A Method for Histological, Enzyme Histochemical and Immunohistochemical Analysis of Periapical Diseases on Undecalcified Bone with Teeth

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Summary

The objective was to examine and apply a method for serial sectioning of undecalcified canine teeth with surrounding jawbone and to analyse it histologically, enzyme histochemically and immunohistochemically. Pulpitis and apical periodontitis were induced in a mongrel dog's premolar teeth by exposing the pulp to the oral environmental influence for 50 days. After animal sacrifice undecalcified bone with the decoronated experimental teeth were embedded in methylmethacrylate and sectioned with a tungsten carbide knife at 5-7 µm. Sections were stained with toluidin blue (TB) for histological analysis and by a method of staining non-specific acid phosphatase (ACP), and tartrat-resistant acid phosphatase (TRAP). Immunohistochemical staining was performed for detection of CD 45+ lymphocytes. Because the specimens were 5-7 µm thick it was possible to perform a detailed cytological analysis of changes in the pulp and periapex in situ *with a light microscope on histological and enzyme histochemical specimens. Immunohistochemical staining was not satisfactory. The method of embedding undecalcified bone and teeth in methylmethacrylate and sectioning with a tungstencarbide knife is satisfactory for histological and some enzyme histochemical analysis in endodontic research. However, immunohistochemical staining needs improvement.*

Key words: *histology, dogs, methyl-methacrylate, periapical diseases, undecalcified bone.*

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Introduction

Analyses of periapical changes on human teeth are directed mainly to the study of occurrences in the soft tissue obtained during surgical removal at the time of apicectomy (1, 2). Periapical pathosis that has to be treated by apicectomy usually occurs in soft tissue in an advanced stage of inflammation, and is large and resistant to conservative therapy. Analysis of the obtained specimens lacks information on the condition of the bone.

Early stages of apical periodontitis or the transitional stage from pulpitis to apical periodontitis can only be analysed *in situ*. Consequently, they are

confined to experimental animals. Rats are the most frequently used experimental animals for endodontic research.

Larger mammals are more suitable for studying the pathophysiology of apical periodontitis *in situ* because the spread of infection from pulp to periapical tissue is slower than in smaller mammals, i.e. rats. Therefore, transitional stages from pulptis to apical periodontitis could be analysed on larger mammals (3, 4).

Certain histochemical and immunohistochemical analyses of bone changes are performed mainly on rat specimens, demineralized with 10% EDTA (5, 6). Small bones of the rat can be demineralized in this way in a few days. Larger bones of the jaw, such as the bones of the dog, in a block with larger teeth, cannot be demineralized in a few days with a chelating agent such as 10% EDTA. In time proteins decompose and cannot be identified. Thus, this presents a problem when protein detection is desired on larger mammals. Specimens of the jaw of larger mammals in the block can be demineralized with stronger acids such as nitric, formic or trichloracetic (7) although these procedures also cause severe alteration in protein content of the tissue (8). Such specimens are therefore unsuitable for histochemical and immunohistochemical analyses (9).

Different methods have been described for the preparation of demineralized bone with teeth. Gross & Strunz (10) described a method which permitted the sectioning of undemineralized tissue in sections 50-200 μm thick. However, the deeper structures in their sections were unstained. Donath & Breuner (11) described "the sawing and grinding technique", which provided thinner sections. This method requires special equipment, and grinding removes a major portion of the specimens.

The aim of this study was to examine the possibility of preparing histological, histochemical and immunohistochemical specimens 5-7 μm thick on undecalcified canine teeth with surrounding jawbone.

Materials and methods

The experiment was approved by the Croatian Veterinary Chamber (approval No. 111/97). The experiment was performed on two mongrel dogs. Two two-rooted mandibular premolars were treated in each animal, i.e. a total number of 8 roots. The dogs were male, between 10 and 15 kg in weight and 4 and 5 years old. On the basis of body weight the quantity of anaesthetics were determined.

They were anaesthetised with ketamine hydrochloride (Ketanest®, 35 mg/kg of body wt; Goedecke /Parke - Davis, Berlin, Germany) and xylazine (Rompun®, 10 mg/kg; Bayer AG, Leverkusen, Germany). The pulps of the mandibular premolars were exposed using a low-speed dental engine. The teeth were left open to the oral environment for 50 days to induce pulpitis and apical periodontitis. After the experimental period the animals were sacrificed. The mandible was removed and resected mechanically into small blocks, so that each block contained up to three premolars. Since enamel is the greatest problem in the preparation of undemineralized teeth for sectioning, the examined teeth were decoronated *in situ* with rotating diamond drills on a low speed dental engine with water-cooling.

The prepared blocks of hard tissue were rinsed in a saline solution and fixed by immersion in 4% paraformaldehyde, pH 7.4 at 4°C for 24 hours. At the end of fixation the tissue was stored in 70% ethanol for at least 48 hours.

