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Histological preparation of teeth and tooth growth

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Abstract

Background: Traditional techniques for preparation of teeth for histological analysis include: 1) Ground section of non-decalcified tooth and view by polarized light.
2) Decalcify the tooth and then section and stain as per soft tissue. 3) Cryomicrotomy -non-decalcified tooth is immersed rapidly in liquid nitrogen and sectioned with a cryostat using a heavy-duty freezing microtome.

Objectives: This study introduces a petrographic technique for histological preparation of teeth, so that thin sections of teeth can be viewed under the microscope without decalcification. In addition the article discusses the use of tetracycline to measure tooth growth in rats.

Methods: Six Wistar rats were given tetracycline as a marker of tooth growth. An incisor tooth from each sacrificed animal was imbedded in an epoxy resin and ground down to achieve a tooth slice, 30 µm thick.

Results: The petrographic preparation technique for teeth for histological analysis clearly demonstrated that thin sections of teeth can be viewed under the microscope with both calcified and soft tissues present in one view. In addition, the mean growth of teeth was 0.010 mm/day during the period of tetracycline administration and 0.016 mm/day when no tetracycline was being administered.

Conclusion: This technique, although a petrographic preparation technique that has been used for more than forty years, is new to dental research and allowed detailed analysis of teeth with all calcified and non-decalcified tissues present in one section. This study also showed that the presence of tetracycline caused a reduction in tooth growth and this finding needs to be taken into consideration when interpreting the results of other studies when tetracycline is used as a histological marker of tooth growth.

Keywords: Non-decalcification technique, teeth, tetracycline, tooth growth, histological analysis

Introduction

The histological processing of teeth is difficult due to the fact that they are made up of both mineralized hard tissues and soft tissues. Traditional preparation techniques and the type of decalcification agents used in processing, commonly lead to mild to severe deterioration in the tissue structure and therefore limitations in staining and examination of all tissues in one histological view.

Traditional techniques for preparation of teeth for histological analysis include Ground Section, Decalcification and Cryomicrotomy.

The ground section of non-decalcified tooth and viewing by polarized light has been traditionally used in the analysis of the crystal structure of teeth. However, sections are very thick and preclude the use of transillumination to analyse tooth crystal structure [1-3].

Decalcify the tooth and then section and stain as per soft tissue. While this technique is useful in analysing the soft tissue component of teeth, such as the pulp and periodontal ligament, it loses the bulk of the crystal structure of teeth, making analysis of hard tissue difficult [4,5].

Cryomicrotomy is used where the non-decalcified tooth and mandible is immersed rapidly in liquid nitrogen and sectioned with a cryostat using a heavy-duty freezing microtome. Both cellular and extracellular structures are well preserved and the sections of tooth and bone appeared to be suitable for optical and scanning electron microscopy and for immunohistochemical analysis. However, there is an overall strong non-specific binding of immunohistochemical reagents to enamel [6,7] therefore making the use of stains and histological examination

definition difficult.

The purpose of this study is to report a petrographic preparation technique for histological preparation of teeth which allows detailed analysis of teeth with all calcified and non-decalcified tissues present in one section. In addition, tooth growth is quantified using tetracycline as marker of hard tissue growth. The laboratory technique for petrographic preparation of samples for histological analysis has been widely used for many years in Geology, Engineering and Archaeology University departments around the world. At the University of Western Australia this sample preparation process is used for nearly all of the geological samples, such as crystals, fossils and meteorites which come to the Geology Department and is also used for analysis of pottery from the Archaeology Department and ceramics and concrete from the Engineering Department. This sample petrographic preparation process was first described by Beauchamp and Williford [8] in 1974. The advantages of polished ultra-thin sections in the study of very fine-grained materials, such as occur in some meteorites, have been further illustrated by Fredriksson et al., [9] using the same preparation process. Barber [10] has also described the same preparation process for extra-thin sections and their use in transmission electron microscopy. The protocol for petrographic preparation of samples is included as no modifications were made for the tooth samples in the current study.

This petrographic technique for preparation of teeth was also successfully used to measure the growth of the lower incisor in a Wistar rat model using tetracycline as a hard tissue marker. The use of low dose tetracycline as a histological hard tissue marker has been well established since the 1960's and allows clear evidence of continued hard tissue growth in experimental animals [11-17].

