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From the histological studies of the Vienna school to today what has changed ?: new proposals for histological processing

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Table of contents

Abstract

Dental histology was born in the 1500s but only at the beginning of the twentieth century, thanks to the members of the prestigious Vienna School, it has raised as true science in all its grandeur and completeness, casting the biological and histological foundations upon which the present dental histopathology giving life to Oral Biology. Thanks to these studies, the various branches of modern dental medicine could be developed such as: periodontology, endodontics, pediatric dentistry and orthodontics. Afterwards, the innovative contributions of this prestigious and pioneering research group, documented and divulged through their articles and books, histological methods and histological knowledge have drastically brake until they are diluted, making it difficult to find good-quality manuals or texts regarding the tooth processing and its supporting tissues.

In this thesis, divided into two parts, we wanted to retrieve the early century's notion of manipulation of histological samples and compare them with a new procedure that sees in microwave utilization a solution for preparing calcified tissue samples while maintaining excellent results both in terms of tissue structure andin the amount of time it takes for this procedure. For this purpose, teeth of bovine origin have been processed because of the ease of finding, chemical composition similar to that of human teeth and the possibility to analyze not only calcified tissues but also large portions of soft tissues.

The second aim of this thesis was to carry out a critical analysis of bacterial staining and how these can be modified to make them more usable, to propose PAS coloration as an adjuvant in dental bacterial topography and how a wise modification of the famous hematoxylin - eosin make this staining much more informative. To obtain the results for the second part of the thesis, series sections were performed from several post extracting teeth with bacterial infections of different typology and severity.

Introduction: when teeth histology begins.

The first dissertation on the biology and pathology of teeth can be tracked down back to the sixteenth century, more precisely in 1578 with the work made by Dr. Petro Monau (Bruziewicz -Mikłaszewska B. 2003) But we have to wait until the eighteenth century to have more accurate studies. Studies were done by John Hunter (Hunter J. 1771), that contributed significantly to the knowledge of dentine morphology. He started to feed pigs with an organic colored food, and in this way, he demonstrated that teeth weren't made of the same tissues as bone and he noted that enamel, dentin, and cementum were separated tissues with their vascularization. During this century there were not many studies on oral histology and many types of research were centered on various technique about teeth treatment like pulp removal and plugging technique (Faucherd P. a, Fauchard P. b).

Between the 1830 and 1906 begins the classical histological era (Cox C.F. et al. 2017). The most important researcher of this period was Professor Johannes E.K. von Purkinje (1787-1869)—founder of the famous Breslau School of Microscopic Anatomy & Histology in Prague Czechoslovakia. He invented a new type of microtome as well a new kind of achromatic microscope. In this "era" the first hand-drawn reproduction of the progressive stage of developing dental tissue was made (Frankel M. 1835, Raschkow J. 1835), as well as the demonstration that the central nerves projected as fine, branching fiber to the periphery of the pulp in the vital pulp. In 1865 the term " odontoblast" was formally introduced and Frankel and Raschow demonstrated that dentin was formed by odontoblast (Frankel M. 1835, Raschkow J. 1835). Sir John Tomes described the tubules that projected form the pulp dentine, the protective layer of secondary dentine, the presence of nerve processes in dentine tubules along other things (Tomes J. 1848). Although this period was filled with new insights and discoveries, the absence of any widely available printed report, makes difficult to recognize the individual who named a particular structure or anatomical component. At that

time most of the information was sent by letters exchanged between the single researchers.

In the first half of the twentieth century, six dentist-scientists contributed in an outstanding way to the development of a stronger researcher establishment and enlarged the scope of oral biology (Kremenak N.W. & Squier C.A. 1997).

Those men were: Bernhard Gottlieb, Balint Orban, Rudolf Kronfield, Albin Oppenheim, Harry Sicher and Joseph Weinmann. They were a group o scientists working at the University of Vienna, but due to the advent of Nazi Party, they were forced to leave Austria and move to the USA after 1938 (Xianghoung L et al. 2007)

Bernhard Gottlieb.

Born in Poland in 1886, he received his medical degree in 1912 at the University of Vienna. He organized his laboratory after the Great War. His works characterized many undescribed microscopic features of oral tissue: the attachment of gingival epithelium to the tooth, the continuous eruption of dentition and a detailed description of the cementum. Much of the biological basis for the evolution of endodontics belong to Gottlieb. He studied the tooth resorption and tissue responses, issues surrounding dental caries and vital pulp therapy, the importance of asepsis during root canal procedure,use of a suction technique to clean root canal, impregnation of dentin with bacteriocidal agents, tissue changes in pyorrhea, structural changes accompanying severe periodontal disease (Gottlieb B. 1921, 1926a, 1926b, 1927, 1939). Gottlieb was more prolific while he worked in Vienna: at that time he made his most significant research, in the United States he settled at Baylor College of Dentistry in Dallas but he struggled to establish a research

program due to limited financial resources, colleagues with limited or no research background and cut off from the main-stream of dental research. In the USA he continued his studies on dental caries: a central issue in dental research (Gottlieb B. 1944, 1947a, 1947b)

Balint Orban

Born in Hungary in the town of Temesvar in 1899, he completed his medical school in 1922 and then joined Gottlieb's staff. He started by continuing Gottlieb works like a study on the effect of nutrition on teeth (Orban B. 1926a) and studies on the structural development of tooth enamel (Orban B. 1926b). He spent two years at Loyola University Dental School of Chicago where was charged to organize a research methodology course. At the end of this period, Orban had given thirty lecture all over the country (Orban B. 1929) and publish "Dental histology and embryology."(Orban B. 1928) That was the precursor of another text: "Oral histology and embryology" (Orban B. 1944), this book is still published and now has come at its fourteenth editions and still contains many pictures taken by Orban himself. Orban returned in the USA in 1937 and continued his studies on the histopathology and surgical treatment of periodontal disease and developed his views on the structure, function, and classification of oral mucosa founding the basis for a nomenclature of the tissues that are used nowadays (Orban B. 1941, 1958). In 1946 he served as editor of a new scientific journal: "the Journal of Endodontia." Although the journal was active only for three issues, its brief life breached the endodontic as a new specialty, and in 1943 a new, and more enduring journal was founded: " The Journal of Endodontics."

Rudolf Kronfield

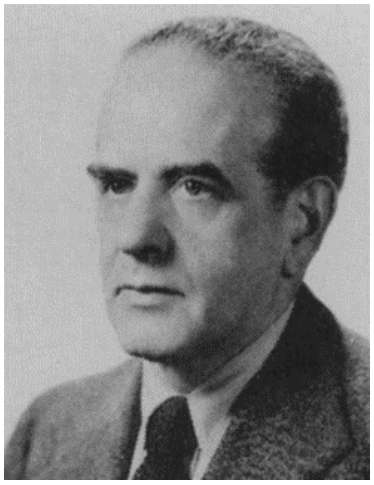
Born in Vienna on 1902, received his degree in 1926. Soon he became inclined toward research and in 1929 took the place of Orban at the Loyola University. He quickly established himself as a participating member of major dental organizations like Chicago Dental Society and the International Association for Dental Research (IADR). In 1933 he published " Histopathology of the teeth and their surrounding structures."(Kronfield R. 1933), thanks to its abundant histological pictures and extensive bibliographies this text became a standard in the dental teaching. With William Logan, Kronfield published another important book:" Development of human jaws and surrounding structures from birth to the age of 15 years" (Logan W., Kronfield R., 1933). A fundamental work entitles:"Research and the future of dentistry" (Kronfield R., 1939) underlying the fundamental connection between the technique of restorative dentistry and the biological foundation on which this technique rests. Kornfield used to say:" Biological research has laid the foundation for correct diagnosis and the proper treatment of dental and oral disease." Kornfield thought that No matter how enthusiastically a treatment method might be endorsed by clinician or manufacturers unless that treatment was based on the fundamentals of biological science it" will fail and soon forgotten."

Sadly in the winter of 1939-40, only one month before Kronfield was to assume the presidency of IADR, he was found dead in his laboratory. He was diagnosed with a serious neurological disease, finding unacceptable the perspective of a life of disability decide to put an end to his life "surrounded by his library, his histologic material and his working in progress for the coming season." (Coolidge 1940).

Albin Oppenheim

Born in Brno in 1875, received his medical degree in 1899 at Ferdinand's University of Prague. Started working as a dentist in Burno from 1905 to 1909 then became an assistant professor. In 1915 he became head of orthodontics Department of Dental Institute of University of Vienna. Until 1935 he made many trips to the USA then after 1936, due to the political situation in Austria, he settles at the University of Southern California in Los Angeles. He was a man dedicated to research; he conducted several experiments on monkeys, dog, and human beings.Researchers from all over the world send him specimens to be analyzed. Thanks to his extensive work on the monkey (Oppenheim A. 1934, 1942), he proposed a new approach to orthodontics procedure: he finds that apply too much mechanical energy in the movement of teeth cause damage to the surrounding structure, for this reason, he proposed new ways of treatment that became wildly accepted.His work leads to the publication of "Tissue Changes Incident to Tooth Movement."(Oppenheim A. 1944), this dissertation is considered one of the all-time contribution to the specialty of orthodontics.

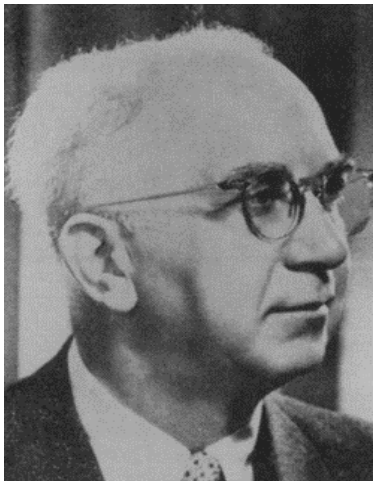
Harry Sicher



Born in Vienna in 1889, Sicher was the eldest member of the Viennese group. He was an associate professor of neuroanatomy for three years at the Chicago Medical School then he joined the faculty of the College of Dental Surgery at Loyola University as associate professor of anatomy and histology. With Weinmann published the text book:" Bone and bones: Fundamental of bone biology"(Weinman

and Sicher. 1947,1955) and the first edition of “Oral Anatomy.” (Oral Anatomy 1949). This text was considered the bridge between theory and practice in dentistry demonstrating that anatomic understanding facilitates clinical work. As well as text book he published eighty paper in oral anatomy and histology. Sicher’s work founded the cornerstone for the teaching of oral anatomy in the dental curriculum for decades.

Joseph Peter Weinmann



Weinmann born in Bohemia in 1896, joined Gottlieb as a research associate in his laboratory in Vienna immediately after gaining his medical degree. In 1938 he moved to the USA and spents one year at the College of Dentistry at the University of Illinois and another year at the Columbia University. Then joined the Dental School at Loyola University. In 1946 he joined the University of Illinois Departement of Histology as associate professor and in 1949 became Professor and Head of the Division of Oral Pathology. Weinmann was a prolific researcher and published more than 160 papers on physiology, amelogenesis, normal and pathologic oral epithelium and periodontal disease. His greater achievement was the creation of a department at the University of Illinois that for twenty years trained many of those who would lead academic dentistry in the USA and elsewhere.

The aim of this thesis

This thesis has the aim to make a critical analysis of the entire process of teeth, from fixation to cutting as well as a critical analysis of some staining technique. In addition to this, some modification will be proposed to enhance current techniques to achieve a better result and simplify different processes.

For what concerns the processing of the sample, in this thesis it will be made a comparison between a classical method of decalcification and a new way to enhance decalcification through EDTA. It will be proposed an integration in the embedding procedure starting from the one proposed by Morse A (Morse A. 1945). and Silva GBA(Silva GBA et al. 2013).

In the second part, concerning staining procedures, it will be proposed a variation of the Brown-Brenn staining method with less steps, the use of PAS staining as method to detect bacteria especially in dentine tubules, different aspects about hematoxylin and eosin staining with an analysis about which kind of staining to use alongside modifications and some consideration on Mallory's trichrome.

There is a substantial lack of available protocol specifically regarding tooth histology, in recent literature. The most recent protocol that can be found on PubMed is the one made by Silva G.A.B (Silva GBA et al., 2013). Even if it is a rather good protocol and can be used to achieve a satisfactory result, has some limitations: the thickness of the section proposed is 6 µm, and requires consecutive reduction of the sample during the decalcification process to obtain a good decalcification in an acceptable amount of time.

To find other protocols it is necessary to retrieve old textbooks, written by Orban or Kronfield (Orban B. 1944, Kronfield R. 1933) or to search in the literature of the first half of the twentieth century: Anna Morse (Morse A., 1945) wrote a pretty good protocol. Another good text is "Manuale di Tecnica histologica dentale" by Carlo Zerosi (Zerosi C. 1986), last time published in 1986 and now only available in some public library. Orban's textbook is still published (Kumar G.S., 2015) but in its last edition, only very few pages are dedicated to the histology techniques.

All these aspects pose two problems: first a lack of improvement in histology technique of sample processing and second the risk to lose all the knowledge that the pioneers of tooth histology gathered in their studies.

After the discussion, a case report will be described in which these techniques were applied.

Part I: samples handling

Decalcification: the root cause of teeth histology problems

Teeth are calcified structure, with a high content of mineral components, higher than bone. For this reason, there are two main ways to obtain histological slides.

Ground sections

The simplest and quickest way is to cut the undecalcified sample to obtain the so-called ground sections: samples are embedded in a resin, mostly epoxy resins or metametilacrylate. Then, sections are obtained with a dedicated bone saw and thinned with glass paper. This technique is quite simple but is very time consuming, in fact to obtain a single good quality slide many hours are needed, and the number of sections obtainable is low due to the thickness of the saw, that cuts away a large part of the tooth substance, and the thinning process, that also removes part of tooth in order to achieve the desired thinness, in order to examine the slice under the microscope. Ground sections can give information about the mineralized structure of the tooth but little o no information about soft tissue. In the images below are displayed some ground sections of a third molar, extracted for orthodontics procedure.

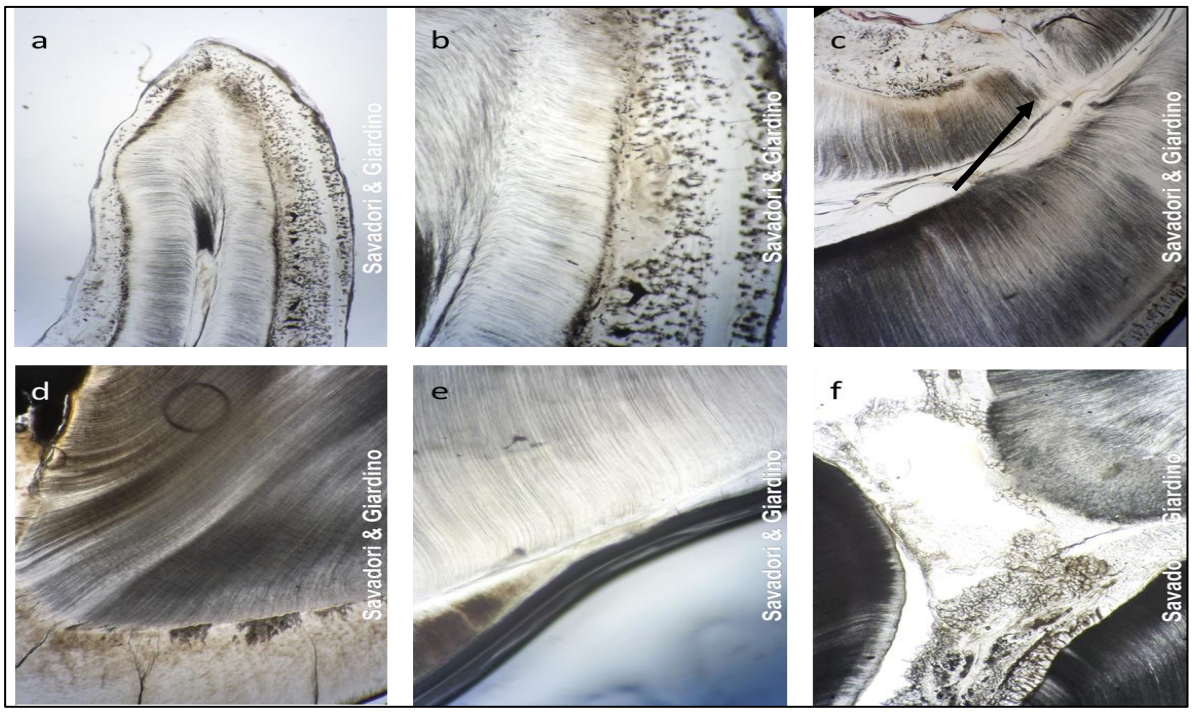


Fig. 1: Ground sections of a third molar. a) molar apex 100x; b) dentin tubules and cementum filled with odontoblasts 200x c) root canal with an accessory canal (arrow) 100x; d) enamel 200x; e) enamel dentin junction 200x; f) pulp chamber: pulp tissue is damaged by grounding procedure. 200x

Decalcification

To study all the structures of the tooth and especially the soft tissue, the sample must undergo a decalcification process. To get rid of the mineralized matrix of teeth, there are different strategies using different chemical substances; there are two types of chemical used: acid and chelating agents.

After performing both of these procedures, the enamel is almost totally lost due to its high concentration of minerals.

Regardless of the decalcifying agent used, the sample must be immersed in a volume equal to 100 times its weight (g/ml), kept in suspension and constant agitation. The solutions must be changed at least every 2 days.

Acids decalcifying agents

Acids dissolve hydroxyapatite producing carbon dioxide; they can be divided into two broad categories: strong inorganic acid and weak organic acid. The main difference is the speed of action and the possible damage to the soft tissue.

Inorganic acids

To this category belongs substance like chlorhydric acid and nitric acid. They present a fast action and can achieve a complete decalcification in few days.

Nitric acid is frequently used at a concentration of 5% or 10%; in some cases, a solution of formalin 10% can be added to continue the fixation of soft tissue.

Using a solution at 5% of nitric acid can a complete decalcification can be achieved in 24-48 hours; instead, a solution at 10% can take 16-24 hours, and a solution of 10% nitric acid plus 10% formalin can take up to 71 hours (Hemal J. et al. 2014).

If, on one hand, these solutions have the advantage of a rather fast action, on the other hand they can cause severe problems of over decalcification and lead to poor staining. To avoid this issue, a careful check must be made.For checking technique see the chapter :” decalcification endpooint”.

Organic acids

To this category belongs substance like formic acids, trichloroacetic acids, citric acid. Their pH is higher than mineral acid, work slower, but there are fewer chances to cause damage to tissue, and the specimen can be left in the decalcifying solution some days after the complete decalcification without damaging the soft tissues.

They can be used alone, as formic acid at a concentration of 5-10%, or, more frequently, they are used in combination with other substances.

Anna Morse's solution (Morse A. 1945), a solution composed of formic acid 22.5% and sodium citrate 10%, can achieve a complete decalcification of a human tooth in a 3-4 week with optimal results. For this reason, this solution has been chosen for running all the staining experiments in the present study.

Chelating decalcifying agents

They are a noncorrosive substance that can bind to metallic ions. For this reason, they don't cause damage to soft tissues but take a very long amount of time to complete decalcification. Human teeth could take up to 60-90 day to achieve a total decalcification (Mattuella L.G.et al., 2007; Gupta S.et al., 2014). The chelating agent most used is Ethylenediaminetetraacetic acid or EDTA

EDTA

The EDTA is a chelating agent that comes in two main variety: disodium EDTA and tetrasodium EDTA salts. They work in the same way, but Tetrasodium EDTA can bind four calcium ions at the same time. Other differences are the pH at which they dissolve: Tetrasodium dissolves well and reach a pH of 12 while disodium dissolves with more difficulties and has a pH of 4. The pH value is very important: in fact, the pH value rules the ability of EDTA to binding calcium ions. A pH near 10 allows EDTA to work at best, displaying the highest chelating activity, while a pH value below 3 doesn't allow EDTA to works at all (Harris E.C., 2007). A standard EDTA solution is 14% at a pH of 7.2.

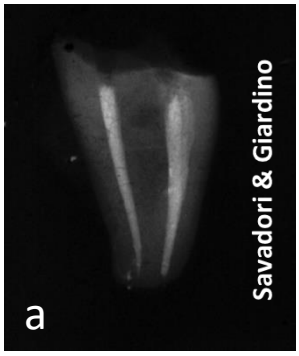
Decalcification endpoint

There are four methods to check the decalcification endpoint; this checkpoint is a crucial step because if the decalcification process is stopped too early a further decalcification is compromised, making cutting impossible. Conversely,, protracting the decalcification for too long time will likely cause damage to the soft tissues (Luna L.G. 1968).

Radiographic check

A radiographic check is considered the most accurate method for checking a complete decalcification: a sample without its mineral content will appear radio-translucent (Fig.2b) while mineral content will be radio-opaque (Fig.2a) and thus easily detectable.

However, this procedure has two possible issues, firstly not all histological laboratories have a radiographic equipment and secondly, an accurate setup of the exposure time and of the distance between the sample and the radiographic source must be done, in order to avoid an overexposure and consequently run the risk to have a false outcome. (Luna L.G. 1968)



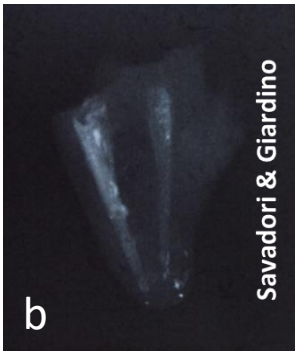


Fig. 2: a) tooth still in decalcification; b) tooth reached a complete decalcification

Chemical check

This method involves the detection of calcium in the decalcifying solution. Five ml of the decalcification solution is brought to pH 7 with ammonium hydroxide, then 5ml of saturated ammonium oxalate is added. If after 30 minutes the solution remains clear it is supposed that the calcification step is concluded. If the solution appears cloudy, this means that calcium hydroxide or calcium oxalate have formed, so it indicates that the decalcification solution is still working. This test cannot be done for a solution with an acid

concentration above 10% because such a high concentration of acid requires a larger amount of ammonium hydroxide and ammonium oxalate while the concentration of calcium in the solution is relatively small and therefore this can reduce the test sensitivity (Clayden E.C. 1952).

This method works well with small samples but is not much accurate because it is always difficult to detect a small quantity of calcium. Moreover, this technique isn't suitable as a chelating agent due to the fact that calcium ions are bound to the chelating agent and not dissolved in the solution. To use this procedure with EDTA first, the solution must be acidified, to release calcium from EDTA, and then ammonium oxalate must be added. One has to keep in mind that when acidifying an EDTA solution, EDTA tends to precipitate and this could make the solution to become cloudy, which and interferes with assay outcome (Callis GM et al. 2008).

Physical check

Physical check is a simple and rapid test to ensure the decalcification occurs in right way. When a sample has reached its complete decalcification, it becomes soft and bendable. Piercing or cutting the sample could give a rather precise idea of the level of mineral loss. This procedure may seem not sophisticated but it is rapid and does not need any particular equipment. When a relatively large sample can be bended without a huge effort this can be the ideal moment to trim it. In this way, besides preparing the sample for further process, it can be checked for decalcification status.

Weight loss

The loss of weight is another "physical" test. This procedure is based on the assumption that during decalcification process the mineral content of the sample diminishes and this produces a loss of weight. The speed of this loss follows an asymptotic curve: more mass is lost at the beginning, while the weight loss rate gets lower with time, until no more mass is lost indicating the complete decalcification. This procedure is not precise but in combination with another method, described subsequently, it could provide a better tracking of decalcification conditions (Verdenius H.H.W. et al. 1957).

Paraffin embedding

After the decalcification process, the samples must be washed carefully for at least 12h-24h in running tap water, this is needed to eliminate all traces of acid, that could interfere with subsequent staining, or prevent EDTA to precipitate during dehydration.

After washing phase, the sample must be dehydrated, clarified with xylene and then infiltrated with paraffin.

Is very important to remove all water content from the specimens. For this reason, successive baths in ethanol with increasing concentration are needed. Ethanol concentration starts from 50% to absolute (100%) alcohol. For each passage, the sample must remain submerged for at least 2-3 hours, though there isn't a maximum immersion time. After dehydration, the sample is placed in xylene and then in paraffin for several hours. (Zerosi 1986)

The last step is done in order to cast the sample in an appropriate mold.