After 48 hours the tissue was ready for embedding in the plastic medium (methylmethacrylate) by application of the technique "Immersion in the solution", according to the following protocol. Tissue was dehydrated in a series of ethanol of increasing concentrations (75%, 96% and absolute ethanol - each for a period of 48 hours). After dehydration the tissue was immersed in Xylene for 72 hours (with three changes of fresh Xylene). The tissue was perfused gradually with a mixed solution of methylmethacrylate (MMA), dibutyl phthalate and benzoyl peroxide. We used methylmethacrylate (Merck, Germany), dibutyl phthalate (Sigma Chemical Co., St. Louis, USA) and benzoyl peroxide (Merck, Germany). These chemicals were mixed in three different proportions of components, so that three solutions were obtained in which the tissue was perfused:

- Solution 1 - 80 ml methylmethacrylate and 20 ml dibutyl phthalate for a period of 24 hours at +4°C.
- Solution 2 - 80 ml methylmethacrylate, 20 ml dibutyl phthalate and 1 g benzoyl peroxide for a period of 24 hours at +4°C.

- Solution 3 - 80 ml methylmethacrylate, 20 ml dibutyl phthalate and 3g benzoyl peroxide for a period of 24 hours at +4°C.

During the soaking of the specimens, the vessels, in which the procedure was carried out, were hermetically closed and kept at lower than +4°C.

Following soaking the tissue was embedded in the final solution for polymerisation, which, according to the composition, was identical to solution 3, except for the fact that it was not kept at +4°C, but in the thermostat at a temperature of +37°C. Tissue blocks were positioned in the plastic to facilitate sectioning. Embedding was performed in glass bottles, which can be hermetically closed, because during MMA polymerisation, the solution for polymerisation evaporates, resulting in a decreased concentration of components. After the final polymerisation (two to three days) the glass bottles were broken mechanically and a hard, transparent plastic block was obtained with embedded tissue that was ready for sectioning. For sectioning a rotatory microtome (Leica RM 2155, Wiena, Austria), equipped with a tungsten carbide knife for cutting hard and mineralised tissue was used (Figure1).

Sections 5-7 μm thick were cut and mounted on silane-prepared slides (Silane-Prep slides, Sigma Chemical Co., St.Louis, USA). The tissue sections were covered with damp cellophane foil, flattened and dried under pressure in a thermostat at +37°C until stained for microscopy.

Before staining, sections were deplastified by 100% acetone twice for 5 minutes and rehydrated. Subsequently, they were ready for further procedure. In this stage of preparing the specimens and during the staining procedure some of the specimens became unstuck from the glass slides. The reason for this phenomenon remains unclear.

Toluidin blue (TB) staining was used for histological analysis. Acid phosphatase (ACP) and tartrat-resistant acid phosphatase (TRAP) were detected histochemically and CD 45+ B-lymphocytes immunohistochemically. Histochemical analysis was performed using diagnostic (Sigma Chemical Co., St.Louis, USA) kit. Mouse anti-dog monoclonal antibodies (donated by Prof. PF Moore, Davis, California, USA) were used for immunohistochemical identification of CD45+ B-lymphocytes. After which universal secondary antibody - biotin (Serotec Ltd. Oxford, UK) was applied. After staining with avidin-biotin-immunoperoxidase, sections were incubated in AEC chromogen and counterstained with hematoxylin.

Results

The technique of embedding undecalcified bone with teeth in methylmethacrylate and sectioning with a tungsten carbide knife in sections 5-7 μm thick could be used in endodontic research. Decoronating of the teeth solves the problem of enamel hardness. Consequently such specimens were suitable for detailed cytological analysis of the inflamed content of soft tissue (Figure 2), and hard tissue of teeth and surrounding jawbone (Figure 3 and 3a). The described procedure enables preparation of a large number of specimens and application of several types of staining on the same tooth, which is difficult to achieve with the grinding method. Apart from basic histological staining, toluidin blue clearly shows anatomical structures, such as pulp tissue in the root canal, dentin, cement, bone, connective periodontal tissue with blood vessels, nerves and other anatomical structures, and a whole spectra of inflammatory cells, exudate and necrotic tissue in the area of the root canal and periapical lesion (Figures 2, 3 and 3a). Apart from the basic histological staining (toluidin blue) it is also possible to carry out enzyme histochemical (Figure 4) and immunohistochemical analyses (Figure 5), which cannot be performed on bones decalcified with stronger acids such as formic, nitric or trichloracetic. Histochemical analysis of acid phosphatase detects osteoclasts and other phagocytotic cells which, like macrophages, perform secretion and absorption. Enzyme tartrat-resistant acid phosphatase is specific for osteoclast series cells. These cells extracellularly secrete the described acids which are stained purple in the analysis which is a sign of osteoclastic activity, as can be seen in Figure 4. Immunohistochemical analysis demonstrated the presence of B-lymphocytes in chronic stages of periapical lesion, by means of CD45 marker on the surface of their cell membrane, which is stained brown in the specimens (Figure 5).

The loss of specimens which became unstuck during the described procedures of staining amounted approximately 10%.