Materials and methods

For the purpose of this research project, six male Wistar rats (250-300gm) were held in a 12 hour light-dark cycle environment at 22ºC+2ºC. A veterinarian-approved tetracycline was administered to all rats as a marker of tooth growth. Alamycin (oxytetracycline 200mg/ml, Diluted 1 in 10 to give 0.25ml/rat, 20mg/kg body wt.)–0.2ml per rat (5mg/rat/day for 3 days), was given, as an Intraperitoneal dose (IP), one dose a day, over 3 days, every 4 weeks, beginning on Day 1, with all animals being sacrificed at Day 231 (33 weeks), using an overdose of Sodium Pentothal.

The two lower central incisor teeth from each animal were removed and placed in formalin and prepared using the following procedure:

1. The teeth were dried overnight in an oven at 40ºC.

2. Each tooth was set in a plastic block using Epo-Tech 301 resin and allowed to set overnight at 40ºC. The Epo-Tech 301 is a two component epoxy adhesive with a very low viscosity and is spectrally transparent. It is primarily designed for optical filters but has many instrument and geological uses as it adheres to many different types of substrates such as glass, quartz, met-

als, most plastics and plastic fibre optics. A similar technique of using methylmethacrylate to embed bone samples has been reported by Erben [18].

3. Each tooth was then ground down to half its longitudinal thickness using 600 grit Carborundum.

4. Each remaining half tooth was then glued to a glass slide using Epo-Tech 301 resin.

5. Each half tooth, glued to the glass slide, was then further reduced in thickness using a diamond bladed rock saw.

6. The tooth slices were then ground down to approximately 40 μm thickness using 600 grit Carborundum.

7. The tooth slices were then polished on 3 grades of polishing cloths (KPM, MSF, +MRE) (KPM, MSF and MRE are the manufacturing companies designations for the polishing cloth types), using 3 grades of diamond paste (6.1+0.25 μm diamond paste). (The KPM cloth is a sprayed on hard flock cloth used with 6 μm diamond paste for 10 minutes, the MSF is a fine woven silk cloth used with 1 μm diamond paste for 20 minutes, and the MRE is a soft velvet type of cloth used with 0.25 μm diamond paste for 5 minutes to finish. All stages also use Kemet brand Type W water based lubricant, which contains glycol).

8. The polishing removes approximately 10 μm leaving the tooth slices 30 μm thick and glued to the glass slides.

Microscopy analysis

1. Magnification of teeth–Images of the teeth were taken with an x2.5 objective. To that is added x10 Magnification (built in the microscope). Then x0.7 adaptor was used with the Nikon Camera. Therefore the magnification of all the teeth images are-2.5x10x0.7=17.5 (Magnification).

2. The mounted tooth sections were viewed at 17.5 magnification using fluorescence microscopy with a narrow band, blue-light filter block (450-490 nanometres [nm]). The fluorescent labelled slides were examined and photographed, using a Nikon Ti-E inverted motorised microscope with Nikon A1Si spectral detector confocal system and transferred to electronic format for analysis, running NIS- C Elements software.

3. The digital images were examined and the following measurements made:

i) The distance between tetracycline growth bands, to determine the growth in 25 days; the time between tetracycline dosages. This measured the growth of tooth structure when the rat was not receiving tetracycline.

ii) The width of the each band was also measured to determine the growth over 3 days when the rat was being given the tetracycline.

Results

The histological tooth sections obtained with this petrographic preparation technique allowed detailed analysis of teeth with all calcified and non-decalcified tissues present in one section. In addition, tooth growth was clearly shown and was able to be measured accurately with tetracycline used as marker of hard tissue growth. Only longitudinal sections were used as a prime requisite of this experiment was to review longitudinal tooth

growth, not the width of the teeth. The photos were taken at a 17.5X magnification. **Figure 1** shows an example of the tetracycline staining of a typical tooth. The photo clearly shows all the internal crystal structures of the tooth and the fluorescent band of tetracycline, indicated by the arrow.