Paraffin embedding procedure used in this thesis

The paraffin embedding procedure used in this thesis work was at first a combination of Anna Morse protocol and Silva's protocol (Morse A. 1945, Silva GBA et al. 2013): samples were put in increasing alcohol concentration for several hours and then submerged in paraffin as indicated in Silva's protocol, but for an extended period. The first protocol follows this timeline:

Ethanol 50% for 24h (In this solution the sample could remain for several days)

Ethanol 70% for 24h

Ethanol 80% for 12h

Ethanol 90% for 24h

Ethanol 100% for 12h

Ethanol 100% for 12h

Ethanol 100% xylene 50:50 for 2-3h

Xylene for 3h

Xylene for 3h

Xylene paraffin 50:50 for 2h

Paraffin for 12h

Paraffin for 4-5h

This protocol produced a good result, but in some cases, an incomplete infiltration occurred, despite the long time spent in a paraffin bath. For this reason, an additional step was introduced: before the bath in xylene and paraffin 50:50, a bath in xylene paraffin 80:20 has been introduced. This modification has proven to enhance the infiltration, hence improving the cuttability of the sample

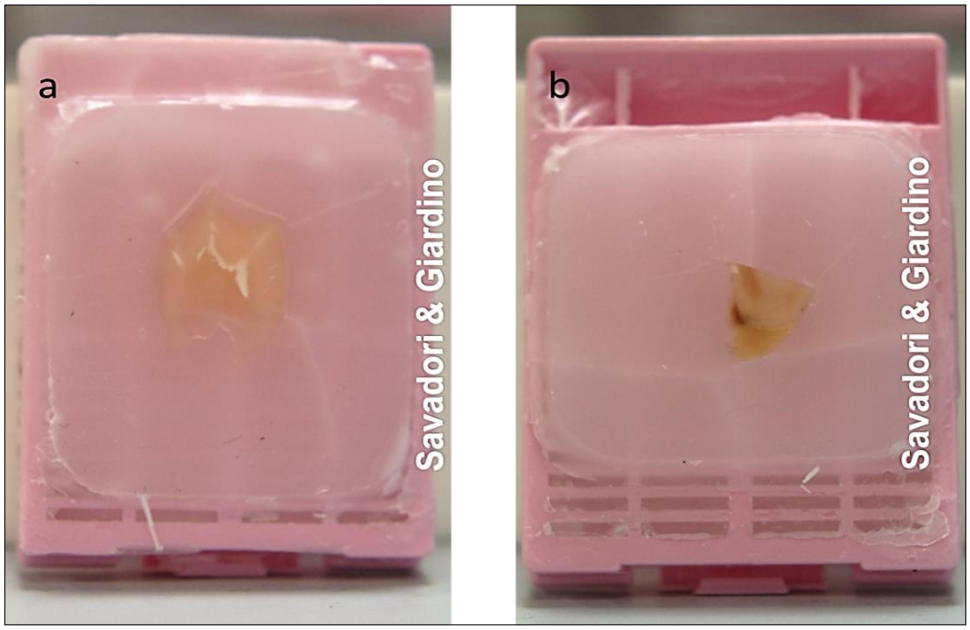


Fig.3: a) Sample embedded with only xylene-paraffin 50:50, the sample has some white spot, especially in the pulp region, showing that an incomplete infiltration has occurred; b) sample embedded with the two xylene-paraffin dilutions: the sample appears uniformly infiltrated leading to a better cuttability

After the passage in xylene it is useful to check the sample to see if the decalcification process is ended correctly: xylene makes the sample

translucent, but if some mineral deposits are still present they will appear as white spots. If these spots are present it is still possible to rehydrate the sample and continue with decalcification; after paraffin embedding you could go back again to decalcification but the sample could be compromised, it will be probably lost or will give poor results.

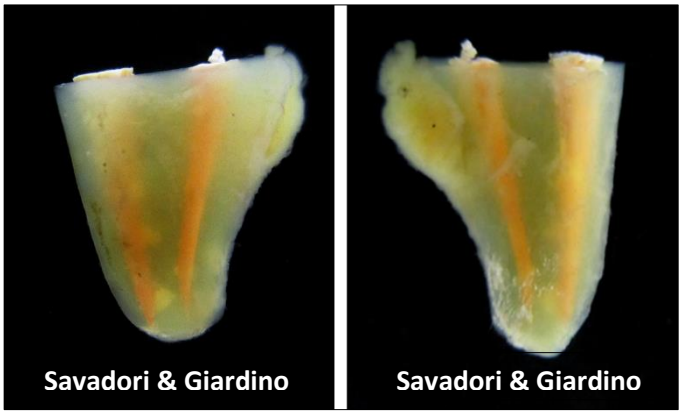


Fig. 4: Tooth clarified with xylene: decalcified tissue appears translucent and the roots are visible

Cutting the sample could be tricky even if the decalcification ended perfectly and the paraffin embedding is done correctly.

Teeth are very dense and compact, for this reason, they represent quite a challenge to the blade. The choice of the blade is certainly one of the most crucial aspects: standard blade like Leica 819 or Feather S35 aren't a good choice, they aren't fit to cut hard tissue, and even if it is possible to cut few ribbons, this kind of blade becomes dull in a very short amount of time.

Blades like Feather R35 work really well but, probably, the best blades fit for this job are N35 or N35HR. This kind of blades are designed to cut hard tissue and can resist for several ribbons. Due to the hardness of teeth, blades don't last for long but for the same reason, the blade's trim time is shortened.

The paraffin block must be cold: the sample is rather hard and compact and to ensure a strong support the paraffin must remain cold to enhance its resistance to compression. Ribbons must not exceed 4-5 slices, mainly for two reasons: first, the friction of the blade warms the paraffin block; second,

the teeth are relatively heavy and a long ribbon can break when translating to the water bath.

Water bath must have a temperature of 45-50°C and the ribbon must allow staying for some minutes. There is not a maximum time of staying: if the ribbon has many wrinkles a longer time could properly stretch the slices

Fig.5: Some models of blades used in this thesis

Glass slides

Untreated glass slides aren't fit for collecting paraffine slice: they will surely detach during staining, probably just after deparaffinization. Commercially available polylysine glass slides are quite good and relatively inexpensive, but they aren’t infallible. Super Frost slides are very good in holding the slice but are extremely expensive.

Alternatively, the slide may be coated with a homemade chromium gelatin. The reagents required are inexpensive, but the procedure is time-consuming. In this thesis commercial polylysine glass and glass coated with Fol’s chromium gelatin was used.

Fol's chromium gelatin

Chromium can form up to six covalent bonds, usually chromium bonds to oxygen or nitrogen. For this reason, on the surface of the glass, a matrix of chromium-gelatin is formed leaving many possible sites of attachment for the carboxy groups of proteins.

Fig. 6: Chemical structure of chromium gelatin on Glass slide (Kiernan, J A., 1999)

The Fol's gelatin is prepared by mixing 4 grams of gelatin in 250ml of ethanol 70% acidified with 20ml of glacial acetic acid. When gelatin is completely dissolved, 4-8ml of 5% chrome potassium sulphate aqueous solution is added. Some crystal of thymol could be used to prolonge the shelf life of glass slide. Then clean and dry glass slide can be submerged in the solution for 30-60 seconds with gentle agitation. It's extremely important to drain excess gelatin until a uniform film is created: an excess of gelatin will absorb colorants leading to poor result during staining procedure. Glass can be air or oven dried. This chrome coated slides have proven to be extremely effective, and no section was lost using them.

Comparison between two decalcification method: organic acid and EDTA in microwave oven

In this thesis work a big role has been played by an investigation about decalcification procedures. Two methods were compared: decalcification made by Anna Morse solution (AM) and decalcification made by EDTA in a microwave oven. A sample was also decalcified with nitric acid, with the sole purpose to show how harmful a strong acid can be.

AM is a 1:1 mixture of formic acid 22,5% and sodium citrate 10%; this is a buffered organic acid solution with a pH around 2-2,5. Usually, according to the size of the sample, three to four weeks are needed to achieve a complete decalcification of a human tooth, and the sample seems to be not damaged even if the decalcification endpoint is exceeded for some days. Samples decalcified with this solution present a good stainability and cuttability.

We have to remember that AM is an acid solution, hence tissue damage is possible due the corrosive nature of formic acid.

It is known that a treatment with EDTA is the best way to remove calcium from specimens, minimizing the possibility of altering the samples. But the action of EDTA takes an extremely long time. A method for speeding up the process is to raise the temperature: acid and EDTA work faster with an increasing temperature, but this also increases the risk of damaging the tissues.

A simple and interesting way to accelerate the decalcifying process is the use of microwave radiations. Microwave radiations interact with polar molecules, like water or any other molecules with a polar group. increasing their kinetic power. If polar molecules are embedded in a crystalline structure, the increment of kinetic energy causes a destabilization of the structure. This aspect helps the activity of EDTA in binding calcium molecules because their chemical bonds are weakened by microwave radiation. The histological use of laboratory microwave devices was proposed by Mayers (Mayers C.P. 1970) for tissue fixation, while Madden and Hensen (Madden V.J. et al. 1997) published the first scientific paper detailing the use of microwave-assisted decalcification of temporal bones with preservation of the ultrastructure. There are some publications dealing about the application of this method on teeth (Vongsavan N. et al. 1990, Sangeetha et al. 2013, Raj A.T. et al. 2016) but they have the common issue to propose very poor quality histological samples, so it's impossible to state if this method preserves the structure and the stainability of soft tissues of the teeth. Another problem is

that in those studies expensive microwave ovens or commercial ones were used but with the need to be hand-operated, reducing the hours of usage.

In the following chapter it will be proposed a protocol using a microwave oven and EDTA (EDTA-MW), with a solution to automatize the duty cycle. An extensive histological comparison between AM solution and EDTA-MW has been made to demonstrate the feasibility and the effectiveness of the technique. Furthermore, one sample was decalcified with nitric acid with the purpose to assess and show how strong mineral acids could damage tissue structure and why they should be avoided whenever possible.

Material and method of decalcification techniques' comparison

Samples and solutions used

A total of eight slices of cow molar teeth with periodontal tissue still attached, one cow incisor and two mice' full head were processed. The cow teeth were procured during an autopsy made to investigate the sudden death of the animal; the mice heads came from carcasses of laboratory animals destined to disposal.

The decision to chose cow teeth was dictated by the fact that their mineral composition is quite similar to the human teeth composition in comparison to other animals like pig or sheep (de Dios Teruel J. et al. 2015) . Heads of mice were used to test the decalcification effect of AM and EDTA-MW on other tissues like the nervous tissue: the fragile structure of the brain can be used to measure the aggressiveness of the two decalcification procedure.

The cow teeth sample was divided into the following group:

Group 1: two cow molars decalcified via AM

Group 2: two cow molars decalcified via EDTA-MW, EDTA 14% pH 10

Group 3: one cow molar decalcified via EDTA-MW, EDTA 20% pH 10

Group 4: one cow molar decalcified via EDTA-MW, EDTA 14% pH 7.2

Group 5: one cow molar decalcified via EDTA-MW, EDTA 14% pH 10 for 60 days

Group 6: one cow molar decalcified via EDTA-MW, EDTA 14% pH 10 for 90 days

Group 7: one cow incisor decalcified via nitric acid 5%

Head 1: mouse head decalcified via AM

Head 2: mouse head decalcified via EDTA-MW, EDTA 14% pH 10

The EDTA used is tetrasodium hydrate, the choice was made due to the low cost of this kind of EDTA and its capacity to dissolve easily in high concentration. pH was set at 10 because at this value EDTA presents the highest activity; a comparison with a solution at pH 7.2 was made to check if the alkaline pH may induce some damage to the tissue. The concentration of 14% was used because this value is commonly used, and a comparison with a concentration of 20% was made to check if the decalcification speed is increased and if it affects tissue structure in any way.

Decalcification done via nitric acid was made with the sole purpose to show the damage that a strong mineral acid could induce to a tissue even if the process is closely monitored.

Decalcification procedure and microwave oven setup

Specimens decalcified via acid, were put in suspension in a plastic jar filled with solution in a proportion of 1:100. Constant agitation was applied. Specimens decalcified via EDTA-MW were put in suspension in a plastic jar like those decalcified via acid, but agitation wasn't possible. In both processes, the decalcification solution was changed every two days. The decalcification endpoint was physically checked.

For this aim a commercial oven was used, SILVERCREST Mikrowelle SMW 700 B1, set at a power of 700 watt, the samples were irradiated for 10 seconds every hour 24h/24h until complete decalcification. To find the proper spot where to place the samples, the microwave irradiation pattern was drawn with an array of mini neon lamps. Neon gas struck by enough microwave energy enters in an excited state and emits light, in this way cold spots (spots slightly hit by microwave) can be avoided.

Fig 7: a) model of microwave used for decalcification procedure; b) Arduino board and relay; c) neon lamps array for the detection of cold/hot spots

The duty cycle was regulated by a relay attached to an Arduino board, in this way the decalcification process was automated and the operator was only required to renew the solution every two days.

After decalcification, the samples were processed for being sectioned. The sections were 4um thick, and the samples were stained with iron hematoxylin

and trichromic eosin (see the hematoxylin and eosin chapter for more details about the staining procedure)

Results

Teeth decalcified with Morse solution (AM), achieved complete decalcification in 15 days while using EDTA-MW the time required was 25 days. The samples treated with EDTA 14% pH=7.2 and EDTA 20% pH=10 requested the same amount of time of EDTA 14% pH=10 but due to the lack of a numerous samples set this aspect is not significant: the variation of pH and concentration were mainly done to investigate if this modification could cause some alteration to the cellular structure rather than to test decalcification speed. The sample decalcified with nitric acid took only 3 days to complete the process.

The entire head of two mice was also decalcified: one with Anna Morse's solution and one with EDTA-MW 14% pH=10. The first method completed the decalcification process in one week and the second took two weeks to reach satisfactory results. On the following pages detailed histological pictures of the treated samples will be shown.

Fig. from 8 to 10 show cow teeth stained with iron hematoxylin and trichromic eosin. Fig. 11 to 13 show cow gingival epithelium stained with ferric hematoxylin and trichromic eosin. Fig. 14 shows mouse cerebellum stained with iron hematoxylin and trichromic eosin. Fig. 15 shows mouse oral cavity, 15a stained with hematoxylin and trichromic eosin, 15b iron hematoxylin and trichromic eosin. Fig. 16 shows cow tooth decalcified with nitric acid 5% and stained with hematoxylin and eosin.

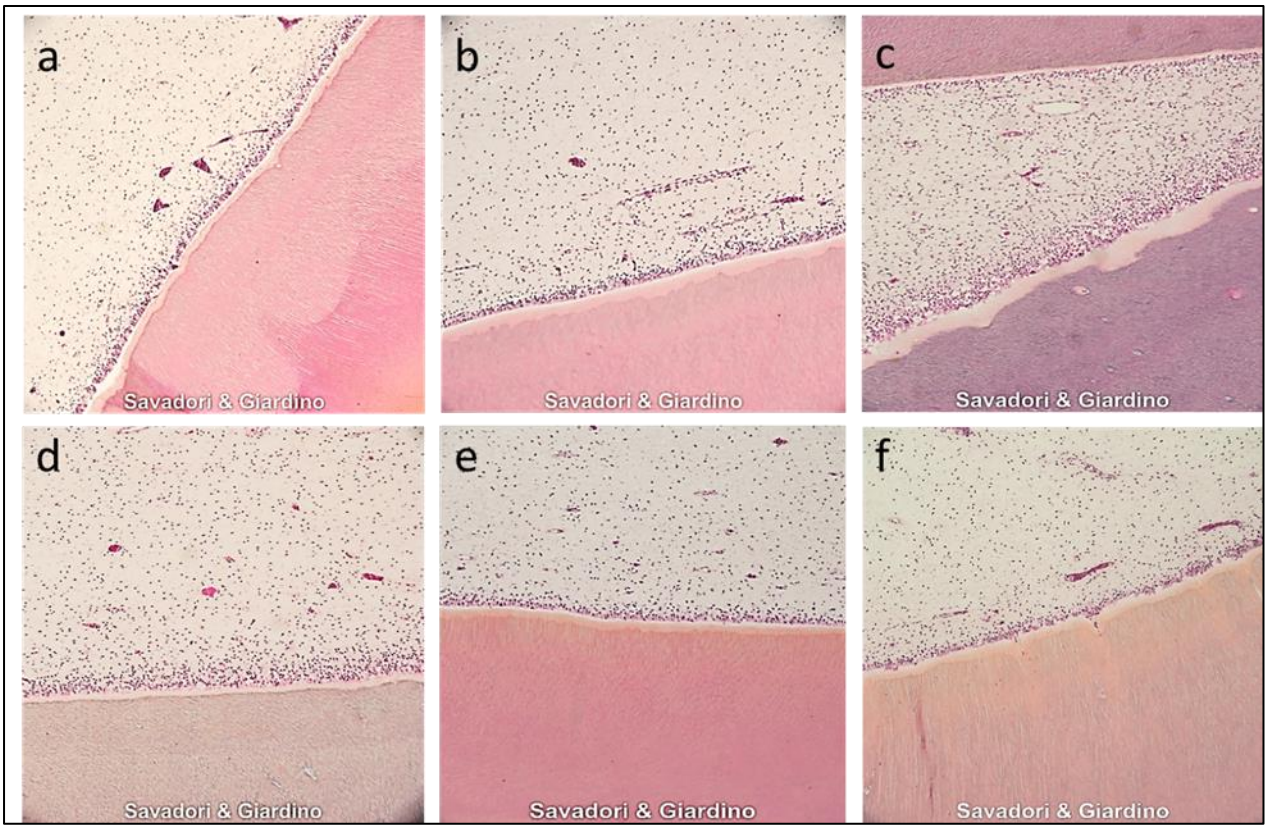


Fig 8: a) cow tooth AM, 100x; b) cow tooth EDTA-MW 14% pH 10, 100x; c) cow tooth EDTA-MW 20% pH10 100x; d) cow tooth EDTA-MW 14%, pH 7.2 100x; e) cow tooth EDTA-MW 14% pH 10 60 days 100x; f) cow tooth EDTA-MW 14% pH 10, 90 days, 100x

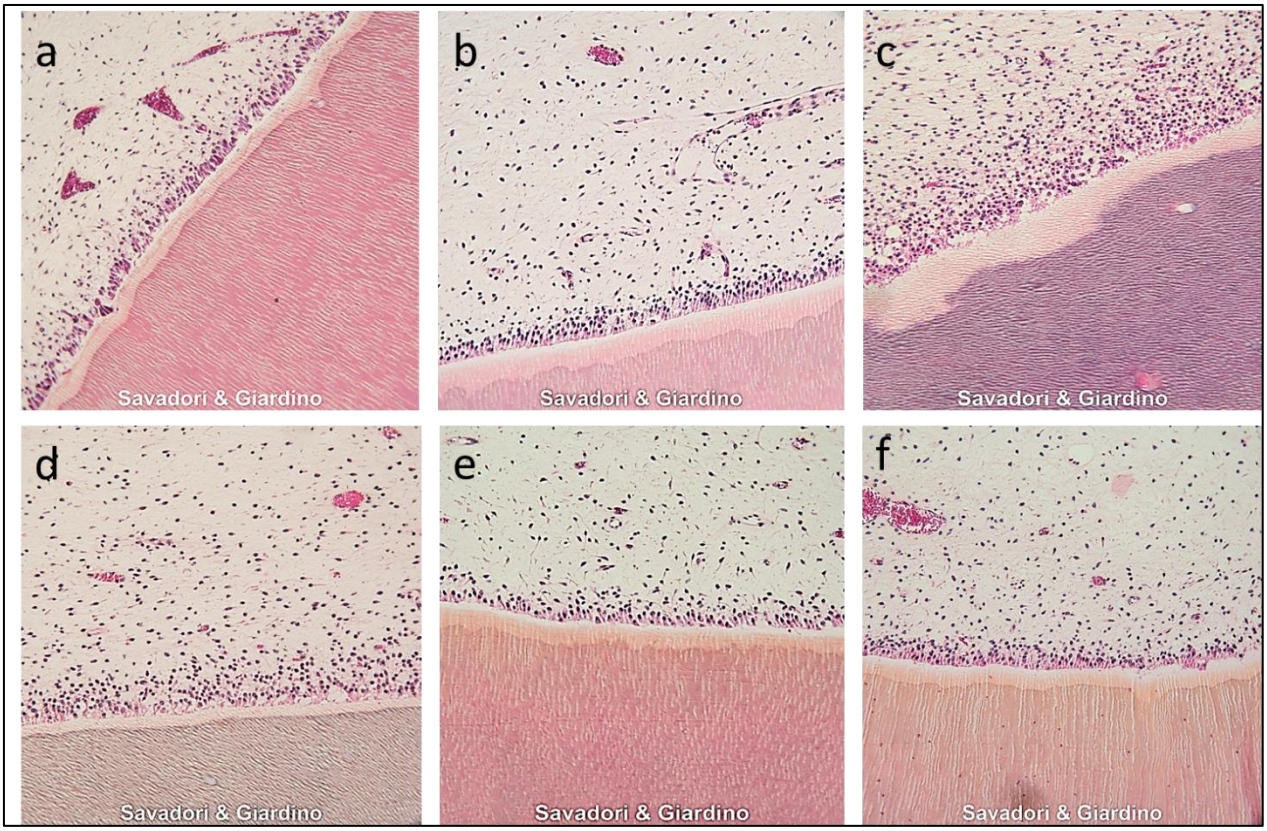


Fig 9: a) cow tooth AM 200x; b) cow tooth EDTA-MW 14% pH 10 200x; c) cow tooth EDTA-MW 20% pH10 200x; d) cow tooth EDTA-MW 14% pH 7.2 200x; e) cow tooth EDTA-MW 14% pH 10 60 days 200x; f) cow tooth EDTA-MW 14% pH10 90 days 200x

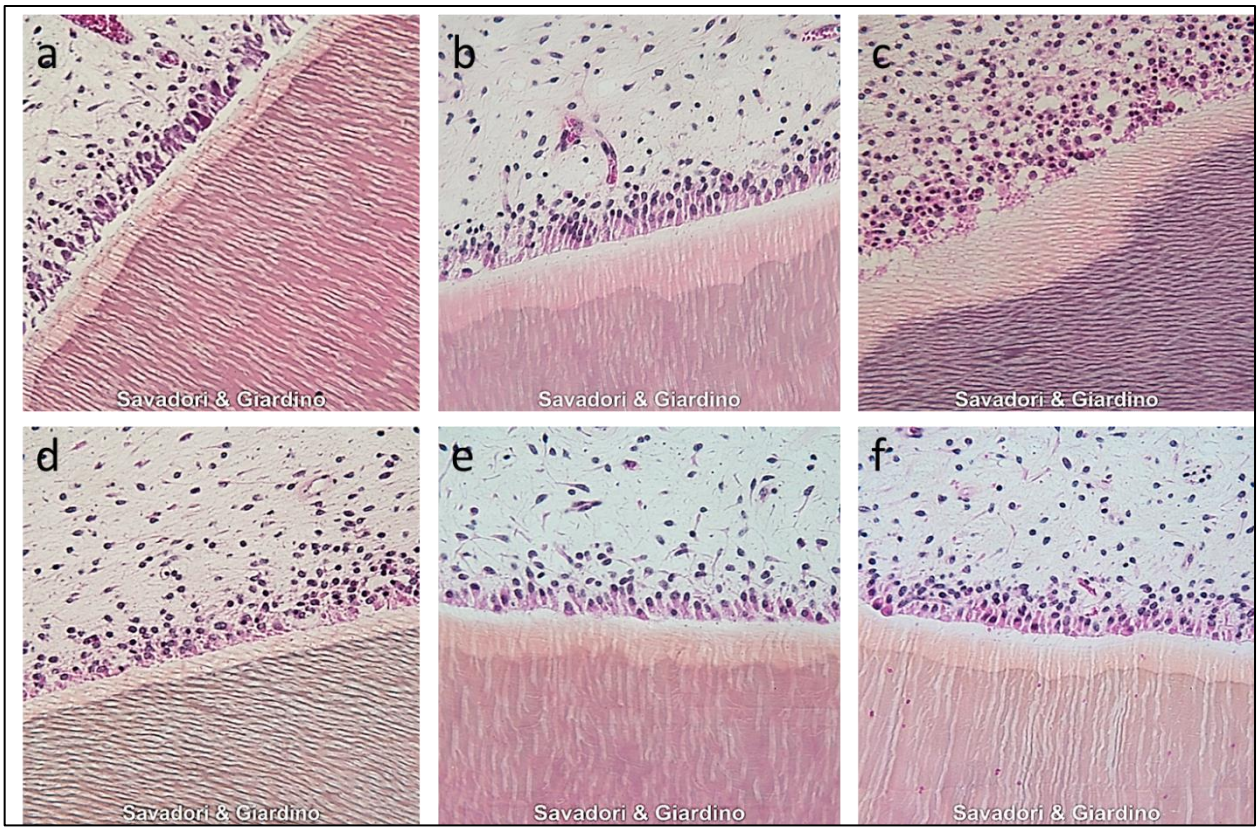


Fig 10: a) cow tooth AM 400x; b) cow tooth EDTA-MW 14% pH 10 400x; c) cow tooth EDTA-MW 20% pH10 400x; d) cow tooth EDTA-MW 14% pH 7.2 400x; e) cow tooth EDTA-MW 14% pH 10 60 days 400x; f) cow tooth EDTA-MW 14% pH10 90 days 400x

