništvo na ev. pomanjkljivosti, posebej še v rezultatih.

Ni potrebno, da se recenzent ukvarja z lektoriranjem in korigiranjem, čeprav ni napak, če opozori na take pomanjkljivosti. Posebej Vas prosimo, da ste pozorni na to, ali je naslov dela jasen in koncizen ter ali ustreza vsebini; ali izvleček povzema bistvene podatke članka; ali avtor citira najnovejšo literaturo in ali omenja domače avtorje, ki so pisali o isti temi v domačih časopisih ali v ZV; ali se avtor izogiba avtorjem, ki zagovarjajo drugačna mnenja, kot so njegova; ali navaja tuje misli brez citiranja; ali so literaturni citati točni. Preveriti je treba dostopne reference. Prav tako je treba oceniti, če so slike, tabele in grafi točni in da se v tabelah ne ponavlja tisto, kar je že navedeno v tekstu; da ne vsebujejo nepojasnjenih kratic, da so številčni podatki v tabelah ustrezni onim v tekstu ter da ni napak.

Če recenzent meni, da delo potrebuje dopolnilo (komentar) ali da bi ga sam lahko dopolnil (s podatki iz literature ali lastnimi izkušnjami), se lahko dogovori z urednikom, da se tak komentar objavi v isti številki kot ocenjevano delo.

Recenzij ne plačujemo.

Zdravniški vestnik

GLASILO SLOVENSKEGA ZDRAVNIŠKEGA DRUŠTVA ZDRAV VESTN, LETNIK 67, JUNIJ 1998, str. 1–94, Sup

ŠTEVILKA OB 10. OBLETNICI CENTRA ZA STOMATOLOŠKE RAZISKAVE

UVODNIK

ČLANKI