Discussion

Gross & Strunz (10) described a method of sectioning undemineralized hard tissues in sections as thin as 50 to 200 μm. Deeper structures in the sections were unstained and there was only one specimen that could be prepared through the apical foramen into the centre of the periapical lesion on the same tooth.

Enamel hardness is a unique problem in the use of undemineralized specimens. Donath & Breuner (11) described "The sawing and grinding technique" that permitted preparation of very thin sections of demineralized dental tissues including enamel. However, during this sawing and grinding, major parts of tissue were discarded. This resulted in only one or two specimens being prepared through the apical foramen. Furthermore, significant amounts of unused tissue were lost.

In contrast to the above described proceedings on undecalcified tissue, use of this method enables the preparation of a larger number of specimens of one tooth with its surrounding tissue, and to stain it with several different techniques which increases analytical possibilities and could increase pathophysiological understanding of the beginning, formation and velocity of expansion of a periapical lesion.

The concept of embedding undemineralized bone in methylmetacrilate has been improved (12-14) by the use of a special tungsten-carbide knife, which enables serial sections 5-7 μm thick. Decoronating the tooth used in this study eliminated the problem of sectioning the hard enamel with the above knife.

Experimental models on animals, for the purpose of studying the early stages of periapical changes, have, for practical reasons, been mainly restricted to rats (5, 6, 15). However, the tooth of the rat, because of its small size, morphology and biological characteristics, such as permanent tooth growth, becomes a questionable model when there is a need to carry out analogy with pathophysiological processes, which occur during the occurrence of apical periodontitis in humans, in whom this characteristic is different. Consequently, these two populations are statistically different and inapplicable with the characteristics of human tissue. For instance, occlusal loading of the tooth delays progression of apical periodontitis in the rat (16), while in humans it causes the process to deteriorate (17). Also, the tooth of the rat, because of its size, is unsuitable for studying endodontic cleaning and shaping techniques and their evaluation, while endodontic technique on canine teeth is more suitable for comparison with work on human teeth .

Because of their biological characteristics, larger mammals, such as the dog, cat, monkey etc, are more suitable for studying the pathophysiology of apical periodontitis *in situ*, because their teeth retain a macroscopically permanent shape and relation of the root and apical periodont after formation of the apex (4). Also, spread of infection from the pulp to the periapical tissue is slower than in rats (3).

Enzyme histochemical and imunohistochemical analyses of rat bone is mainly carried out on bone decalcified with 10% EDTA (5, 6, 15). This mild organic acid can successfully decalcify, within a few days, very small bones, or very small pieces (10 x 6 x 2 mm) of larger bones (18). There is a problem of bone overheating during sectioning of larger bones into small pieces, which could be suitable for decalcification with EDTA. Decalcification with stronger acids such as nitric, formic or trichloracetic causes protein denaturation and is therefore not suitable for enzyme histochemical and imunohistochemical analyses of larger bones with teeth (9).

The results obtained by the method used, apart from histological analyses, also enables enzyme histochemical and immunohistochemical analyses of the pulp and periapical tissue *in situ*, because this method does not cause severe alteration of proteins. Such prepared specimens could be analysed with a standard light microscope.

Specimens prepared by the above method were histologically analysed with toluidin blue staining. Enzyme histochemical staining of acid phosphatase gives satisfactory results. Purple staining of acid-phosphatase and tartrat-resistant acid phosphatase could be seen on the surface of the bone and root cementum resorptive lacunae in osteoclast cells.

Immunohistochemical staining of CD45+ B-lymphocytes gave only approximate results. We could not detect specific antigen on the surface of the cell, but merely identified the region with accumulation of CD45+ B-lymphocytes (Figure 5). The procedure of immunohistochemical staining should be further improved.

In order to obtain optimal fixation we mounted the sections on silane prepared glass slides and stuck them with cyanoacrylate. However, we were unable to completely solve the problem of adhesion of the specimens to the silane-prepared glass slide. This explains the phenomenon of samples which became unstuck, as described in the "Results". Namely, if care was not taken when rinsing during the staining process the specimens occasionally became unstuck. Once the specimen had become unstuck it was impossible to return it to the initial position by pressure, and because of tearing it was unusable. Although it is possible to stain the specimens without deplastification, as stated by Donath & Breuner, the quality of such staining is much poorer. We presume that the poor adhesion is due to the size and weight of the specimens of teeth and surrounding tissue, and attempts have to be made to improve adhesion of the specimens to the glass slides.

Conclusions

The method for embedding undecalcified teeth with surrounding jawbone in methylmetacrylate is suitable for histological and histochemical analysis. Immunohistochemical analysis needs improvement. The advantage of this procedure is the possibility of analysing pulp, dentin, cement and soft periapical inflamed tissue. Furthermore, specimens made in the described manner enable evaluation of the dynamics of periapical lesion development and phenomenon of formation and reshaping of bone tissue which enables deeper pathophysiological perception.

A drawback of this method is immunohistochemical staining in the procedure of cell identification. A disadvantage of the procedure is the problem of adhesion of the specimens to the glass slides.

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