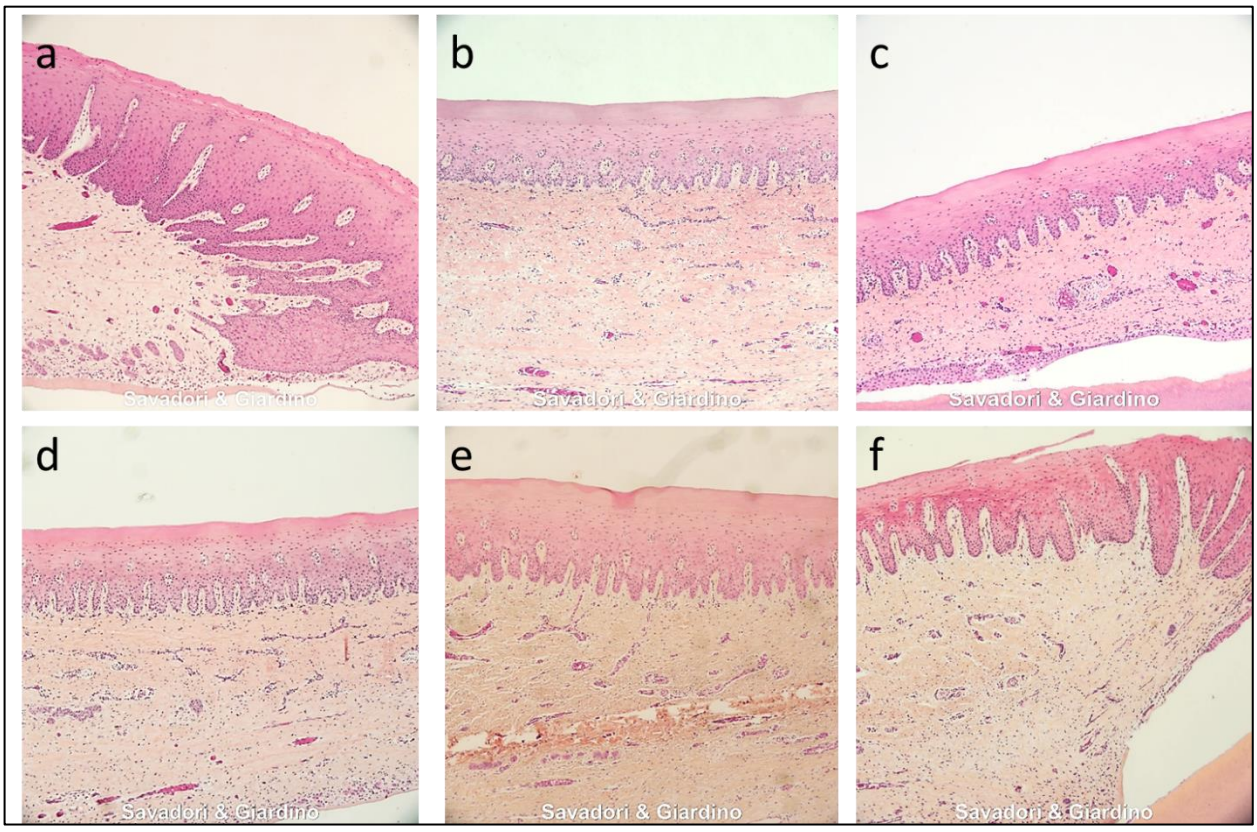


Fig11: a) cow gingival epithelium AM 100x; b) cow gingival epithelium EDTA-MW 14% pH 10 100x; c) cow gingival epithelium EDTA-MW 20% pH10 100x; d) cow gingival epithelium EDTA-MW 14% pH 7.2 100x; e) cow gingival epithelium EDTA-MW 14% pH 10 60 days 100x; f) cow gingival epithelium EDTA-MW 14% pH10 90 days 100x

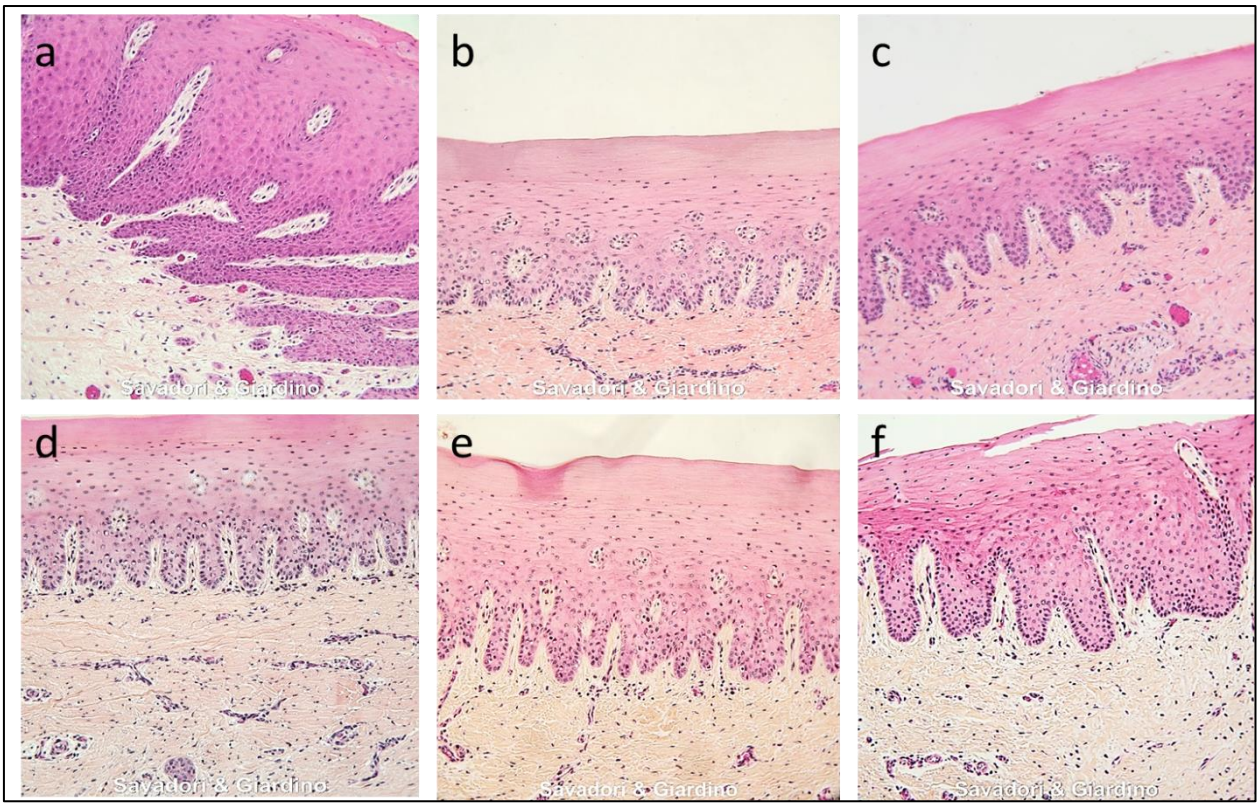


Fig 12: a) cow gingival epithelium AM 200x; b) cow gingival epithelium EDTA-MW 14% pH 10 200x; c) cow gingival epithelium EDTA-MW 20% pH10 200x; d) cow gingival epithelium EDTA-MW 14% pH 7.2 200x; e) cow gingival epithelium EDTA-MW 14% pH 10 60 days 200x; f) cow gingival epithelium EDTA-MW 14% pH10 90 days 200x

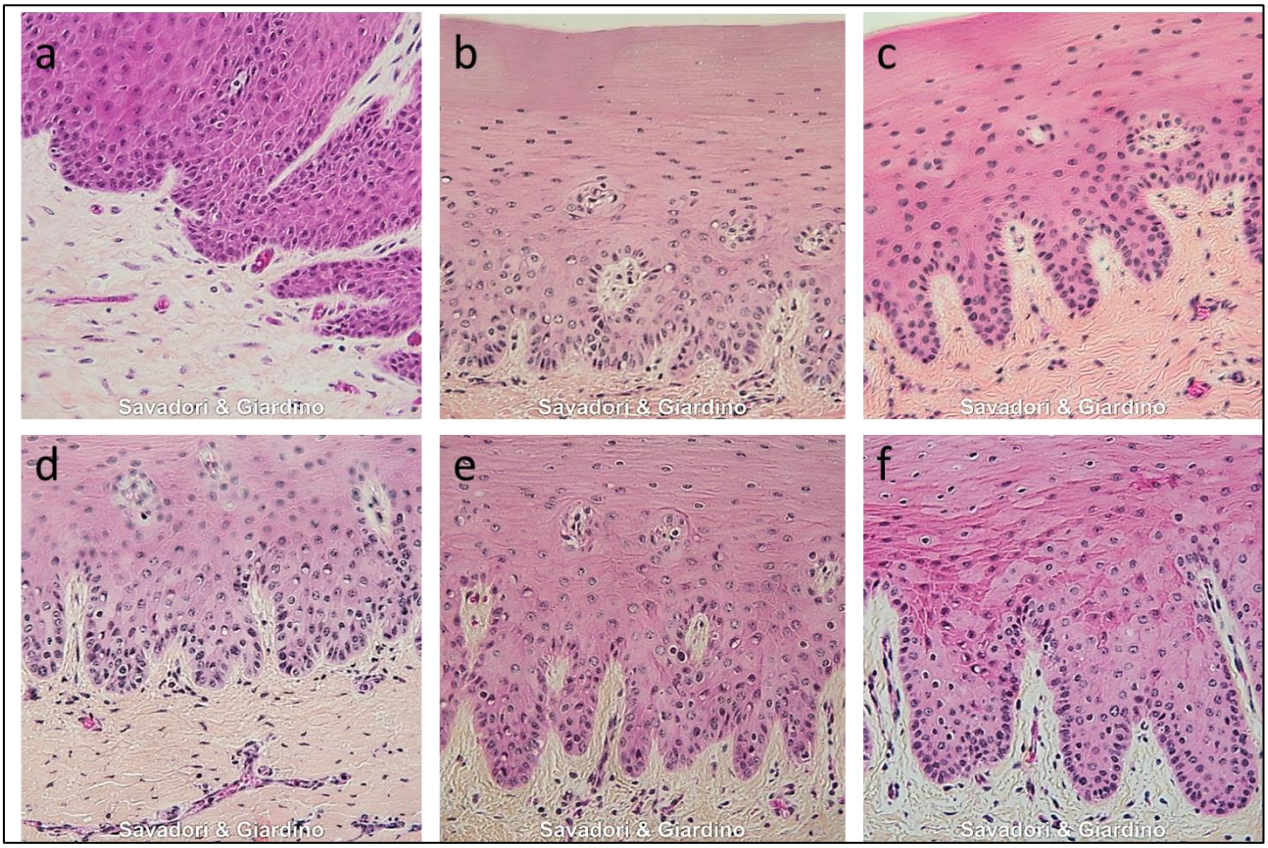


Fig 13: a) cow gingival epithelium AM 400x; b) cow gingival epithelium EDTA-MW 14% pH 10 400x; c) cow gingival epithelium EDTA-MW 20% pH10 400x; d) cow gingival epithelium EDTA-MW 14% pH 7.2 400x; e) cow gingival epithelium EDTA-MW 14% pH 10 60 days 400x; f) cow gingival epithelium EDTA-MW 14% pH10 90 days 400x

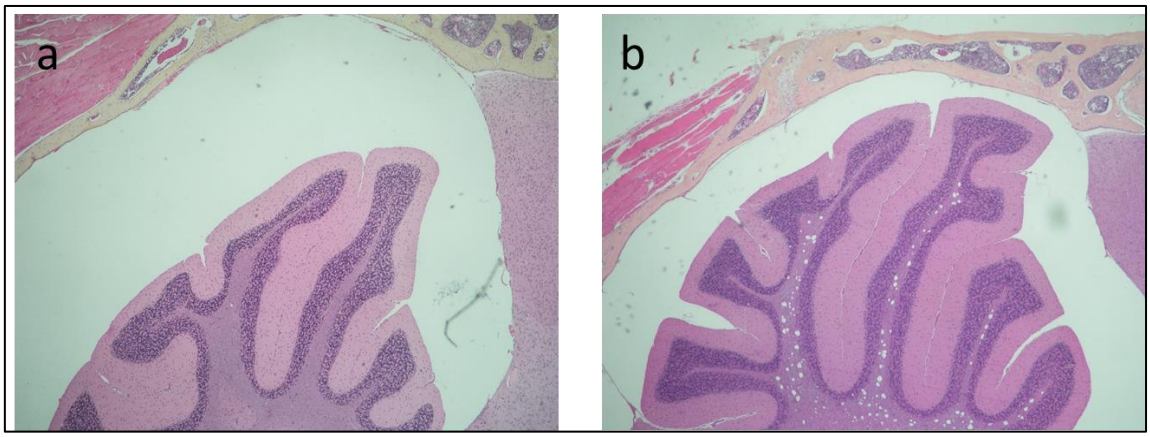


Fig 14:iron hematoxylin –trichromic eosin, a) mouse cerebellum EDTA-MW 14% pH 10 40x; b) mouse cerebellum AM 40x

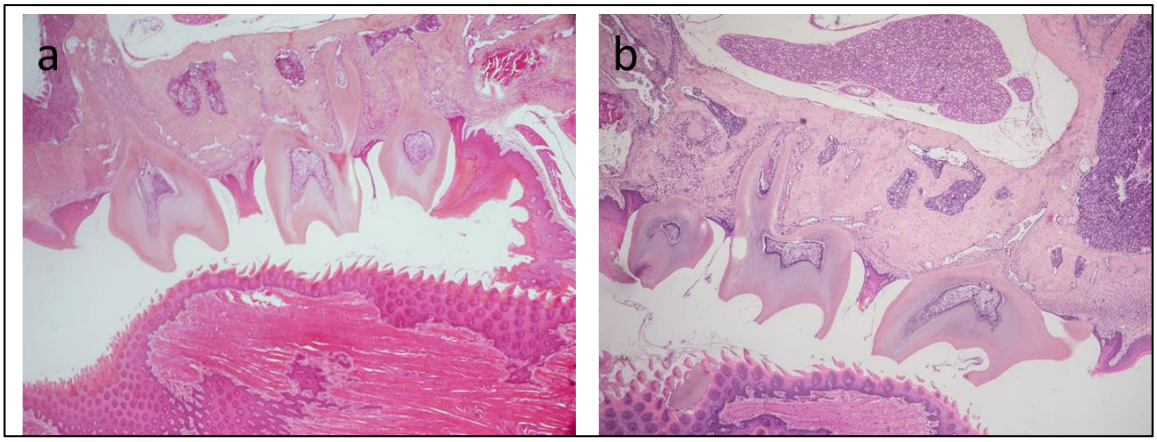


Fig 15 a) mouse oral cavity EDTA-MW 14% pH 10 40x; b) mouse oral cavity AM 40x

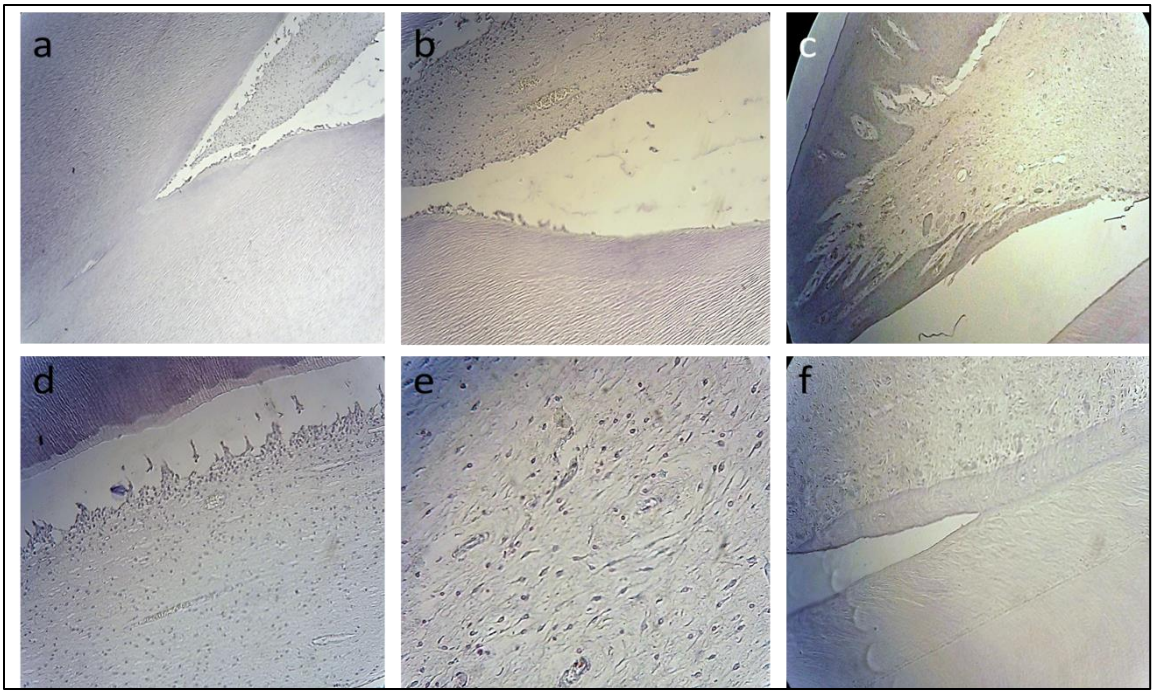


Fig 16: a) cow tooth nitric acid 5% 40x; b) cow tooth nitric acid 5% 100x; d) cow tooth nitric acid 5% 200x; e) cow tooth nitric acid 5% 400x; c) cow gingival epithelium nitric acid 5% 40x; f) cow periodontal ligament nitric acid 5% 40x

In the second part of this thesis, an analysis of different staining will be given. In particular, a bacterial staining technique such as the Brown & Brenn will be discussed, and a modification of this method will also be described. It will be shown how the Brown & Brenn technique could be simplified, maintaining optimal results. The PAS staining technique will be also proposed as a possible complementary bacteria staining, unbinding this stain from being used only for yeasts and fungi. A critical analysis will be made about the use of hematoxylin and eosin, showing that a good staining practice could lead to visualization of more details besides the anatomical structure and in the last place we will make some consideration on Mallory's trichrome staining technique.

The material for this study consisted of 5 non-restorable human teeth affected by apical periodontitis and two rat mandibles. Human teeth were decalcified with AM due to the simplicity of this method and the very good preservation of dental tissue (see discussion below). Each rat mandible was decalcified via EDTA-MW 14% pH=10 due to the extensive presence of soft tissue (see discussion below). Serial sections cut from each sample were 4 µm thick, all staining solutions used were homemade and no kits were used.

Part II: Staining techniques

Bacterial staining

Gram in 1884 (Gram C. 1884) invented a staining procedure to detect bacteria isolated from tuberculosis lesion and other diseases (Hucker G.J. & Conn H.J.1923). This technique is based on the propriety of crystal violet dye to bind peptidoglycan presents in the cell wall, especially bacteria with a big amount of peptidoglycan. When an iodate solution is added, it interacts with the crystal violet forming large complexes that can't be washed away with a differentiation medium. Then a second dye is added, generally Basic fuchsin, to stain the remaining bacteria. In this way, bacteria could be stained and subdivided into the two major categories gram-positive, the first ones colored, and gram-negative, the second ones colored.

Early after the introductions of gram staining, several variants were proposed: changing the dyes and the differentiation medium. This aspect indicates the high level of variability posed by this technique. Moreover, the basic Gram staining and its variants aren't suitable for detecting bacteria in dental tissues, mainly for the lack of a second differentiation.

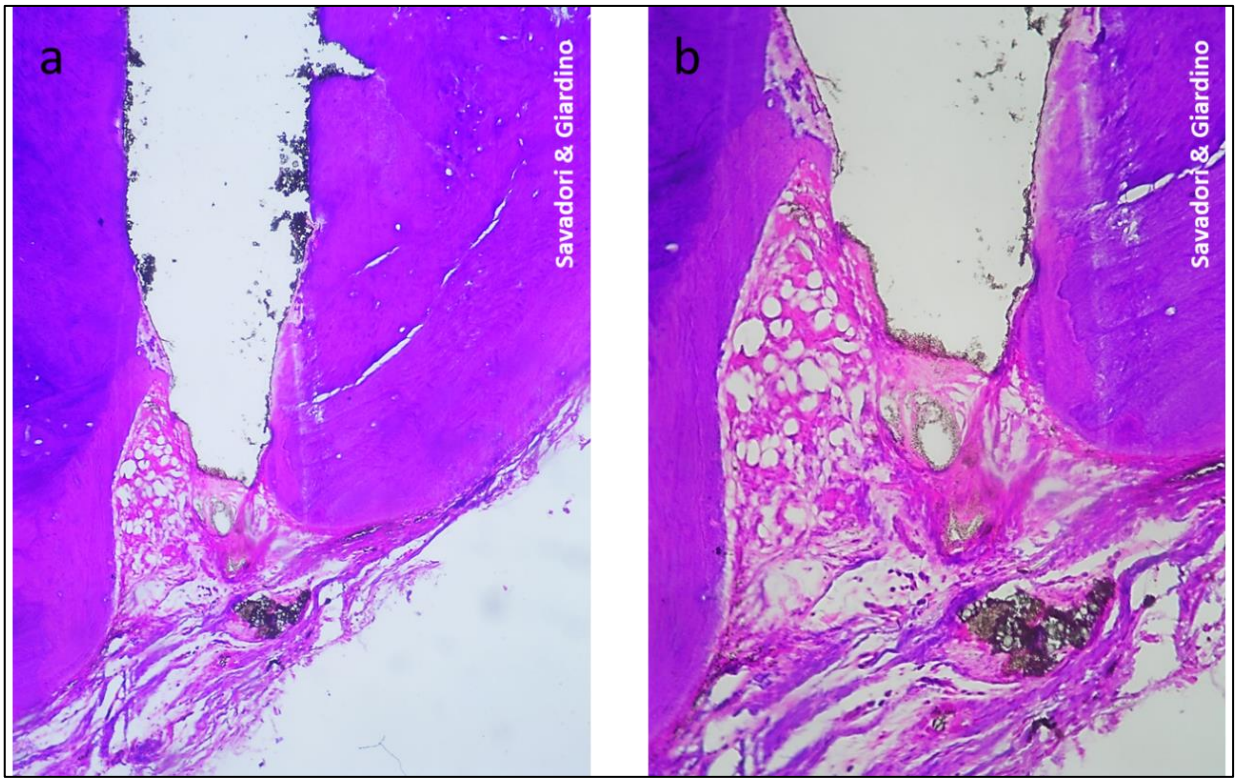


Fig 17: a,b) apex of a first molar stained with gram staining, 40x and 200x

In order to stain bacteria present in the tissues three staining techniques are normally used (Peck M.& Badrick T. 2017): Brown-Brenn (Brown J.H. & Brenn L.A. 1931) Brown-Hopps (Brown R.C. & Hopps H.C. 1973) and Gram-Twort (Twort F.W. 1924). Brown-Brenn and Brown-Hopps add a second differentiation passage after the second dye while Gram-Twort is pretty similar to the standard Gram procedure but with different dyes.