Quantification of tooth growth

In order to quantify the growth of the teeth using the tetracycline banding and to ascertain whether there is any difference in growth rate in the presence of tetracycline, two measurements were taken:

The width of the tetracycline band: This demonstrates the growth of the tooth over the 3 days that the animal was receiving the tetracycline and is shown in the photograph in **Figure 2** and found to be a mean of 0.010 mm/day.

The distance between the tetracycline bands: This demonstrates the growth rate over the 25 days that the animals were not receiving tetracycline and is shown in the photograph in **Figure 3** and found to be a mean of 0.016 mm/day.

Figure 2. The width of the tetracycline band demonstrates the growth rate over the 3 days that the tetracycline was administered to the rats. (Magx100).

Figure 1. Arrow indicates tetracycline staining of a tooth. (Magx17.5).

Figure 3. The distance between the bands demonstrates the growth rate over the 25 days that the animals were not receiving tetracycline. (Magx17.5).
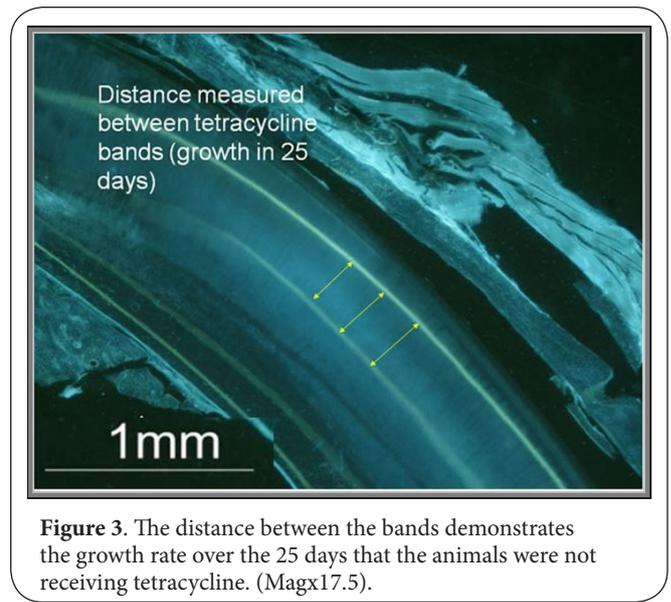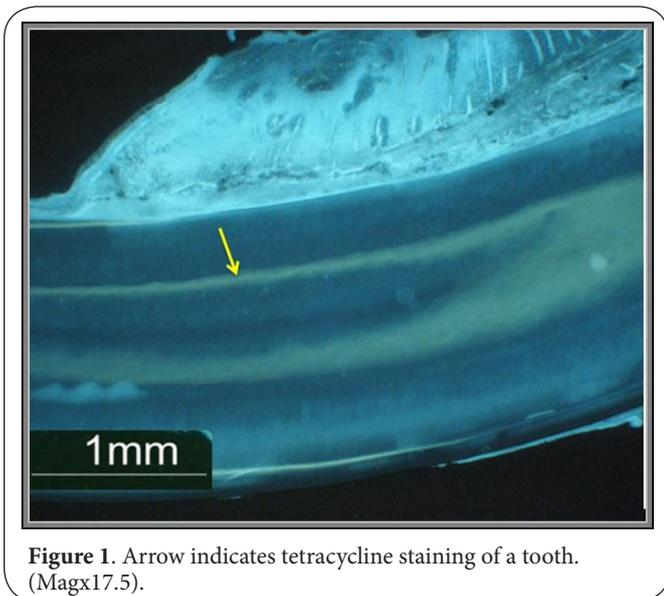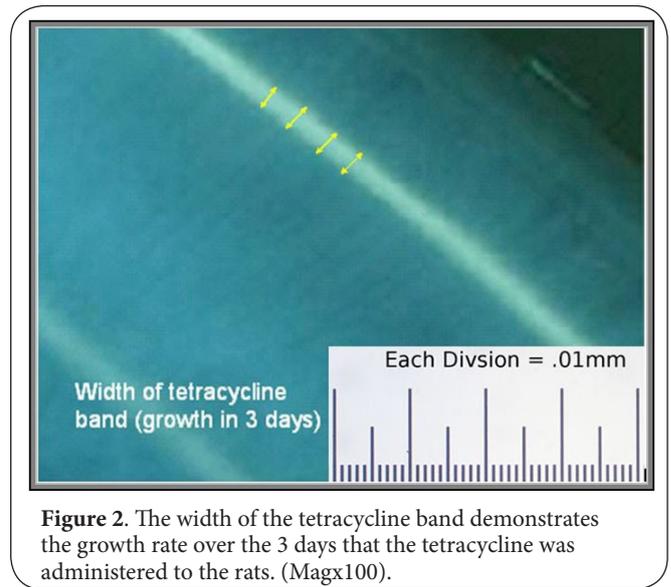
Discussion