Brown and Brenn and its modifications

The Brown and Brenn technique is based on the same assumptions of the Gram technique with an addition of a second differentiation passage. A summarized procedure is given below:

- Deparaffinize the slide

- Dye with crystal violet

- Wash with water

- Add iodated solution

- Wash with water

- First differentiation

- Wash with water

- Dye with basic fuchsin

- Wash with water

- Acetone bath

- Second differentiation with picric acid in acetone

- Acetone bath

- Xylene baths

This procedure, like the original Gram staining, has collected many variants over the years: for example, the standard Brown-Brenn method uses 1% crystal violet solution and sodium bicarbonate 5% as mordant but subsequent modification like Taylor's modified Brown-Brenn (Taylor R.D. 1966) uses Hucker's crystal violet (Hucker G.J. & Conn H.J.1923) that includes ammonium oxalate as mordant; in Luna's Brown-Brenn (Luna L. G. 1968) only crystal violet 1% is used and the sections are allowed to dry before the differentiation passage with acetone. The first differentiation passage is a critical point: an overdtain could lead to false outcome because gram-positive bacteria, bleached by differentiation medium, will be stained with fucsin and then appear as Gram-negative bacteria. Generally all original protocols used a mixture of ether and acetone, but this mixture has been substituted with only acetone or ethyl alcohol and acetone in equal parts. Acetone acts very quickly in removing crystal violet, and this step must be carefully watched. The second dye used is basic fuchsin for nearly all variants, the difference lying into its concentration: Brown-Hopps and Taylor methods use a quite diluted dye (0,01% and 0,07% respectively) while Luna's method uses a more concentrated solution (0,25%). The second differentiation is usually done by a solution of picric acid and acetone: the picric acid has the fundamental function of coloring the background of the tissue slide in a yellowish color allowing the observation of bacteria in the tissue. In some staining kits (Sigma-Aldrich Gram stain HT90) tartrazine is used instead of picric acid and saffron could be used as well (Becerra S.C. et al. 2016). The second passage of differentiation is more crucial than the first because an overexposure to picric acid and acetone will strip off all dyes leaving only a yellowish background color. The last phase of this staining technique consists in washing the excess of picric acid with an acetone bath and then clearing the slide with several passages into xylene.

Below it will be proposed a simplified protocol for Brown-Brenn staining, called GS-Brown-Brenn (GS stands for Giardino-Savadori), that has been developed in the present thesis work, and that aims at standardizing the

timings and shortening the whole procedure, maintaining optimal results with an accurate detection of both Gram-positive and Gram-negative bacteria.

GS-Brown-Brenn

This modification is summarized below

- Deparaffinize the slide

- Dye with Hucker's crystal violet for 3 minutes

- Wash with water

- Add modified Gram's iodine solution for 3 minutes

- Wash with water

- Differentiation with isopropyl alcohol and acetone

- Wash with water

- Dye with basic fuchsin 0.25% for 3 minutes

- Wash with water

- Acetone bath

- Second differentiation with picric acid in acetone

- Acetone bath

- Xylene bath

Hucker's crystal violet was chosen for its stability and the presence of ammonium oxalate in the solution prevents to use other mordants like sodium bicarbonate solution. Modified Gram's iodine solution has a higher

concentration of iodide compound, and this could facilitate the formation of crystal violet-iodine complex. The first differentiation solution is a mixture of isopropyl alcohol and acetone with an excess of isopropyl alcohol: this solution has shown to act gently and to remove the excess of crystal violet in a more controlled way than acetone alone. The timing of staining was chosen in order to be easy to remember and shows optimal results making useless longer exposures. The second step of differentiation start with an acetone bath: only a couple of dips are required and the slide must be put in picric acid-acetone right after the fuchsin starts to run off the tissue. The slide must be dipped in picric acid until the section starts to turn yellow then it must be dipped in the second bath of acetone just a couple of times, only to remove the excess of picric acid. The last step is the passage in xylene, there isn't the necessity of using several changes of xylene: the slide must be dipped several times in xylene until a uniform layer of xylene is formed, then the slide is ready to be mounted or put in xylene if there are other slides to process.

Below some images show several tooth sections stained with this technique.

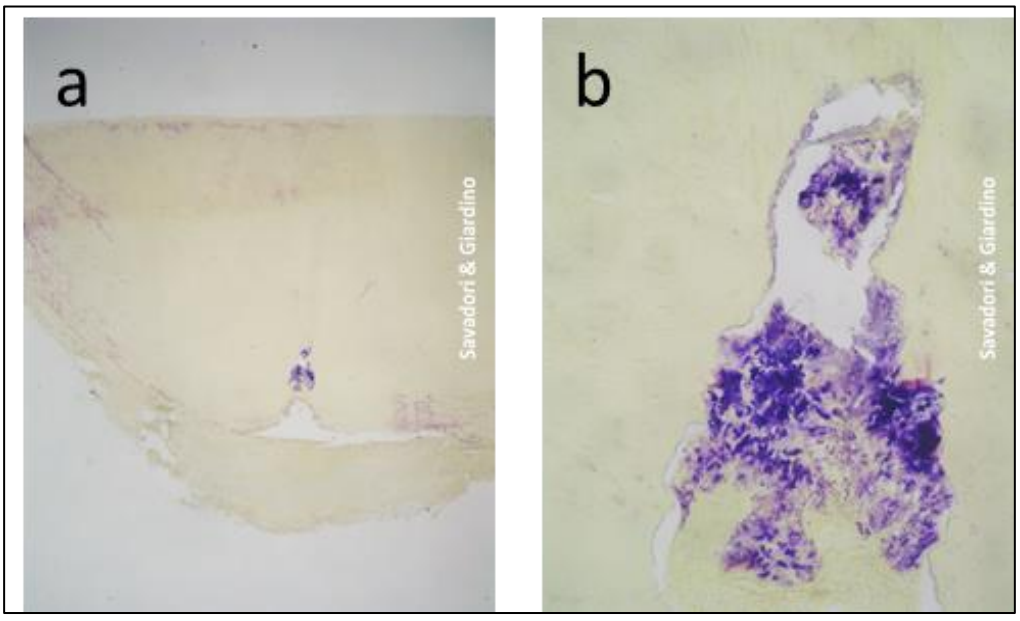


Fig 18: Standard Brown-Brenn. In picture a) is possible to notice some background staining of fuchsine, absent in the GS-Brown-Brenn. Picture b) shows a Gram-positive bacteria population without presence of Gram-negative bacteria. Due to the abundance and morphological difference of the bacterial population, it is difficult, even if not impossible, that there are no Gram-negative bacteria.

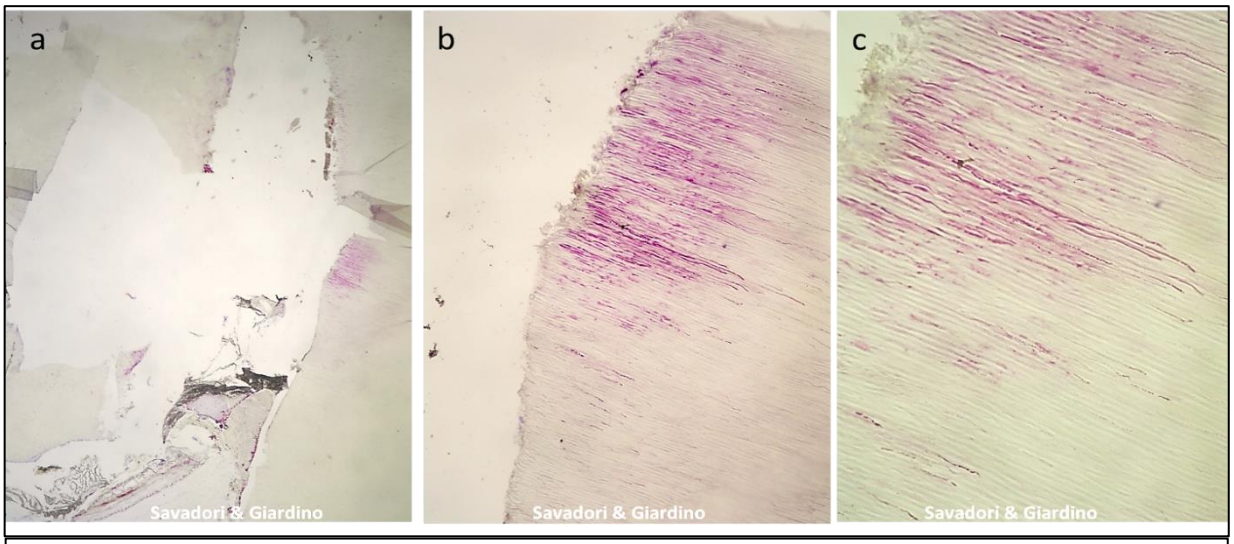


Fig. 18bis) GS-Brown-Brenn stain of molar mesial root, gram positive bacteria in dentinal tubules; a) 40x; b) 200x; c) 400x

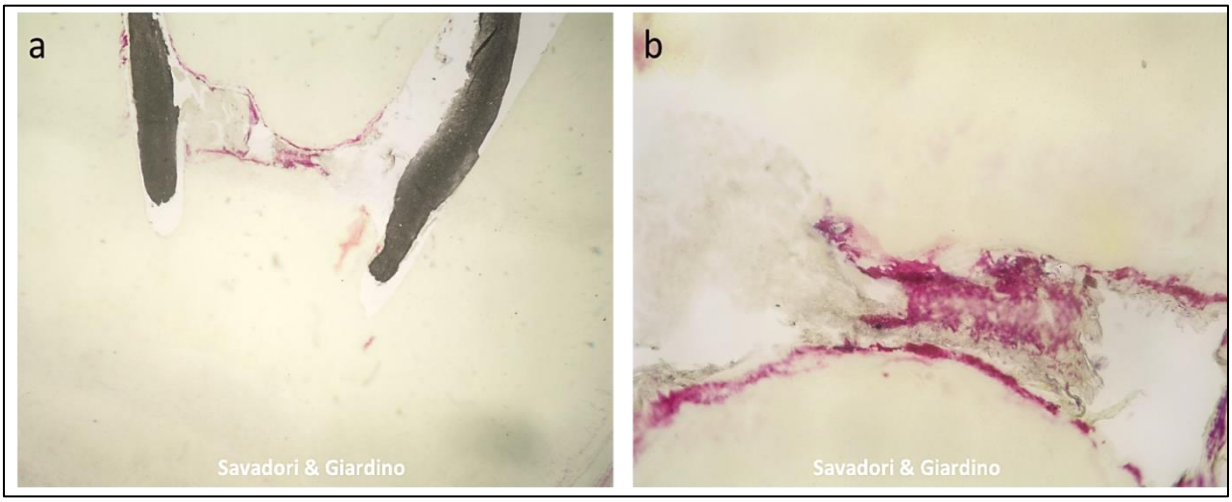


Fig. 19: GS-Brown-Brenn of molar distal root, bacteria population in isthmus between the two main root; a) 40x; b) 100x

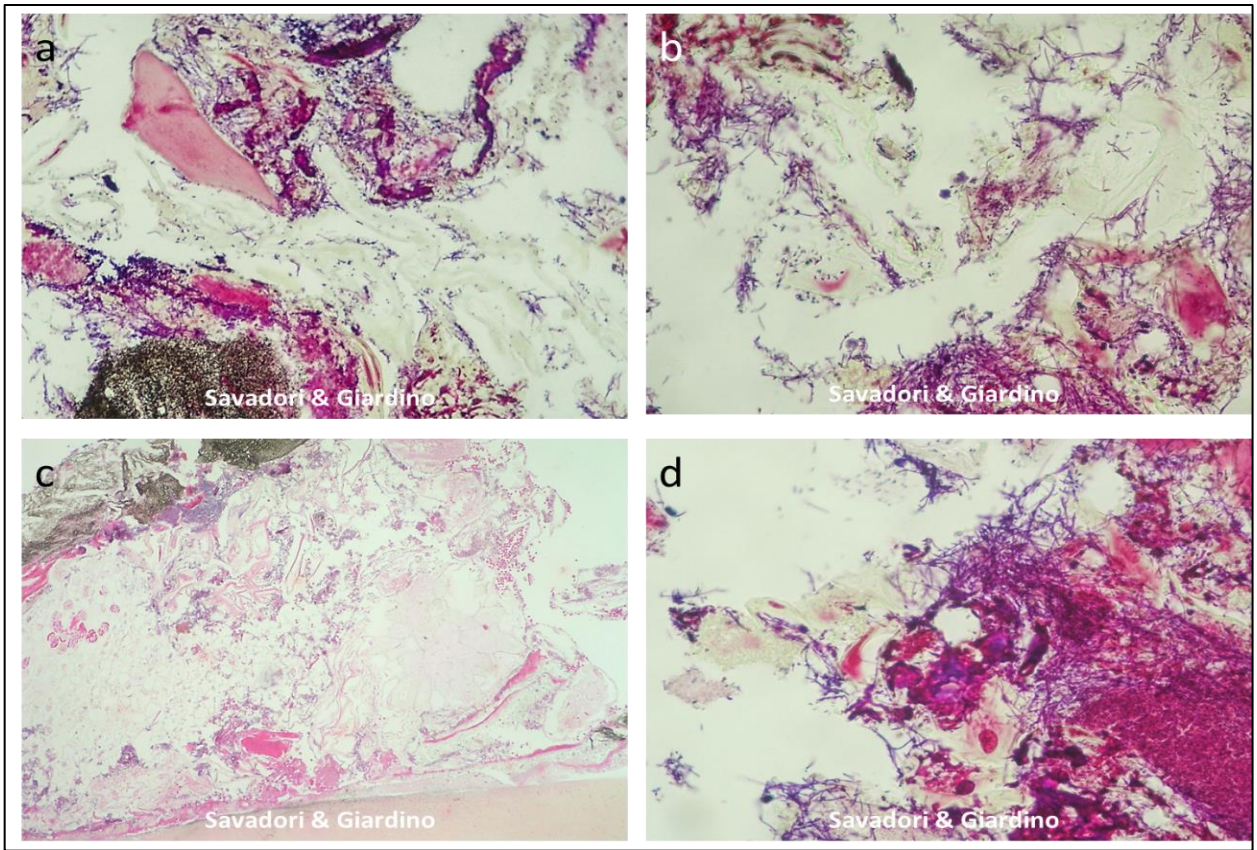


Fig. 21: upper portion of mesial root with the presence of oganic debris. Fig. a), b), d) GS-Brown-Brenn 1000x, presence of gram positive and gram negative bacteria; c) Iron-hematoxylin and trichromic-eosin 40x

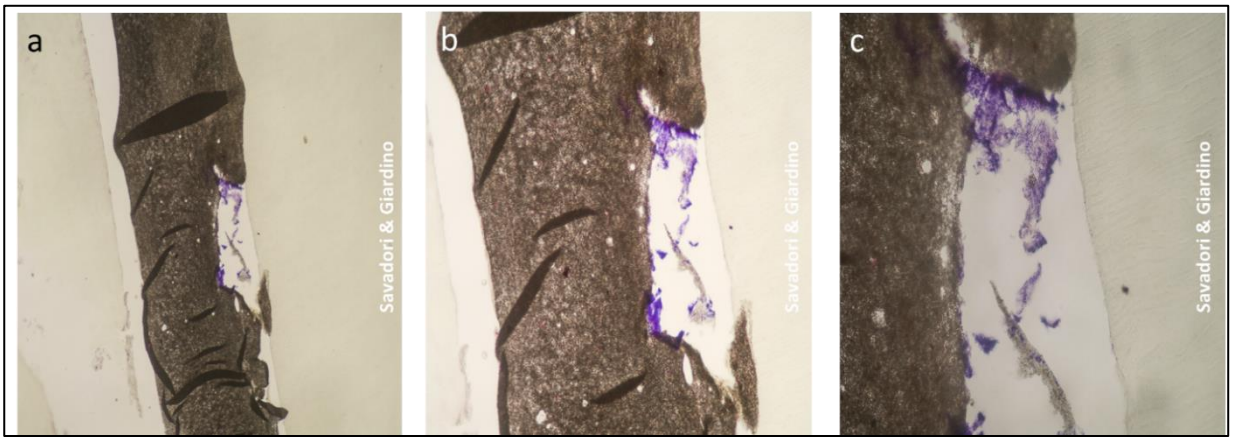


Fig. 20: GS-Brown-Brenn of molar distal canal (same sample of fig 19) Several Gram-positive bacteria attached to gutta-percha a) 40x; b)100x; c)200x

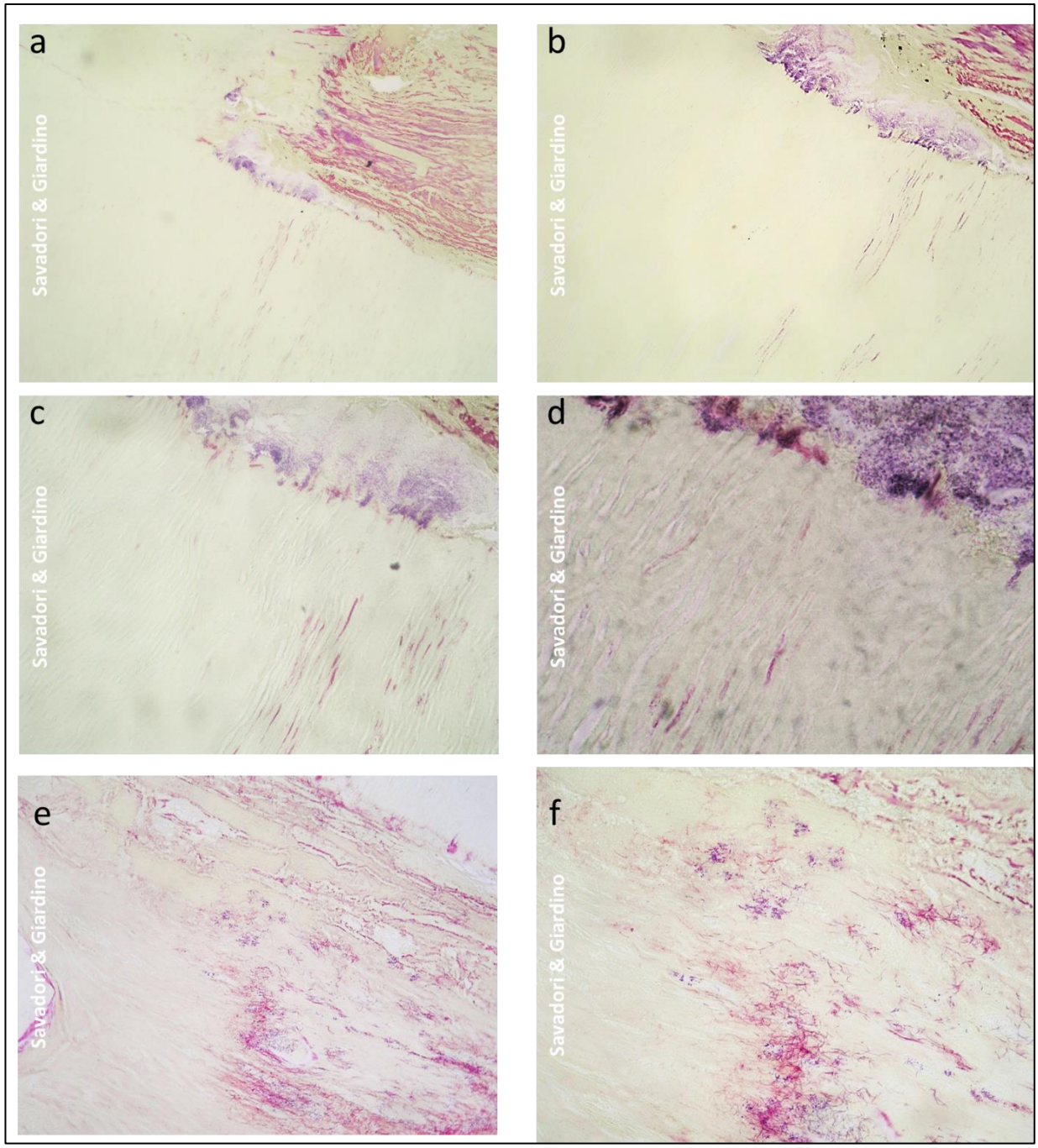


Fig. 22 GS-Brown-Bren, apical portion of a first lower molar mesial root. Gram-positive bacteria attached to the canal wall with sub population of Gram-negative. Gram-negative bacteria inside dentinal tubules. a) 40x; b) 100x; c) 200x and d) 1000x; picture e) and f) show cocci gram-positive and gram-negative filamentous bacteria in the necrotic pulp.

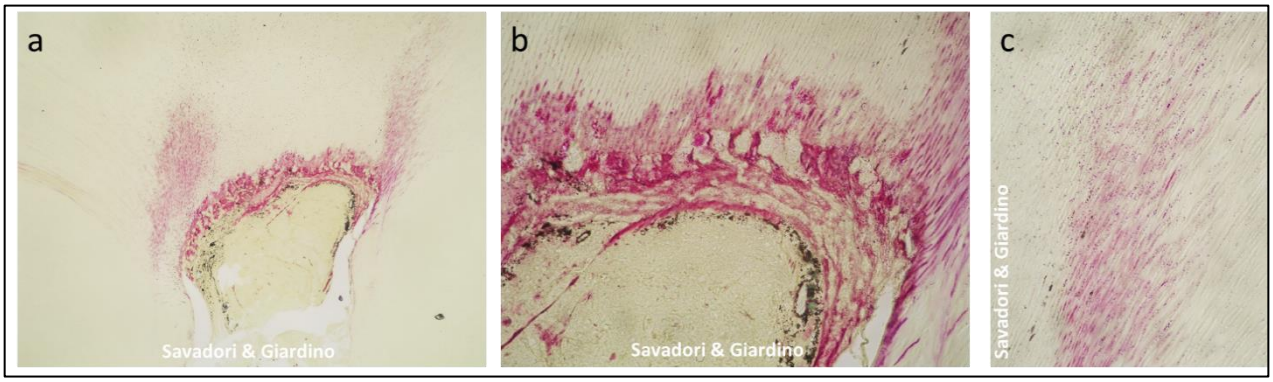


Fig. 23: GS-Brown-Brenn transversal section of a first molar (same sample of figure 22). Gram negative bacteria fill dentinal tubules a) 40x b,)400x

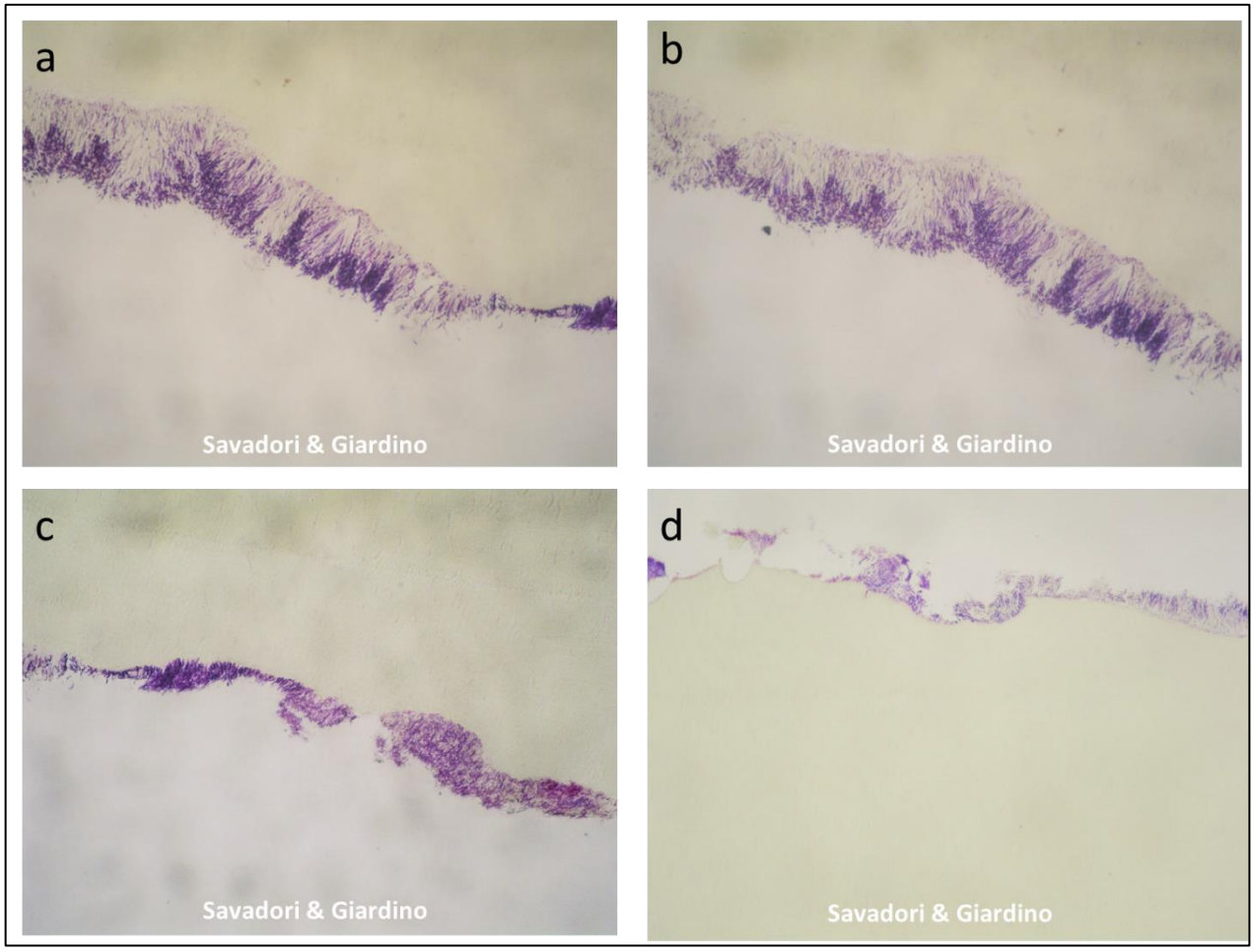


Fig. 24: GS-Brown-Brenn staining. Transversal sections of an incisor. Bacteria organized in biofilm around the outer side of the tooth. a, b) 400x , c,d) 400x

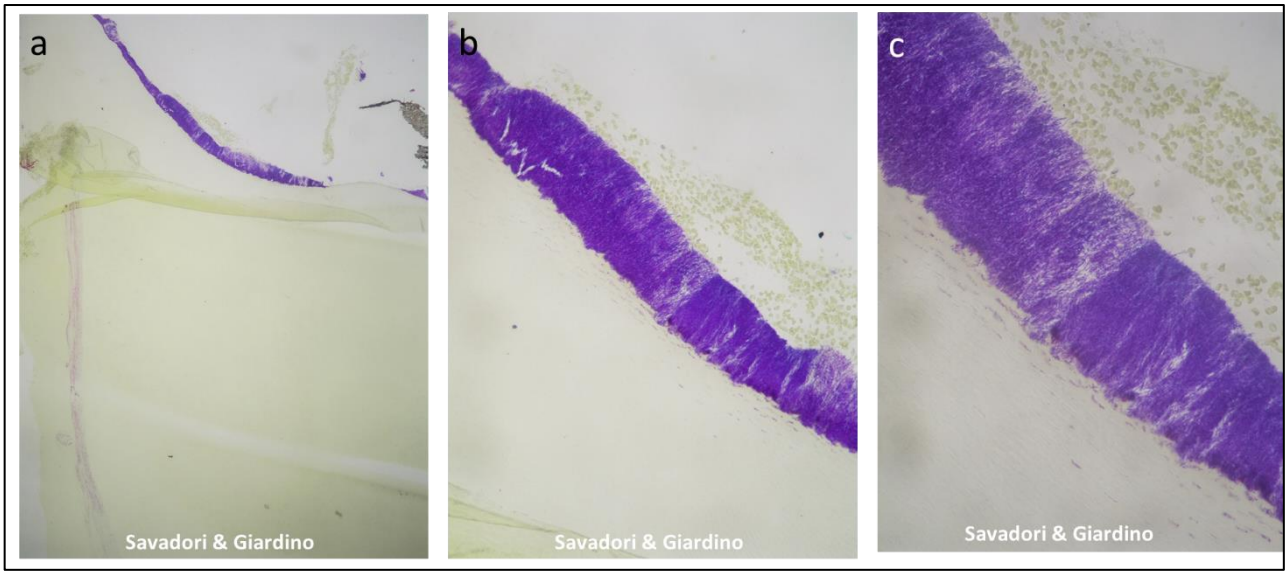


Fig. 25: GS-Brown-Brenn. Premolar root, gram positive bacteria cover the upper portion of the root. A 40x, b)200x, c) 400x.

Gram-Twort

Gram-Twort technique, as the name suggests, is a modified procedure introduced by Twort in 1924 for staining bacteria that is quite similar to a normal Gram staining. The first half of the protocol is the same as the Gram staining or the Brown-Brenn, starting with the usage of crystal violet then iodine solution and differentiation process but as counterstaining it is used the Twort solution made with neutral red and fast green. Neutral red is capable of staining the Gram-negative bacteria while fast green has the role of staining the background and it is used picric acid instead. The whole procedure is easier than Brown-Brenn techniques and has only one differentiation step, but the chromaticity is not as satisfactory as the original Brown-Brenn staining method.

In the following panel samples stained with the Gram-Twort technique are shown.

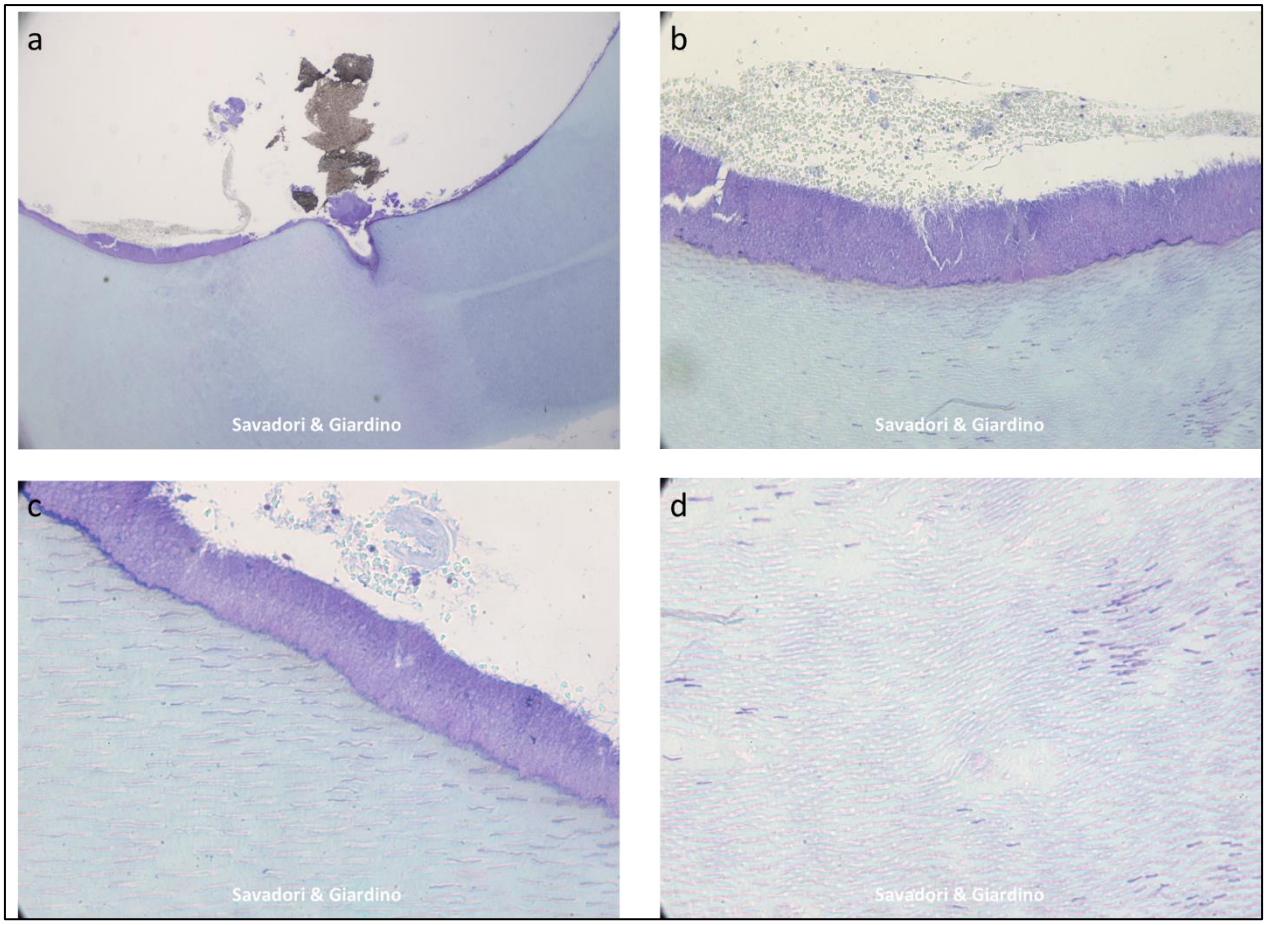


Fig. 26: Gram-Twort stain. Premolar root (same sample of fig.25 subsequent section), bacteria cover the upper part of the root, and bacteria inside the dentinal tubules. a) 40x, b,c) 100x, d) 200x.

PAS

The pas staining technique was developed independently by McManus (McManus J.F.A. 1946), Lillie (Lillie R.D. 1947) and Hotchkiss (Hotchkiss R.D. 1948) with the chemical basis previously described by Malaparte (Malaparte L. 1928, 1934). This reaction has an important role in the detection of carbohydrates. These molecules are detected through a reaction with periodic acid that reacts exclusively with glycols with a vicinal OH group, carbonyl compound where carbonyl group is adjacent to a hydroxyl group. Furthermore, the reaction could take place when the hydroxyl is replaced by a primary amino group or a secondary aminic group (Bangle R. & Alford W.C. 1953). The outcome of this process is the formation of an aldehydic group that can react with the Schiff's reagent: a compound formed

of pararosaniline and HCl that form a bright purple color when entering into contact with the acidic group.

This staining procedure is often used to detect fungal infections (Guarner J & Brandt M.E. 2011) and some glycogen related disease. However, a study made by Khavari (Khavari P.A. et al. 1991) showed that many bacteria are susceptible to PAS reaction, especially the gram-positive bacteria

In this thesis, an analysis of the applicability of PAS staining for the detection of bacteria in teeth has been made. Subsequent slides were stained with GS-Brown-Brenn and then with PAS staining in order to check if bacteria could be detected in the same areas.

McManus's PAS staining procedure was also used due to the presence of light green as counterstain that provides a good contrast

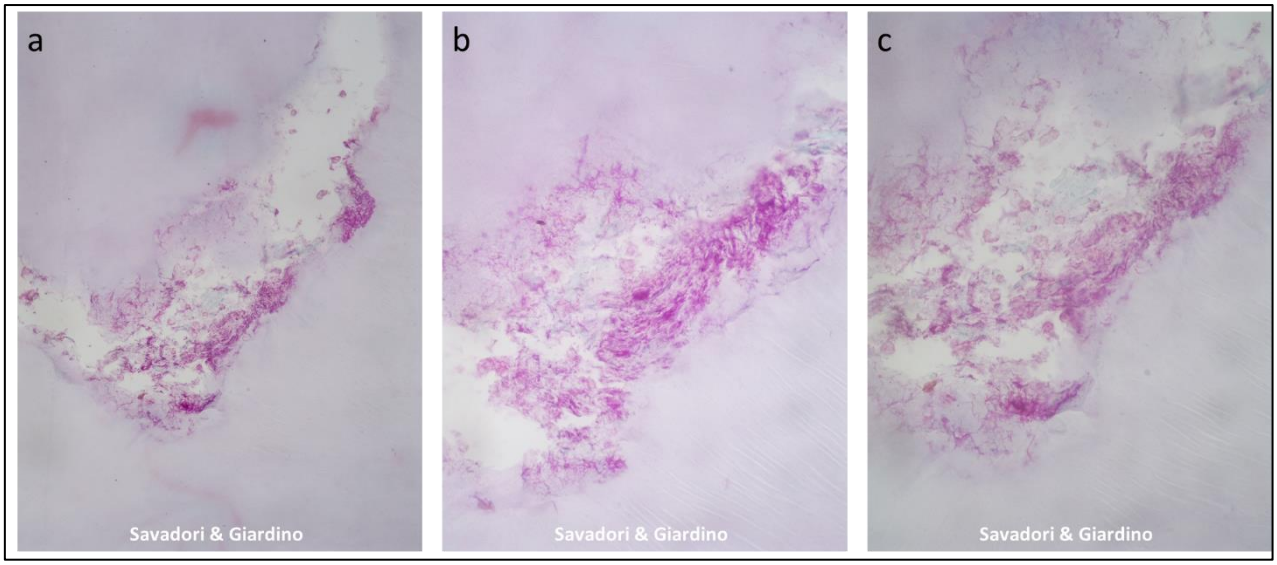


Fig. 27: PAS-light green. Distal root of a first molar (same sample of figure 19 subsequent slide). Isthmus between the two main canal, abundance of PAS positive substances probably bacteria. A) 100x, b) 200x, c) 400x

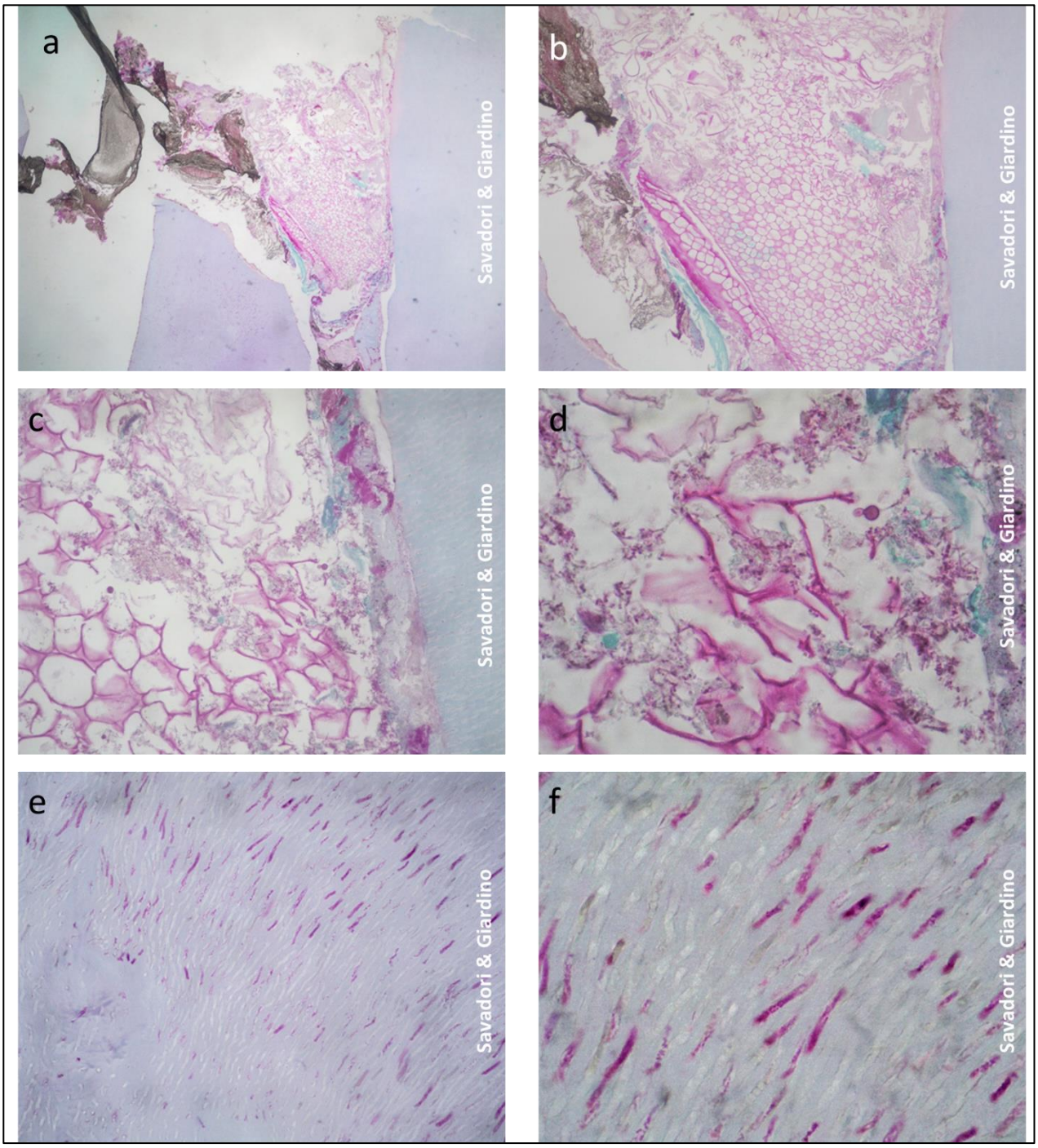


Fig. 28: PAS-light green of a molar mesial root (same sample of fig 18 and 21 subsequent sections). A, b and c (40x, 100x and 200x respectively) show some organic debris mainly of vegetable origins. d) (1000x) plants membrane, bacteria and a yeast in active division. e, f) 400x and 1000x, bacteria in dentinal tubules

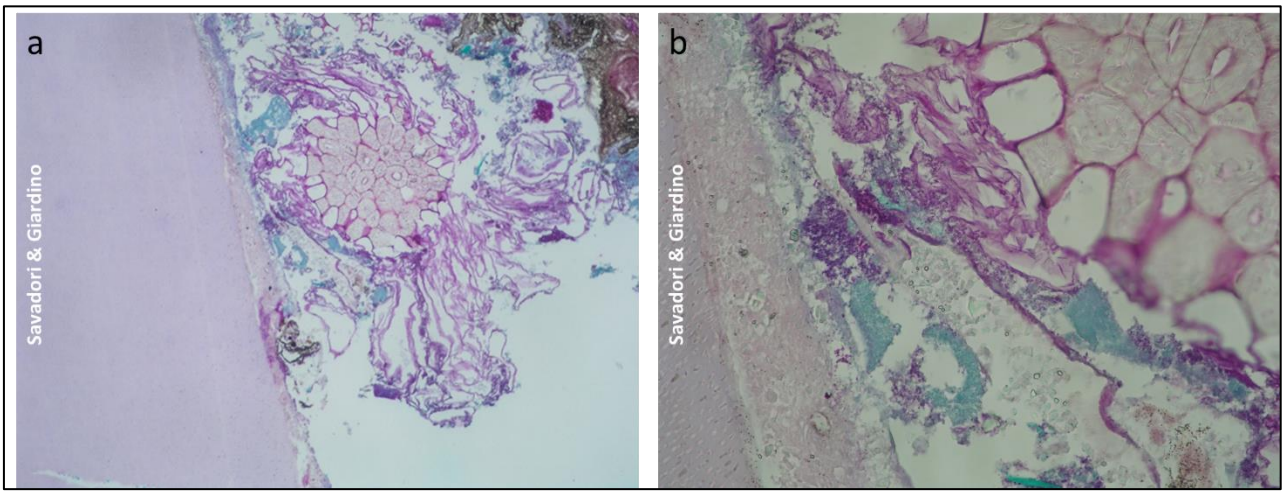


Fig. 28: PAS-light green, mesial molar root (same sample of fig 18, 21, 28 subsequent section). a, b) 200x and 400x respectively show plant's cells structure

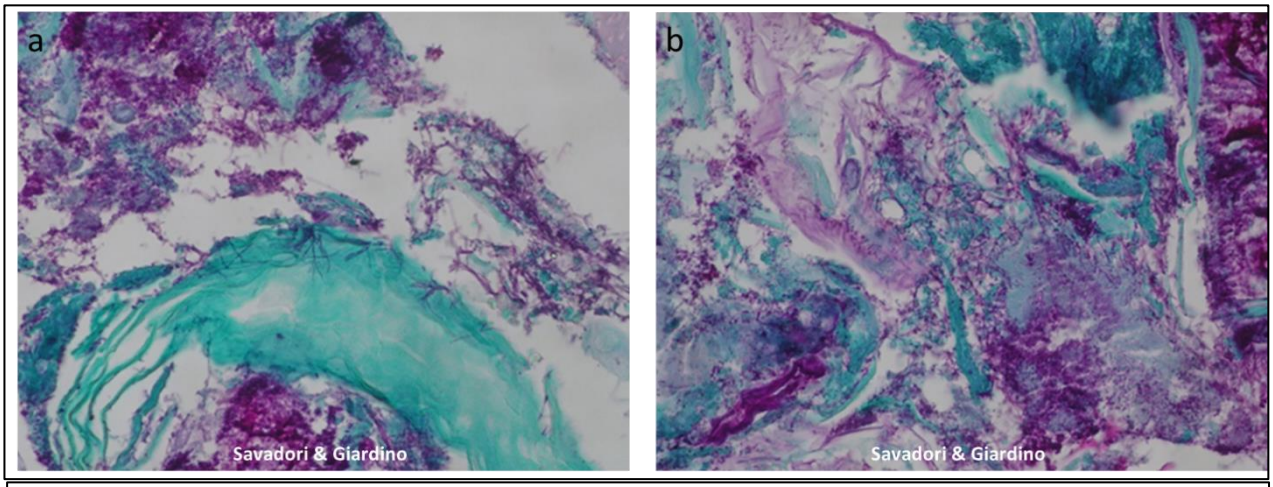


Fig. 29: PAS-light green, mesial molar root (same sample of fig 27). a, b) 1000x bacteria between organic debris

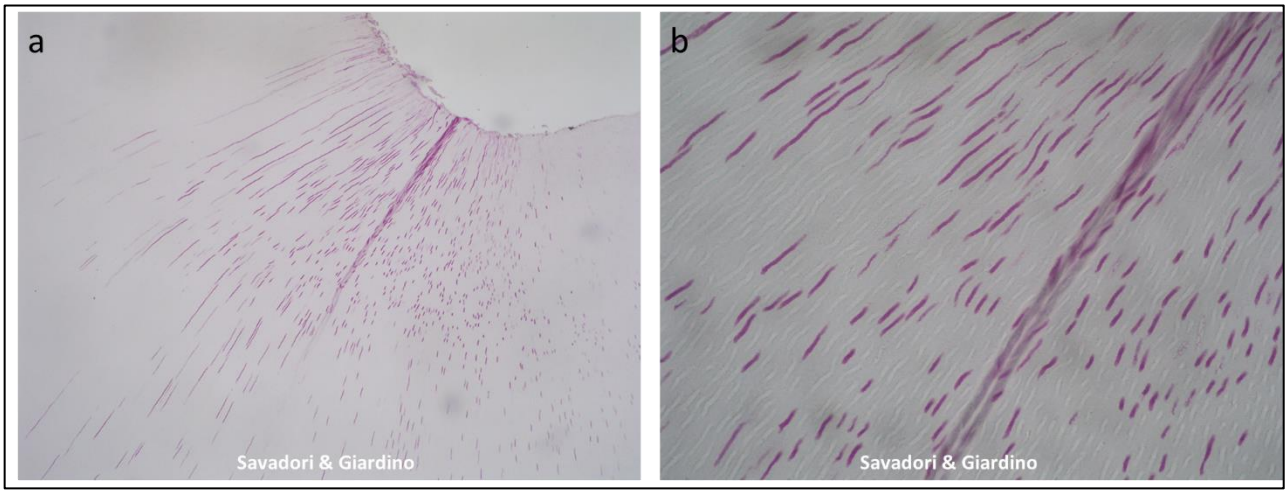


Fig. 30: PAS-light green. Transversal sections of incisor, a,b) 100x and 400x respectively, bacteria in dentinal tubules.