The main aim of this is study is to introduce a petrographic technique for histological preparation of teeth which includes all dental tissues so that thin sections of teeth can be viewed under the microscope without decalcification. In addition the use of tetracycline to measure tooth growth in rats was demonstrated using fluorescence microscopy.

Traditional techniques for preparation of teeth for histological analysis include Ground Section, Decalcification and Cryomicrotomy. These techniques provide useful information for researchers but have the disadvantage of not being able to histologically examine both hard and soft tissue structures at the same time.

The ground sectioning of a non-decalcified tooth and then being viewed by polarized light has been traditionally used in the analysis of the crystal structure of teeth. However, sections are very thick and preclude the use of transillumination to analyse tooth crystal structure [1-3]. In dried ground sections of dentine the odontoblastic processes disintegrate and the empty tubules are filled with air appearing black in transmitted light and white in reflected light [19]. Again, in dry sections, the structural elements may be lost and spaces result giving the black coloured structures in transmitted light, and these are like, Tome's granular layer, interglobular dentine and dead tracts [20]. Tome's layer can on only be discerned in thick ground sections as a result of a superimposition phenomenon and the ability to focus within

the depth of the sections of root dentine and middle layers of coronal dentin [21-23].

With traditional decalcification techniques, the enamel, being over 90% mineral in composition, is lost after preparation. Dentine consists of 35% organic matter and water and 65% inorganic material, so again much of the basic tooth tissue is lost with either decalcification. Decalcification techniques use a variety of acid chemicals to demineralise the tooth structure and this often takes more than a week to undertake. Depending on the type of acid used, mild to severe deterioration of the tooth structure will occur limiting examination and staining of the dental tissues [24]. The paper by Keklikoglu and Akinci [24] from 2013 reviewed three different techniques of histological tooth preparation and compared the histological staining differences in the preparations from decalcified and undecalcified tooth roots by three different embedding materials and techniques. They concluded that the best histological detail was obtained from the decalcified, paraffin-embedded sections. Here again all calcified material would be lost from the specimen with resulting reduced histological information.

The Cryomicrotomy technique, using a heavy-duty freezing microtome, is useful for examination of both hard and soft tissue structures. Both cellular and extracellular structure are well preserved and the sections of tooth and bone appeared to be suitable for optical and scanning electron microscopy and for immunohistochemical analysis, However, it has the disadvantage of there being an overall strong non-specific binding of immunohistochemical reagents to enamel [6].

The use of the petrographic preparation technique by investing a non-decalcified tooth in an Epo-Tech 301 resin and grinding it down to 30 μm thick slices and then using fluorescence microscopy was found to be a most useful method for tooth analysis of both hard and soft tissues and should be commended to researchers. The petrographic preparation techniques are also particularly useful for histologic sections and microradiography of tissues containing manmade implants (metallic, ceramic, polymer, and composite) which cannot be sectioned with a microtome. In this study this technique provided very clear histological sections for analysis of all dental tissues in one section and allows for a variety of tissue stains.

In addition the use of tetracycline to measure tooth growth in rats was demonstrated using fluorescence microscopy. The use of low dose tetracycline as a histological hard tissue marker has been well established and allows clear evidence of continued hard tissue growth in experimental animals [11-17].

The tetracycline is taken up by growing teeth and can be seen histologically as bands of growth, like growth rings in a tree section. Tetracycline is available, as a Veterinary Approved Drug (Alamycin), in vials of 200mg/ml for Intravenous/Intramuscular/Intraperitoneal use in Australia and was used in this study. Oral tetracycline is very poorly absorbed (only 2% being absorbed) and dose depends on the amount of water an animal is drinking and therefore was not used in this study.