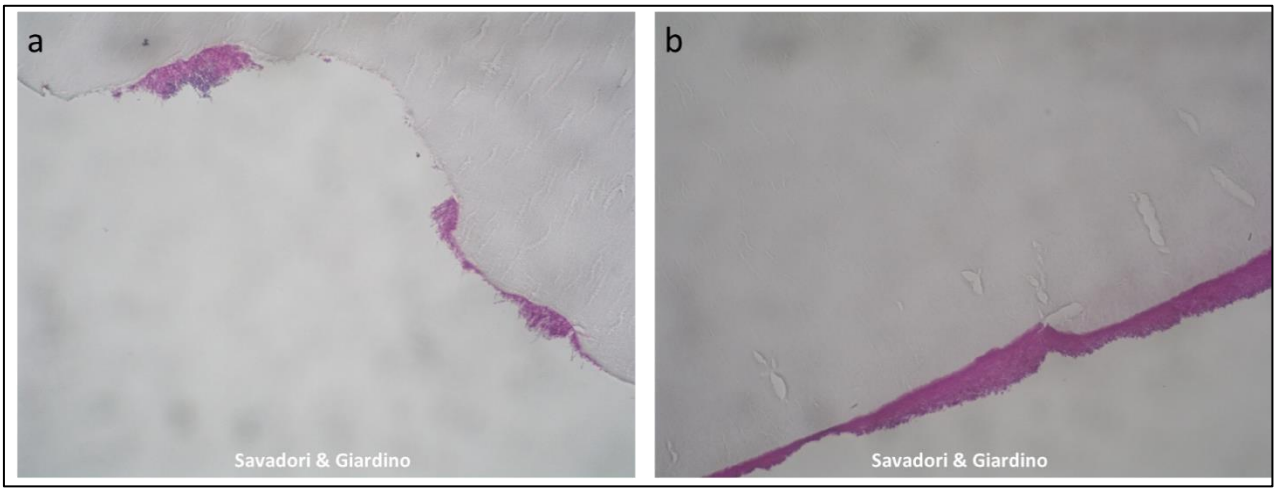


Fig. 31: PAS-light green. Transversal section of incisor. Bacteria attached on outer side of tooth. a,b) 200x

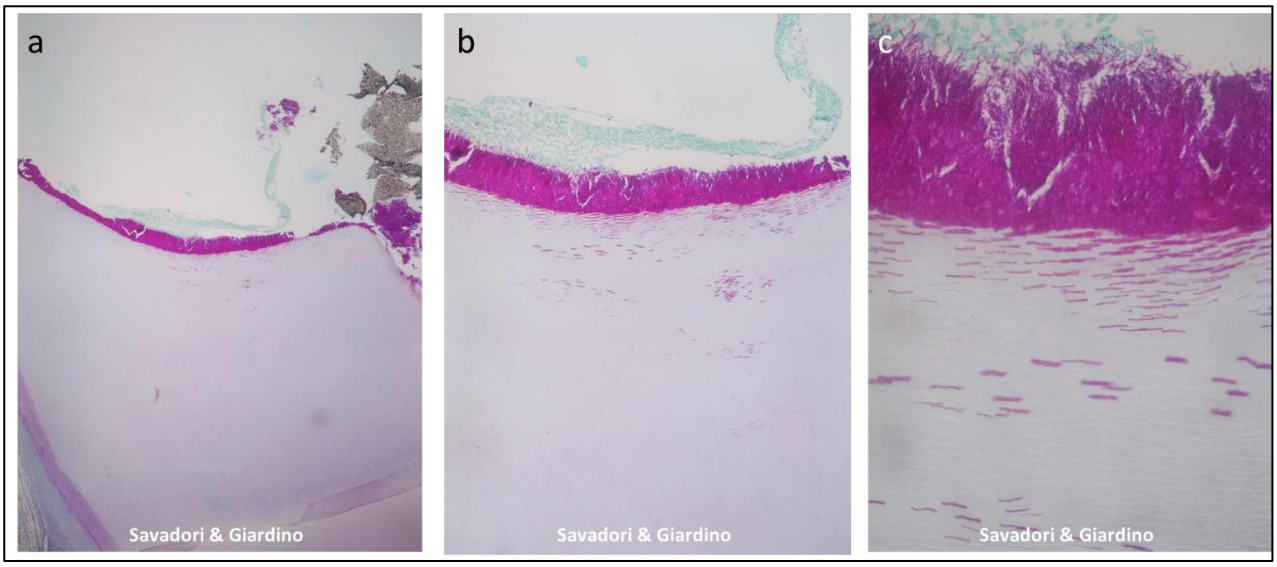


Fig.32: PAS-light green. Premolar root (same sample of fig 25 subsequent section). Bacteria cover the upper part of the root and inside the dentinal tubules beneath the bacteria vegetation. a)40x, b) 100x, c) 400x.

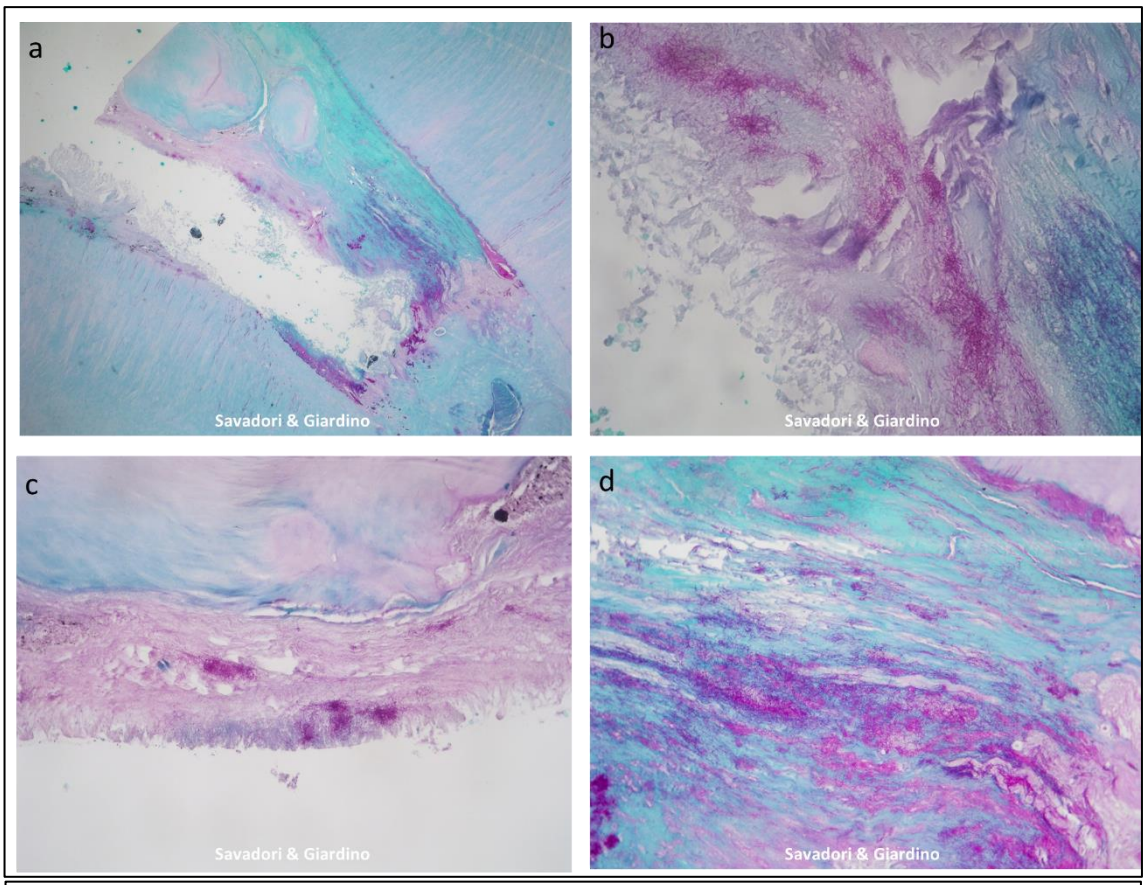


Fig. 33: PAS-light green. Apical portion of a first lower molar mesial root (same sample of fig 22 subsequent section). Calcified and necrotic pulp with bacteria population; a) 40x, b,d 200x) c)100x

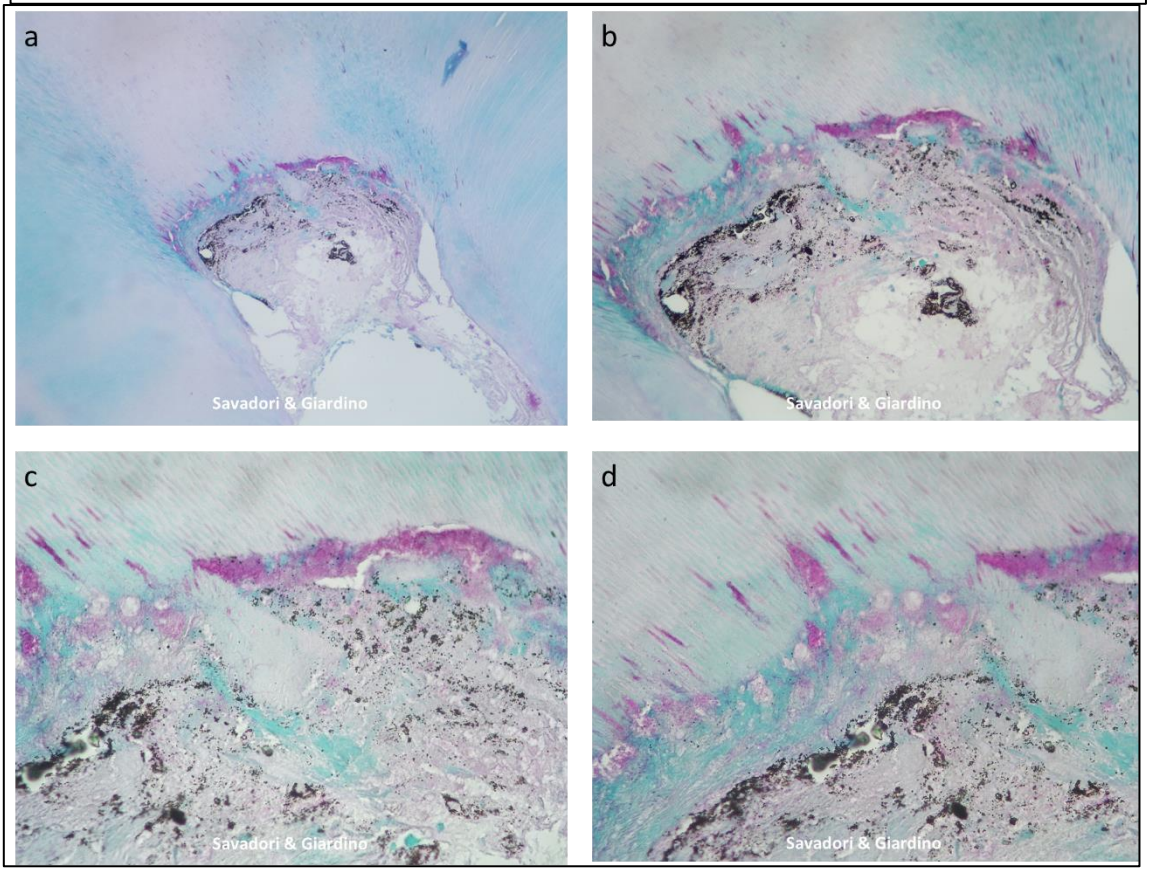


Fig. 34: PAS-light green. Transversal section of a first molar mesial root (same sample of figure 23 subsequent section). Bacteria inside the dentinal tubules a) 100x, b) 200x, c,d) 400x

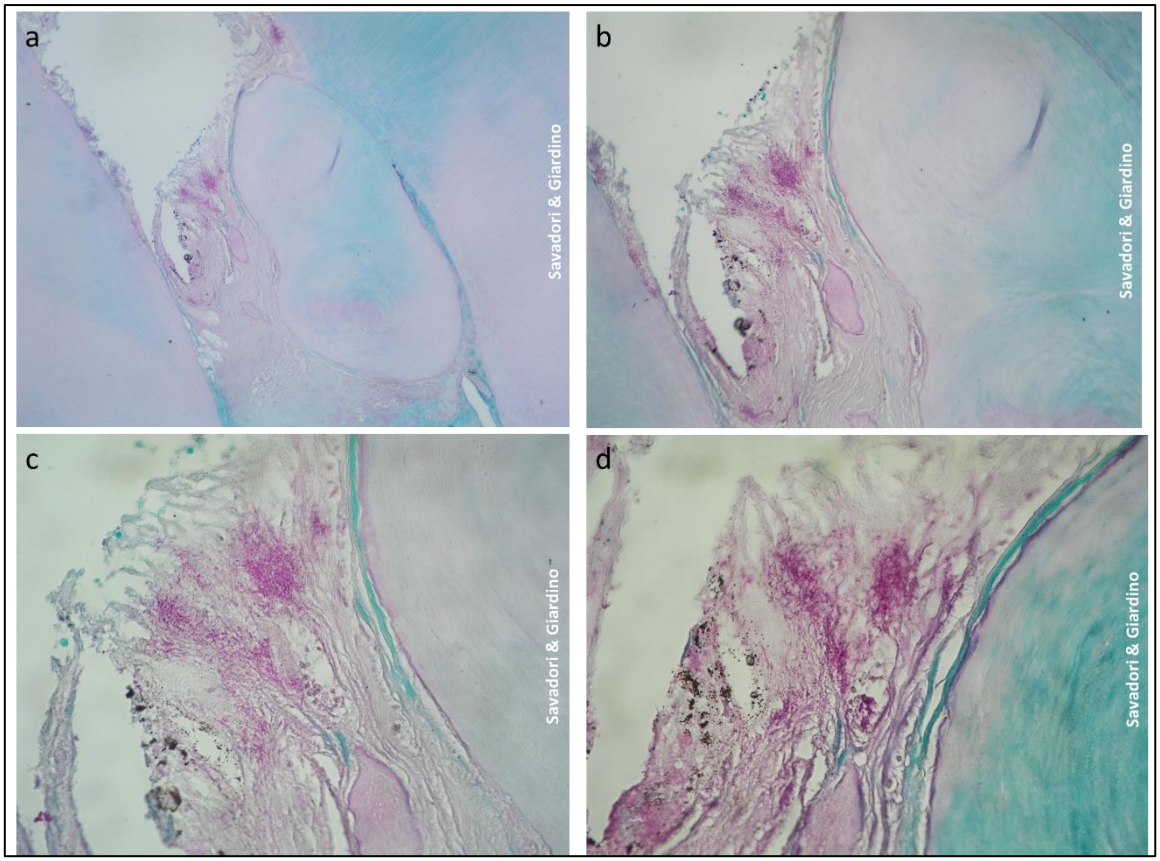


Fig. 35: PAS-light green. Transversal section of a first lower molar (same sample of fig 34), pulp stone surrounded by necrotic pulp with bacteria. a) 40x, b) 100x c) 200x d) 400x

Hematoxylin and Eosin

Hematoxylin and eosin (H&E) stain is one of the most known staining procedure. It has been used since the end of the nineteenth century (Titford M 2005). This staining is very important because it can recognize various tissue and cell types and this is the first step in every histological investigation.

There are two ways to perform the hematoxylin-eosin stain: the first one is called progressive, invented by Mayer (Mayer P. 1903) and the second one regressive, invented by Harris (Harris H.F. 1900). The Mayer's method is called progressive and all the passages are done in sequence while Harris's method is called regressive and requires a differentiation passage after the staining in hematoxylin. Both procedures give the same result, staining the nuclei in blue-purple and the remaining tissue stained in shades of pink.

Despite the large use of this technique, H&E is confined to morphology study and often used with the standard procedure. Rarely it is used a variant of hematoxylin, like iron hematoxylin that can color nuclei more intensely, or eosin variant with the addition of more dyes.

In this thesis it will be proposed a variation of H&E using a iron hematoxylin and a trichromic eosin composed with eosin y, phloxine b and orange G. This staining has proven to give exceptional details about tissue organization, far more compelling than normal H&E; moreover, this modification has demonstrated to be capable of detecting bacteria inside the dental tubules with greater clarity as compared to previous techniques.

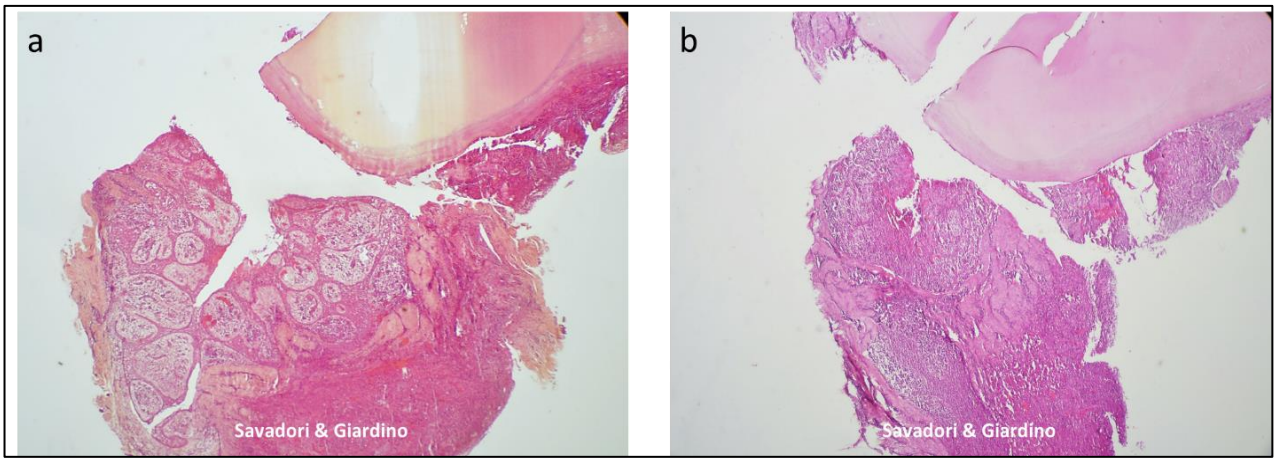


Fig 36: first molar root , transversal section. a) 200x hematoxylin eosin trichromic, b) 200x standard hematoxylin and eosin

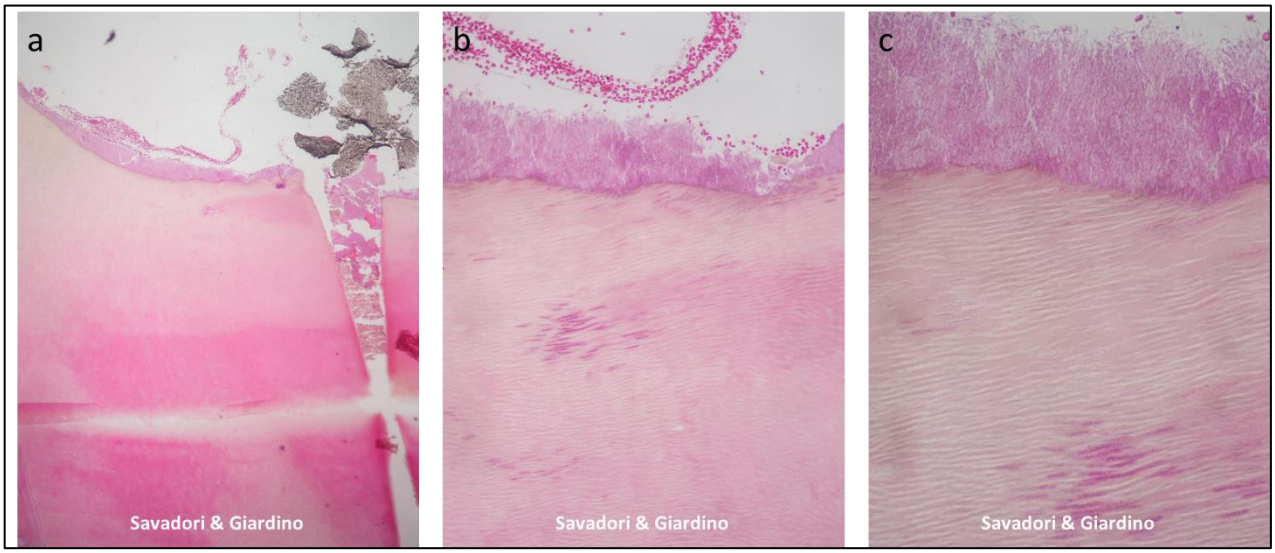


Fig. 37 Iron hematoxylin- trichromic eosin. Premolar root (same sample of fig 25 subsequent section). Bacteria cover the upper part of the root and presence detectable in dentinal tubules. a) 40x, b) 200x c) 400x

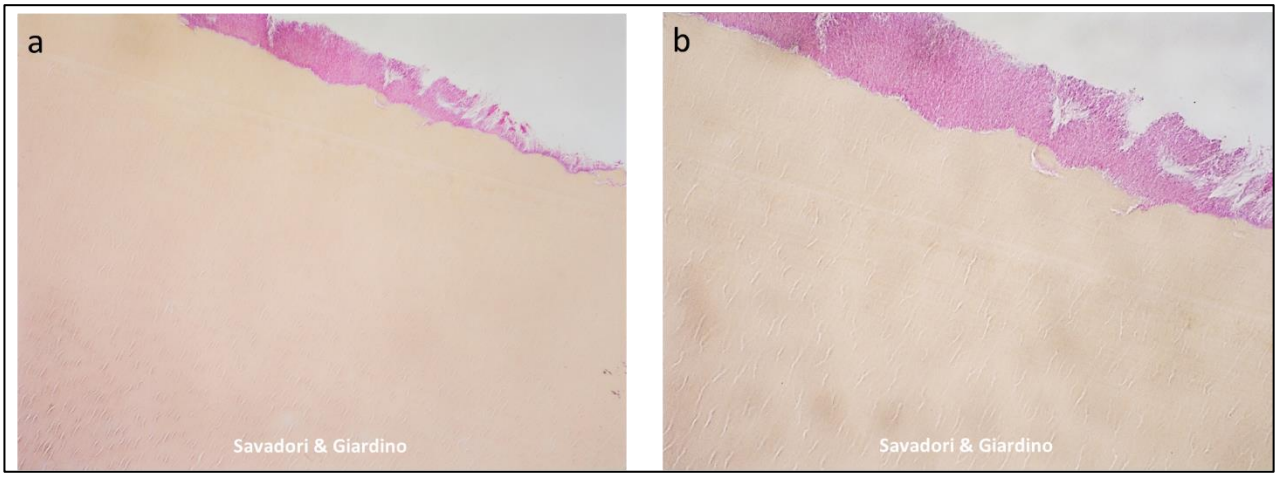


Fig.38 Iron hematoxylin-trichromic eosin. Transversal section of an incisor. (same sample of fig 24 subsequent section). Bacteria organized around the outer side of the tooth. a) 20x, b) 400x

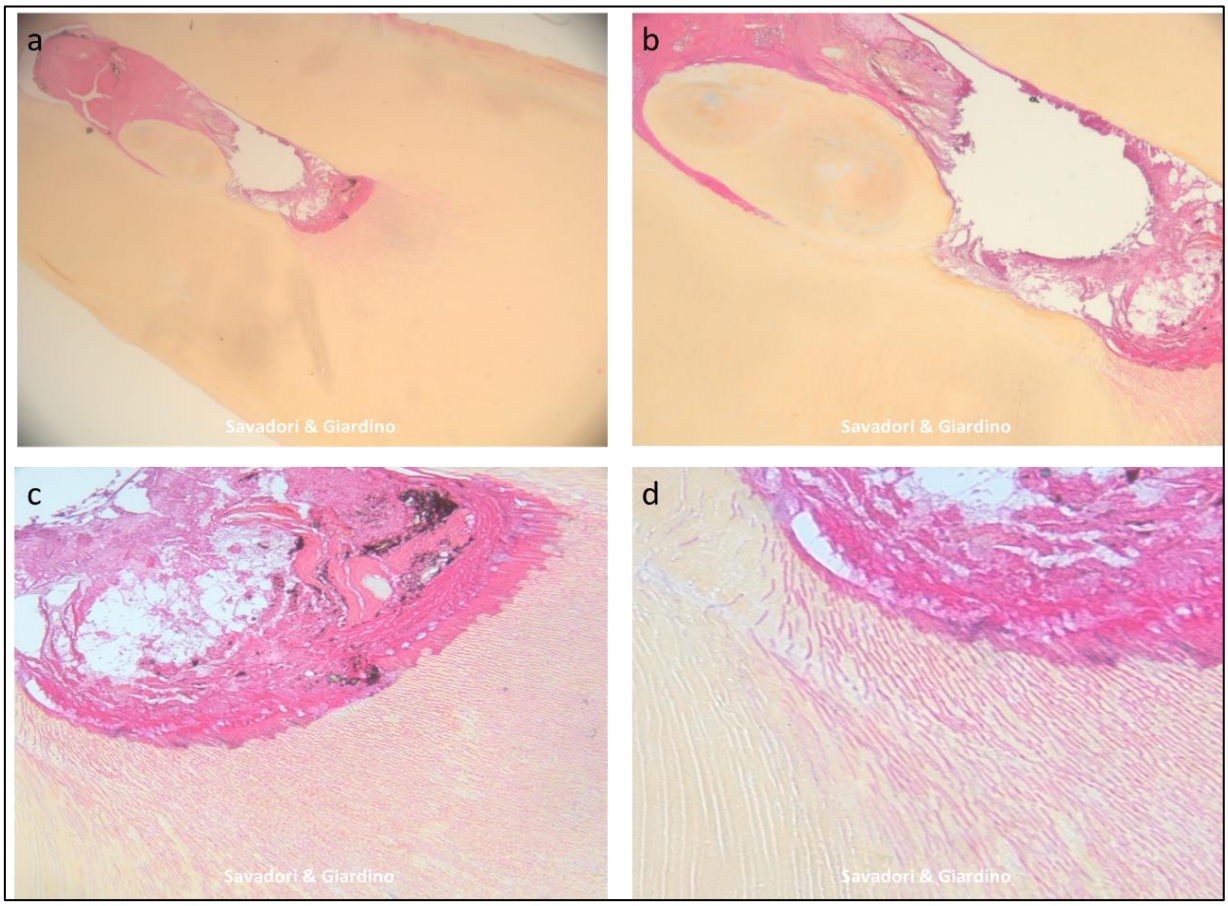


Fig. 39: Iron hematoxylin – trichromic eosin. Transversal section of a first lower molar (same sample of fig 23 and 34 subsequent slide). Calcified pulp surrounded by necrotic pulp. Bacteria visible in dentinal tubules. a) 40x, b) 100x c) 200x, d) 400x

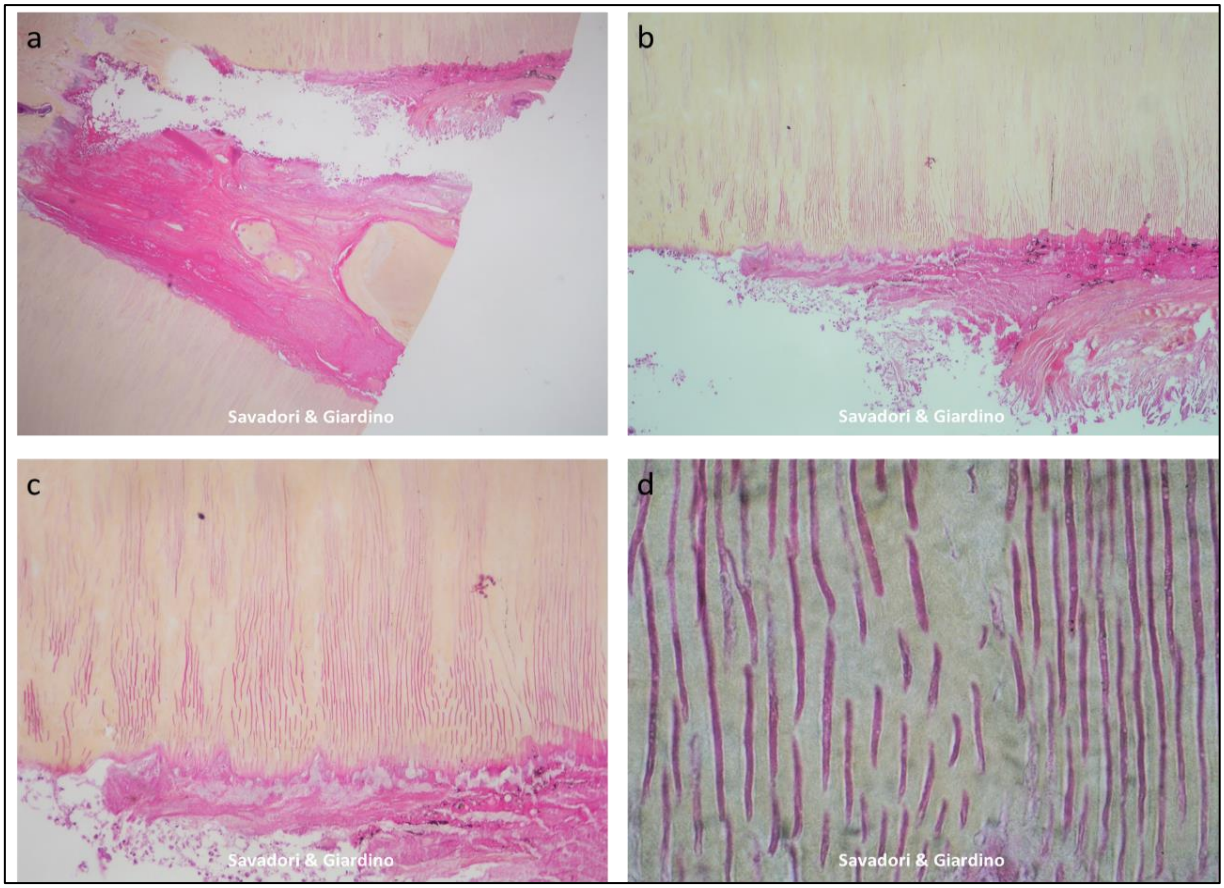


Fig. 40: Iron hematoxylin – trichrome eosin. Apical portion of a first lower molar mesial root (same sample of fig 22 subsequent section. Pulp stone surrounded by necrotic pulp. Bacteria clearly visible inside dentinal tubules. A) 40x, b) 100x, c) 200x and d) 1000x

Mallory trichrome

This trichromic method is designed for studying the connective tissue, especially collagen fibers, mucus and ground substance (Mallory F.B. 1936).

In tooth histology Mallory's trichrome doesn't found large applications while in the study of periodontal tissue it could be useful. This staining technique can point out well red blood cells and so gives a good overview of the bloodstream. For example in the images below it is shown a rat mandible that presents many blood clots caused by a massive intravascular coagulation.

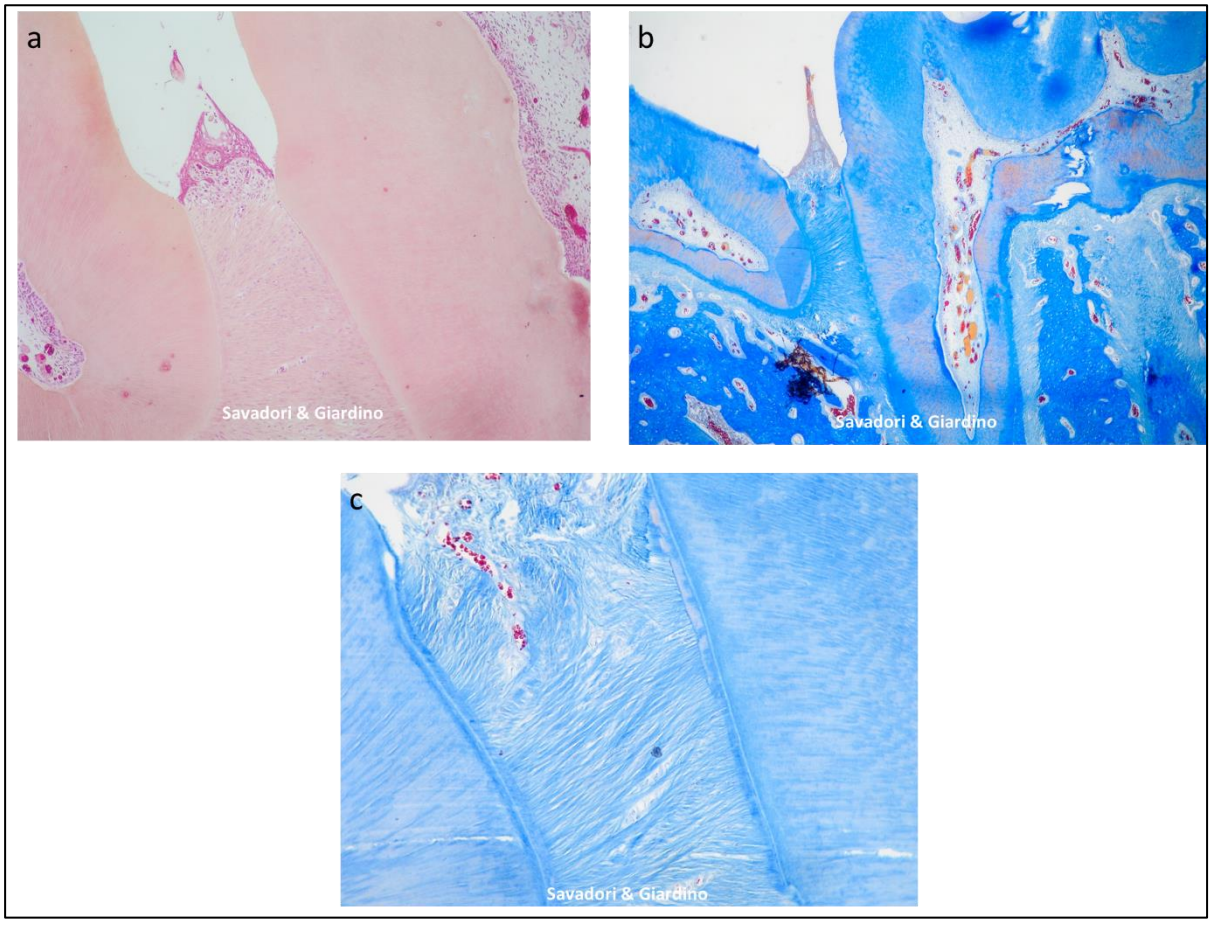


Fig. 41: a) hematoxylin – trichrome eosin of a molars rat 100x. b,c) 40x and 100x respectively, Mallory's trichrome of the same sampele of fig a subsequent slide.

Discussion

About samples handling

Sample processing is a difficult task due to the characteristics of teeth. The simplest way to obtain histological slide is to make ground sections. These sections can provide an overview of the calcified structure and enamel. In order to study the enamel, the ground sections are the only way to obtain histological slices because during the decalcification process the enamel is completely removed. Ground sections can't give any information about soft tissue, to study them a tooth must first undergo decalcification.

In fig. 1 are shown ground sections of a third molar; these images show well the organization of the dental tubules (fig 1e) as well as dentin and cementum (1a), an accessory canal is visible in the figure 2c. Cementoblasts are visible, entrapped in their lacunae, and their prolongations can be appreciated as well (1b). Enamel (1d) and enamel-dentine junction (1e) is well represented, but the pulp in the pulp chamber (1f) is barely visible and appears very damaged.

Decalcification has always been a struggle between speed and preservation of the tissue: the strongest the decalcifying agent is, the fastest the process will be. But using a strong mineral acid like nitric acid 5%, that can complete decalcification in few days, lead to very poor results in terms of preservation of tissue integrity. In fig. 16 it is shown a cow tooth decalcified via nitric acid; the quality of histological slide is very poor: the structure of dentine and cementum is fairly preserved, and it's possible to distinguish between dentine and predentine (16d). Conversely, soft tissues are severely damaged, and the pulp is completely detached from the canal walls. The gingival tissue isn't preserved, and in the fig. 16c is clearly visible the basal laminae detached from the surrounding connective tissue. Overall the stainability is poor with an excessive uptake of hematoxyline due to the low pH of the sections. The only advantage of using nitric acid is that the sections are easy to cut, but this aspect doesn't overcome the overall poor results of this procedure.

For obtaining the best outcome EDTA is the best option, but it requires a considerable amount of time to reach a complete decalcification. Though, the time needed could be markedly reduced using the microwaves. Microwaves have been used in the past years to this purpose, but the protocols proposed lack of a solid histological description of the result, and the decalcification process required that the duty cycle was controlled by a person, or a very expensive instrumentation like lab-dedicated microwave ovens had to be used.

Using an Arduino board can turn a cheap commercial microwave into an automated device allowing continuous work 24 hours a day, further reducing the decalcification time. It is relatively easy to setup this kind of device, not many competences in the electronic field are required, and this board is not expensive at all. This approach was compared with a conventional method of decalcification: AM solution. AM solution is inexpensive and easy to prepare and because it is a buffered weak organic acid, it isn't harmful like the minerals one, isn't very fast but still significantly faster than EDTA.

The mineral content of bovine teeth is less than human's teeth and for this reason, the decalcification time is faster in cow teeth than in human. Using animal teeth gives the chance to analyze the surrounding tissue; cow teeth are characterized by a large amount of dental pulp, and this is an advantageous aspect that permits to analyze the status of soft tissue after decalcification.

Figures 8, 9, and 10 show a comparison between decalcification done by AM and some variants of EDTA-MW. At first sight, it is noticeable that there aren't major differences between the different methods: the pulp structure is well-preserved with odontoblasts well attached to the canal walls and disposed in a fine organization forming their characteristic palisade. In the sample treated with EDTA-MW, 20% pH=10 the odontoblasts appear to be less organized probably because the sample wasn't perfectly parallel to the microtome blade. Dentine and predentine are easily recognizable as well as the blood vessels and red blood cells along with some white blood cells.

The same clear structure could be observed for soft tissues. Figures 11, 12 and 13 show gingival tissue. As can be observed, each decalcification method seems to preserve the structure. Connective tissue and tissue cells are bound together, and the cell organization appears undisturbed by the decalcifying agent: particularly in figure 13 it is possible to see the cell progression from the basal lamina to the outer layer and the progressive denuclearization of the cells. Microcirculation in the connective tissue is preserved and blood cells are clearly visible.

Overall stainability is good as well as the cuttability.

An interesting aspect is that prolonged treatment with EDTA-MW doesn't cause any damage: as it can be seen in the related figures, samples processed for 60 and 90 days don't show any trace of damage and these samples were the easiest to cut.

A true difference between AM and EDTA-MW can be found in the handling of very delicate tissue like cerebellar matter: figure 14 shows two mouse heads decalcified with AM (14b) and EDTA-MW (14a), in this case, the acidic action of formic acid contained in AM solution causes damage to the cerebellum's with matter while in the case of EDTA-MW this kind of damage isn't present.

Figure 15 shows the oral cavity of a mouse and independently from the method used the tissue structure are well preserved.

This comparison between AM and EDTA-MW evaluated so far in the present work leads to some potentially useful consideration:

- AM solution, despite its long age, is a simple and reliable method to obtain a complete decalcification in a reasonable amount of time.
- Microwaves may considerably accelerate the speed of action of EDTA leading to a complete decalcification in only a week instead of months.

- Automatization of microwave oven, either self-made or professionally accomplished, is a really helpful advance that may improve standardization and efficiency of the technique.
- Samples could be left in EDTA-MW well after the decalcification end-point without harming the tissues
- EDTA-MW causes no damage even to very delicate tissue like central nervous system
- Anna Morse solution can be used in the routine practice while EDTA-MW could be adopted when there is the need to decalcify large specimens and/or when a large amount of soft tissue is present within calcified tissue. Large samples usually require a great amount of time, and decalcification is faster on the outside of the sample and lower on the inner parts; so, if the time required to achieve a complete decalcification is long, the acid could start to damage the already decalcified tissue.
- Anna Morse and EDTA-MW don't interfere with stainability and allow a good cuttability, achieving excellent results.

It must be underlined that decalcification is only one-step, even though essential, of sample handling during the histological preparation. Much care has to be observed also during the dehydration and embedding. These are two steps that require time and haste isn’t a wise option. Diafanizaation is a key point because is the last call to check if a proper decalcification has occurred, if there is some white spot there is a chance to rehydrate the sample and continue the decalcification, after this point there isn’t the chance to turning back.

Microtome blade and glass slide are also important: standard blades aren’t fit to cut hard tissue in general and are less effective in cutting teeth, it is possible to obtain some ribbons but the blade will not last long. Glass slide has to be coated in some way otherwise the slice will be surely lost.

About tissue staining

Bacteria staining technique

Staining bacteria in calcified tissue is a complicated task for two main reasons: first the staining technique is quite complicated, and the multitude of different protocols doesn't helps, second the decalcifying process damage bacteria lowering the amount that can be detected. A study has shown (Wijnbergen & Van Mullem P.J. 1987) a significant decrease of stainable bacteria after exposure to weak and strong acid as well as EDTA, even if in a minor entity.

To overcome the first problem, in this thesis a brief and easy modification of Brown-Brenn staining has been proposed. Brown-Brenn staining is an evolution of the classical Gram procedure: the normal version of the Gram isn’t suitable for tooth tissues. Fig 17 shows an apex of a first molar stained with normal Gram and is clearly visible the absence of details and the overall overstaining. Brown-Bren, with a second differentiation passage, can resolve the overstaining problem turning the background to a neutral color. This technique has its problem: it has many passages, and Gram-negative bacteria are somewhat difficult to detect. Two works proposed by Larry (Larry K 1984) and Churukian (Churukian C.J. & Schenk E.A. 1982) proposed a modification of Brown-Hopps staining but increasing the number of passages and so the chances of errors.

GS-Brown-Brenn is a shorter version of the standard protocol, its key features are:

- modified Gram iodine that can lead to a stronger bound between crystal violet and the Gram-positive cell wall
- first differentiation made by a mixture of isopropyl alcohol and acetone in order to mild the action of the acetone and have a more controlled differentiation
- rather concentrated fuchsin solution
- one bath of xylene after the last differentiation

This procedure has proven to be reliable and easy to perform, the samples tested weren't many, so this work has to be considered a qualitative study rather a quantitative one.

Analyzing the sections colored with this technique we can see a good stainability of bacteria independently from the region of the tooth-

Fig 18bis and 23 show a population of Gram-negative bacteria inside the dentinal tubules. Figure 19 shows a secondary canal that links the two main canal, the presence of necrotic pulp renders the detection of bacteria more difficult due to the presence of a background coloration, figure 20 shows a portion of canal of the same sample of fig. 19, in this case a hole in the gutta-percha filling has been colonized by Gram-positive bacteria.

Fig.22 shows a mixed population of Gram-positive and negative bacteria: Gram-positive bacteria spread from the canal wall to the center of the canal while the Gram-negative are more adherent to the canal wall and penetrate in the dentinal tubules. These pictures show the ability of this technique to identify both kinds of bacteria even if they are tight pack together.

This procedure works well in more “opened” spices: fig 21 is relative some organic debris, manly plant origins, infiltrated in the upper portion of a mesial molar root. Gram-positive bacteria are easily recognizable.

Biofilm is also well colored: fig 25 shows a dense biofilm formed on the upper portion of a premolar root. In the fig 4c are visible some Gram-positive bacteria that have colonized the dentinal tubules beneath the bacteria layer. Fig 24 shows two structures of biofilm: 24a and 24b are similar to the type shows in fig. 25 but less dense. The biofilm represented in fig 23c and 23d are more compact and less defined, the color tends to read and probably indicates a mixed population of Gram-positive and negative bacteria.

Overall, this staining procedure has shown to be reliable and give information about both type of bacteria with a very good definition. It is easy to use and

relatively fast to complete, for this reason this technique is a good choice for a routine procedure.

The Gram-Twort stain is a modification of the Gram stain and has some similarity with Brawn-Brenn. Gram - Twort is relatively easy to make but the lack of second differentiation and the use of fast green as counterstain doesn't give a proper contrast. As it is possible to observe in fig.26, all the section has the chromaticity that goes to purple to green bluish. The biofilm is detectable but appears more uniform if compared to the fig. 25. Bacteria in dentinal tubules are visible, but a high magnification is requested. This technique is still useful but not meet the quality of a Brown-Brenn o GS-Brown-Brenn.

Gram, and its modifications are not the only way to detect bacteria: PAS staining, particular if combined with light green has shown to be particularly good in detecting bacteria.

PAS staining is often used to detect fungi and glycogen, very rarely to detect bacteria. Is true that many substances are PAS positive (a substance that reacts with the Schiff reagent), but inside a tooth the number of this substrates are fewer compared to those that can be found in a soft tissue. Moreover, a PAS-positive signal found inside a dentinal tubule is very probable that is a bacteria due to the really small dimensions of this tubules.

Fig. 27 show a subsequent section of the sample shown in fig. 19, in the section stained with GS-Brown-Brenn the detection of bacteria is difficult due to the presence of background stain but in the one stained with PAS-light green, the shape of bacteria are more define. The Same thing is found in a comparison between fig 22a and 33a: necrotic pulp in PAS-light green is stained in green and bacteria, stained in purple, are easily identifiable. In fig. 33b and 33d bacteria are detectable even if the background turns to a deeper blue-green. Fig 35. Shows bacteria population in the necrotic pulp

surrounding a pulp stone: In this case, the background is light colored increasing the contrast with the bacteria.

One thing that can be noticed is the minor quantity of bacteria stained in fig 34., this can be dependent on the composition of the bacteria membrane: as has been mentioned earlier not all kinds of bacteria respond in the same way to Schiff reagent. This should not be necessarily a flow: find bacteria stainable with a Gram technique but not with PAS-light green can be used to draw some bacteria category groups.

PAS staining has demonstrated to be more sensitive than a Brown-Brenn staining. Fig.32 shows the same sample of fig.25: the large layer of bacteria is well defined in both cases but bacteria in dentinal tubules are more represented in the sections stained with PAS. Im any case bacteria detected in dentinal tubules, when are stored, are very defined: fig. 30, 28e and 28f show bacteria stained with this method with the optimum result, in particular in the fig. 28f thanks to the high magnification, the shape of singles bacteria are noticeable.

PAS-light green has some limitations when many different substrates are stained in the same moment like fig.28 a,b,c,d and in particular fig 29. In fig 29a and b is clearly visible some vegetable tissues, vegetable walls are PAS positive. In fig. 28d are detectable some bacteria, they can be identified by their conformation, and a yeat is seen in the active division. The presence of vegetable cell walls somehow masks the presence of bacteria due to their pas positivity.

The Same situation can be found in fig.29 where organic debris poses some difficulties to a clear vision of bacteria without, however, preventing their observation.

PAS-light green should not be considered an alternative to Brown-Brenn technique or its replacement but a method to use alongside Brown-Brenn.

Both stains give information about bacteria but differently and the results can be combined to have a clearer view of a bacteria colonization.

Morphological stains

Hematoxylin and eosin is the most popular stain in histology: is the base of almost all histological investigation. Maybe due to its widespread and routinary usage, this coloration may be undervalued.

Hematoxylin and eosin are usually used with its standard protocol, but adding some modification the result can be significantly improved. Fig. 36b shows a standard hematoxylin-eosin and fig 36a show a hematoxylin and a modified eosin with orange G and phloxine B. The section stained with the modified eosin show more details than the normal one in particular in the lesion tissue.

There is some variation of hematoxylin too, for example, iron hematoxylin: fig 15a shows a mouse oral cavity stained with a hematoxylin-trichromic eosin and fig 15b iron hematoxylin has been used the normal one instead. Iron hematoxylin stain the nuclei with a darker color than normal hematoxylin and this increase the definitions of the singles cell. In some cases the overall color tends toward a purplish staining but not always: in fig. 14 the use of iron hematoxylin doesn't interfere with the bone staining, which remains orange-pink, and muscular staining, that remains bright pink.