The dosage of 20mg/kg of tetracycline for animal use has

been reported by Frost [15] and was most effectively given as an IP dose as correct dosage could be assured and there was minimal effect on the animal's intestinal flora.

Tetracyclines were introduced in 1948 as broad-spectrum antibiotics that may be used in the treatment of common infections in children and adults [25]. The use of low dose tetracycline as a histological bone marker has been well established since the 1960s with papers by Bevelander et al., [12], Ibsen and Urist [13], Faccini [14], Frost [15], and more recently by Ranggard [11], Engstrom [16], Sun et al., [17]. It was discovered that tetracycline is incorporated into hard tissues calcifying at the time of their administration and subsequently could be studied in undecalcified sections by fluorescence microscopy [12,14]. When ultraviolet (UV) light (450-490 nanometres [nm]) is applied to tetracycline-stained hard tissues, it exhibits a yellow Fuchsin Fluorescence. Hence tetracycline compounds are utilized in hard tissue research as a vital fluorescent dye for measuring the rate of hard tissue formation [11-16]. The tetracycline tends to remain incorporated into the newly formed hard tissue areas for some time after systemic administration [26]. The dosage of 20mg/kg of tetracycline for animal use, reported by Frost [5], was successfully used in this study to demonstrate growth in the rat incisor.

The biologically active tissue in continuous tooth growth in rodents is dentine with enamel being acellular. Therefore, most studies on rat tooth growth relate to dentine. A review of the literature reveals that the mean eruption rate of the rat mandibular incisor has been measured by many authors [27-31], using a number of techniques with a range of results (0.015 mm/day [27] to 1.0 mm/day [28]). The mean unimpeded eruption rate of the rat mandibular incisor, measured by Law et al., [28], using an image analysis technique, is reported as 1.0±0.1 mm/day. Harari et al., [31] reported a mean growth of 0.542±49 mm/day in young rats compared to 0.443±25 mm/day in mature rats. Chiba and Yamaguchi [32] reported the average eruption rates of the rat incisor to be between 0.406 and 0.516 mm/day. Fatani and Raja [27], using radioautographs, measured the thickness of dentine laid down per day as 0.015 mm. It is clear that the growth rate of teeth is effected by many factors, including age of animal [28,31], the physical properties of the diet [33], and medications [11]. In this study the mean growth of teeth was 0.010 mm/day during the period of tetracycline administration and 0.016 mm/day when no tetracycline was being administered. This compares with the studies by Fatani and Raja [8] who reported a rate of 0.015 mm/day, using radioautographs. The growth rate of the teeth in this study was also seen to be less in the presence of tetracycline which compares with Ranggard [11] who reported that even a single low dose of tetracycline (2 mg oxytetracycline per 100 g) caused a disturbance in normal amelogenesis in the rat incisor. The question then is "How does this affect the interpretation of the results when tetracycline is used as a histological marker for other studies?" As there is a significant difference between tooth growth of 0.010 mm/day during the period of tetracycline administration and 0.016 mm/

day when no tetracycline is being administered, this consequence needs to be taken into consideration when interpreting the results of other studies when tetracycline is used as a histological marker of tooth growth.

Conclusion

Within the limitations of this study, the use of the petrographic preparation technique by investing a non-decalcified tooth in an Epo-Tech 301 resin and grinding it down to 30 μm thick slices and then using fluorescence microscopy provided excellent results for tooth analysis as can be seen in **Figures 1-3**. This technique preserves all dental tissues for histological analysis in one viewing field without the chemical alterations of traditional techniques. The petrographic preparation techniques are also particularly useful for histologic sections and microradiography of tissues containing manmade implants (metallic, ceramic, polymer, and composite) which cannot be sectioned with a microtome. This technique should be commended to researchers with further studies needed to assess its full application in the area of dental research.

This study further commends the use of tetracycline as a clinical marker of growth in hard tissue research. However, this study also showed that the presence of tetracycline caused a reduction in tooth growth which was consistent with the findings of Ranggard [11] and Fatani & Raja [27]. This finding needs to be taken into consideration when interpreting the results of other studies when tetracycline is used as a histological marker of tooth growth.

Competing interests

The author declares that he has no competing interests.

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