Iron hematoxylin-trichromic eosin is capable of detecting biofilm as is shown in fig. 38 and fig. 37, but is good to stain bacteria in the dentinal tubules. The presence of orange G gives a good contrast allowing to have a good picture of bacteria stained with phloxine b. Fig. 40b, c, d and fig. 39c and d give an excellent view of bacteria inside the tubules, the number of bacteria stained is over than the other techniques.

Because hematoxylin-eosin staining is a general stain, there isn't the certainty that all the substrate colored are living fixed bacteria, perhaps even cellular debris and biofilm residue are colored but the results obtained with this procedure are encouraging and worthy of further investigations.

In conclusion, adding some modification to a routinary staining, like hematoxylin-eosin, could increase the information obtainable.

Mallory's trichrome is a common staining used to study collagen and connective tissue. There isn't much application for teeth but can be useful for a periodontal tissue. Fig 41a shows an hematoxylin – trichrome of trans-settal fibers, with this technique they aren't much visible but with a Mallory's trichrome this fiber are well defined, fig. 41b.

Mallory's trichrome is very suitable for the study of blood circulation: red blood cells are colored in bright purplish-red and are easily observable. Fig. 41b shows a rat molar with an altered blood circulation, in fact the rat was affected by a massive intravascular coagulation and the blood clots are stained in orange.

Case report

In 2013, a 59-year-old man was referred to the at School of Dentistry, the University of Magna Graecia at Catanzaro, for evaluation and endodontic retreatment of the lower left first molar because of loss of coronal restoration. A clinical examination revealed extensive destruction of the hard tissues, and the tooth was tender to occlusal percussion and palpation of the vestibular margin. A periapical radiograph (Rx) showed short root canal fillings in mesial and distal roots (fig. 42a). Then, endodontic retreatment was suggested to the patient and consent form was obtained. In the first visit the remaining carious tissue was eliminated until only sound tooth structure remained. After cleaning the decayed dentin, a pre-endodontic build-up was necessary to obtain four-walled cavities for proper isolation and to preventing microleakage. After building the walls, the tooth was isolated with a rubber dam and the endodontic treatment was initiated (fig. 42b), filling of root canals was performed using gutta-percha Continuous Wave of Condensation (fig. 43c). At six months, a Rx control showed no lesion and good periodontal health with composite build-up integrity maintained (fig. 42d). Then the tooth was prepared prosthetically and temporized with a provisional restoration. After one year of absence due to work outside Italy, the patient returned to our attention without the provisional restoration on the restored tooth, pain after mastication (dated about one month), deep probing defect and fracture line localized mesio-vestibularly (fig. 42f, g, h). A periapical Rx showed enlargement of the lamina dura and little periapical lesion in the mesial root (fig. 42e). These clinical and radiographic signs confirmed VRF of the mesial root, then the mesial root was extracted after tooth hemisection (fig. 42i, l).

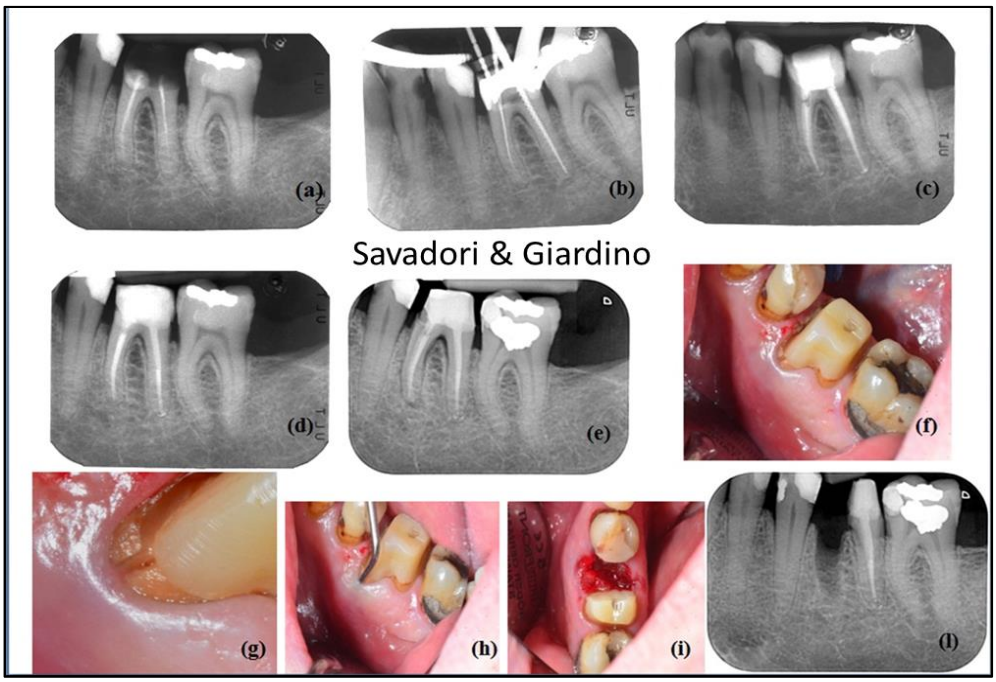


Fig. 42: clinical pictures of the case report. a) Rx before the first treatment; b) Rx during treatment; c) Rx after the endodontic procedure; d)control Rx taken after six months since the treatment; e) Rx taken after one year, showing a periapical lesion and an enlargement of the lamina dura,; f,g) tooth fracture; h) periodontal probing depth measurement; i) tooth hemisecting; l) final Rx after tooth extraction

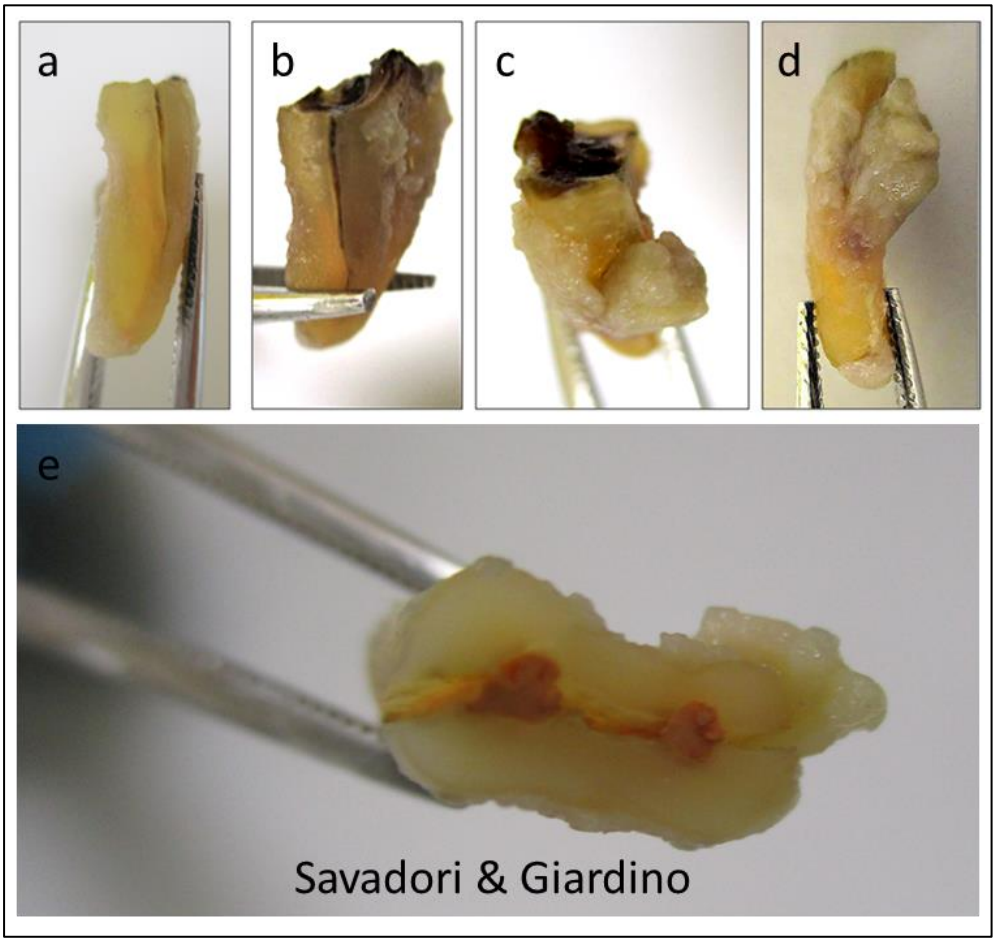


Fig. 43: mesial root after extraction. a,b,c,d) side wiev of tooth, two fractures are present, one on each side of the tooth; e) occlusal wiev of the mesial root.

The upper two/third of the tooth were cut transversely and then stained with PAS. Despite the fracture was extending throughout the diameter of the tooth reaching both canals, bacteria could be found only in the margin (fig. 44b,c,d,e) of the fracture line but not inside the canals system (fig. 44a).

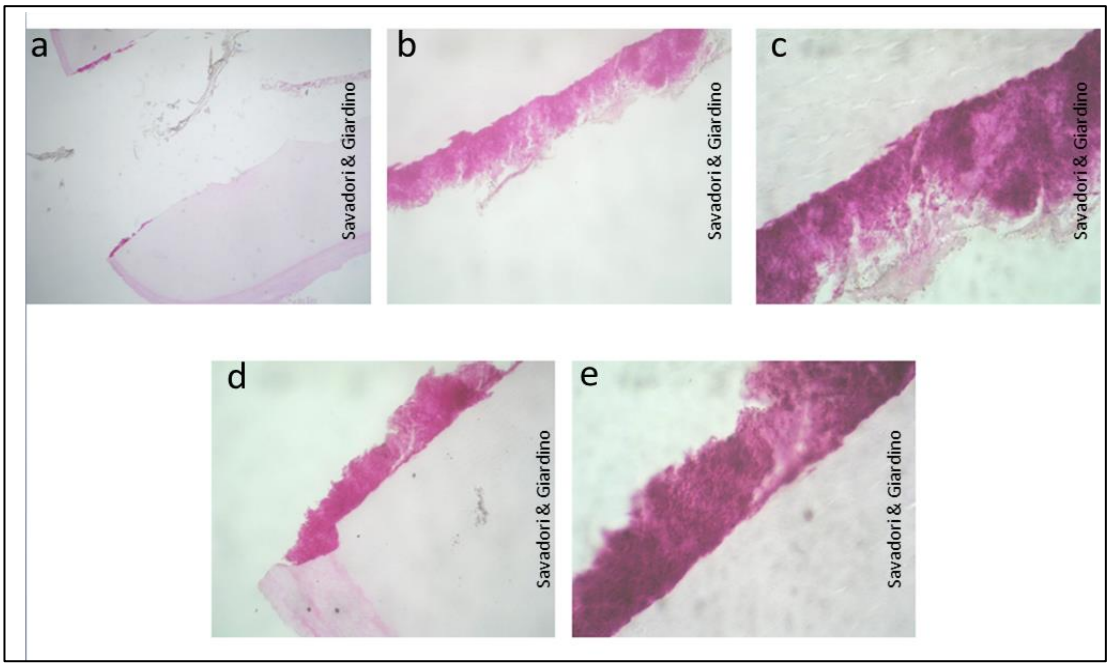


Fig. 44. a) bacteria in the fracture line 40x PAS; b,) biofilm on the upper side of the fracture line 100x PAS; d,e) biofilm bacteria on the lower side of the fracture line 100x PAS.

The sectioning of the apical portion in bucco-lingual direction, has shown the presence of a lateral canal not clean and the presence of a bacteria population.

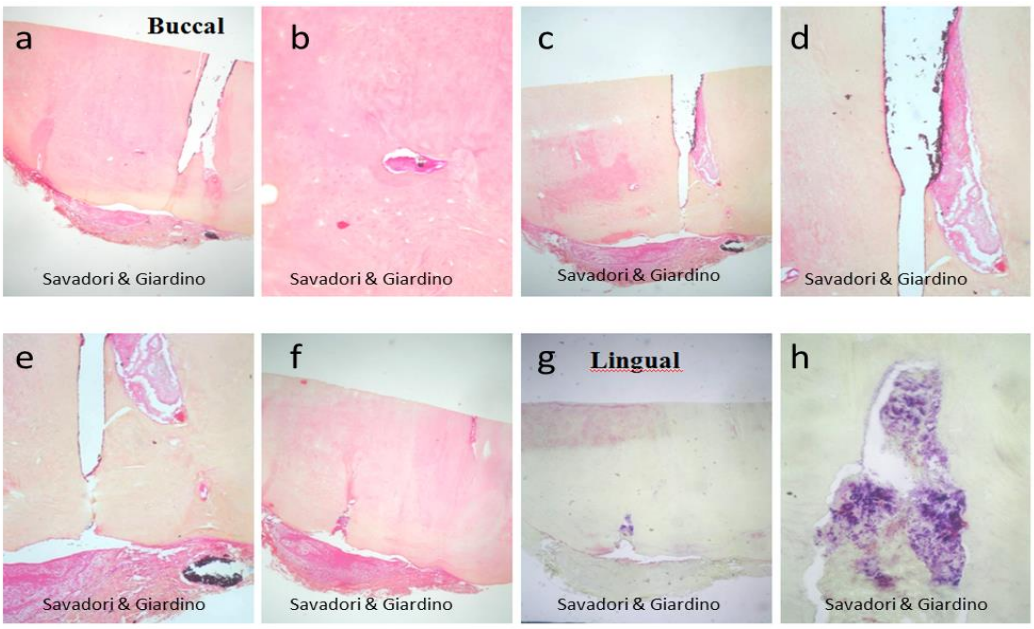


Fig. 45. a) apical lateral canal and lesion tissue 40x H&E; b) detail of lateral canal filled with necrotic pulp 100x H&E; c) subsequent tissue section better showing the lateral canal not completely clean 40x H&E; d) detail of lateral canal 100X H&E; g,h) B&B staining showing bacteria infection in the last 2mm of the dental apex

The histological study of this tooth has allowed to characterize two different clinical situations that led to tooth loss: the first was the formation of the tooth fracture but did not lead to a generalized infection although there were ample spaces for a bacterial infiltration; the second was the presence of a chronic apical periodontitis.

Conclusions

Tooth histology has come a long way since its introduction but in the last decades has, somewhat, slowed its course. In literature are used the same techniques and little has done to improve the overall technique. Furthermore all the knowledge gathered by the members of the school of Vienna begins to be lost and proper information about how to handle teeth for histological propose is difficult to find.

There is plenty of room to improve the histological techniques but is a process that requires much time and even more dedication. Histology can be seen as a routinary procedure but this lead to routinary results or can be considered a sort of a scientific-art form in which the scientific result blends with the careful research of the right dyes combination and staining techniques. To see histology in this way not only can produce good scientific outcomes but also images beautifully to watch.

This work is dedicated to my mentor Dr. Luciano Giardino that, thank to his expertise and guide during all this long and tiring path, has rendered possible this thesis. This work is also dedicated to the spirit of the members of the school of Vienna that have been forced to leave their country to fulfill their scientific vocations, an exodus that, without the tragic implications of their time, continue today with exceptional scientists that are forced to leave their home in order to follow their dream called science.

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Bibliography

Brown J.H. and Brenn L.A. (1931). “A method for the differential staining of gram-positive and gram-negative bacteria in tissue sections.” Bulletin of the Johns Hopkins Hospital. 48:69-73

Brown R.C. and Hopps H.C. (1973). “Staining of bacteria in tissue sections: A reliable gram stain method.” American Journal of Clinical Pathology 60:234-240

Bruziewicz-Mikłaszewska B. (2003) :”Peter Monavius (1551–1588) - physician from Wroclaw and his doctor's degree thesis (1578) "De dentium affectibus" - the oldest work Stomatology about topics in Europe”.Advances in Clinical and Experimental Medicine.

Clayden E.C. (1952). “A discussion on the preparation of bone sections by the paraffin wax method with specieal reference to the control of decalcification.” Journal of Medical Laboratory Technology. 10:103–123

Coolidge E.D. (1940). “Rudolf Kronfield, BS, MS, DDS.” Journal of Dental Research. 19:266-276

Cox C.F., Hiroyasu Y., Suzuki S., Akimoto N., Ruby J.D., Maeda N., Moimoi Y. (2017). “How histology enriched endodontics as a specialty and nurtured pulp biology: our endodontic continuum from 1528 into the 1900’s – Part I.” Dental, Oral and Craniofacial Research. 6: 1-9

Fauchard P (1728) b. “Le chirurgien dentiste ou traite des Dents.” The Surgeon Dentist.Mariette, Paris.

Fauchard P. (1746) a. “Methods for removing decay and restoring teeth.” Le chirurgiendentisteou traité des dentes”. 2:346.

Fraenkel M (1835) “On the observations of the intrinsic structure of the human teeth”.University of Breslau. Friedlander

Gottlieb B (1927). “Tissue changes in pyorrhea.” Journal of American Dental Association. 14:2178-2207.

Gottlieb B. (1921). “Der Epithelansatz am Zahne. Dtsch Monatschr” ZHK 39:142-147.

Gottlieb B. (1926a). “Tissue changes in pyorrhea.” Federation Dentaire Internationale. Seventh international dental congress, Philadelphia, August 23-27. Transactions, pp. 373-402.

Gottlieb B. (1926b). “What is a normal pocket?” Journal of American Dental Association. 13:1747-1751.

Gottlieb B. (1939). “The aetiology of dental caries.” British Dental Journal. 67: 377-387

Gram C. (1884). “Ueber die isolirte Far bung der Schizomyceten in Schnitt und Trockenpraparaten.” Fortschritte der Med. 2, r85-r89.

Guarner J. and Brandt M.E. (2011). “Histopathologic diagnosis of fungal infections in the 21st century.” Clinical Microbiology Reviews. 2:247-280

Gupta S., Jawanda M.K. Manjunath S.M., Bharti A. (2014). “Qualitative histological evalutiation of hard and soft tissue components of hard and soft tissue components of permanent human teeth using various decalcifying agents –a comparative study.”

Harris D.C. (2007). “Quantitative Chemical Analysis.” W.H .Freeman and Co 7th edition. pp 228-245.

Harris H.F. (1900). “On the rapid conversion of haematoxylin into haematein in staining reactions.” Journal of Applied Microscopy. 3:777-780

Hotchkiss R.D. (1948). “A microchemical reaction resulting in the staining of polysaccharide structures in fixed tissue preparation.” Archive of Biochemistry and Biophysics. 16:131-142

Hucker G.J. and Conn H.J. (1923). “Methods of Gram staining.”Technical. Bulletin. No. 93 New York Agriultural Experiment Station.

Hunter J (1771) “Treatise on the natural history and diseases of the human teeth.” 1st edition, J. Johnson, London UK.

Khavari P.A., Bolognia J.L., Eisen R., Edberg S.C., Grimshw S.C., Shapiro P.E. (1991). “ Periodic acid-Shiff-positive organism in primary cutaneous *Bacillus cereus* infection.” Archives of Dermatology. 4:543-546

Kiernan, J A., (1999). ” Strategies for preventing detachment of sections from glass slides.” Microscopy Today. 6: 22-24

Kremenak N.W and Squier C.A. (1997). “Pioneers in oral biology: the migrations of Gottlieb, Kronfeld, Orban, Weinmann and Sicher from Vienna to America.” Critical Reviews in Oral Biology & Medicine. 2:108-128

Kronfeld R. (1933). “Histopathology of the teeth and their surrounding structures.” Philadelphia: Lea & Febiger.

Kronfield R (1939). “Research and the future of dentistry.” Bulletin of Chicago Dental Society. 19:17,21 Also published in Journal of the California Dent Association 15:207, 239; Journal of the Michigan State Dental Association 21:205-206; Texas Dental Journal 57:279-280;Washintong State Dental Journal 7:15, 17-18.

Kumar G. S. (2015). “Orbans’ Oral Histology &Embriolgy” 14th Edition Elsevier India 2015

Lillie R.D. (1947). “Studies on the preservation and histologic demonstration of glycogen.” Journal of Tech Methods. 27:23-61

Logan W.H.G and Kronfield R. (1933).” Development of the human jaws surrounding structures from birth to the age of fifteen years”. Journal of America Dental Association 20:379-427

Luna L.G. (1968) “Manual of histologic staining methods of the armed forces institute of pathology.” 3rd edition. New York, Blakiston Division, McGraw-Hill

Madden V.J., Henson M.M. (1997). “Rapid decalcification of temporal bones with preservation of ultrastructure.” Hearing Research. 1-2: 76-84.

Malaparte L. (1928). ”Action des polyalcools sur l’acide periodique. Application analytique.” Bulletin de la Societe Chimique de France Series 4. 43: 683-686

Malaparte L. (1934). “Etude de l’action des polyalcools sur l’acide periodique et les periodates alcalins.” Bulletin de la Societe Chimique de France. Series 5. 1:833-852

Mallory F.B. (1936) “The aniline blue collagen stain.” Stain Technology. 11:101-102

Mattuella L.G., Bento L.W., Vier-Pelisser F.V., Araujo F.B., Fossati A.C.M. (2007). “Comparative analysis of two fixating and two decalcifying solution for processing of primary human teeth with inactive dentin carious lesion.” Journal of Dental Science. 22:55

Mayer P. (1903). “Notiz uber Hamatein und Hamalun.” Zeitschrift Fur Wissenschaftiche Mikroskopie und Mikrokopische Technik. 20:409

Mayers C.P. (1970). “Histological fixation by microwave heating.” Journal of Clinical Pathology. 3: 273-275.

McManus J.F.A. (1946). “Histological demonstration of mucin after periodic acid.” Nature. 156: 202

Morse A. (1945). ”Formic acid-sodium citrate decalcification and butyl alcohol dehydration of teeth and bones for sectioning in paraffin.” Journal of Dental Research 24:3-4

Oppenheim A. (1934). “A practical suggestion.” International Journal of Orthodontia and Dentistry for Children. 3. 894-895

Oppenheim A. (1942). “Human tissue response to orthodontic intervention of short and long duration.” American Journal of Orthodontics and Oral Surgery. 28-263-301

Oppenheim A. (1944). “A possibility for physiologic orthodontics movement.” American Jpurnal of Orthodontics and Oral Surgery., 30: 277-328

Orban B (1926a) “Nutrition and teeth.” Federation Dentair Internationale. Seventh international dental congress, Philadelphia august 23-27- Transactions, pp.257-268

Orban B (1926b) Histology of enamel lamellae and tufts. Federation Dentaire Internationale. Seventh international dental congress, Philadelphia. August 23-27. Transactions, pp 482-492

Orban B. (1928). “Dental histology and embryology.” Chicago: Rogers Printing Co.

Orban B. (1929). “Two years' activity of the Research Department, Chicago College of Dental Surgery, Dental Department of Loyola University. Bur 29:83-88.

Orban B. (1941). “Biologic considerations in restorative dentistry.” Journal of America Dental Association 28:1069-1079.

Orban B. (1944). “Oral histology and embryology.” St. Louis. Mosby.

Orban B. (1958). “Periodontics: A concept-theory and practice.” St. Louis: Mosby.

Raschkow I. (1835) ”Meletemata circa mammalium dentium evolutionem. Diss Inaug Anatomico- Physiologica. Friedlaender, Vratislaviae.”

Raymond B and Alford W.C. (1953). “The chemical basis of the periodic acid Schiff reaction of collagen fibers with reference to periodate consumption by collagen and by insulin”. Journal of Histochemestry & Cytochemestry 1:62-67

Sangeetha R., Uma K., Chandavarkar V. (2013). “Comparison of routine decalcification methods with microwave decalcification of bone and teeth.” Journal of Oral and Maxillofacial Pathology. 17:386–391.

Sicher H. (1949). “Oral anatomy.” Dental Items of Interest. 71:551

Taylor R.D. (1966). “Modification of the Brown and Brenn gram stain for the differential staining of gram-positive and gram-negative bacteria in tissue sections.” American Journal of Clinical Pathology 46:472-476

Teruel J., Alcolea A, Hernández A, Ruiz A.J. (2015). “Comparison of chemical composition of enamel and dentine in human, bovine, porcine and ovine teeth.” Archives of Oral Biology. 5:768-775.

Titford M.(2005). “The long history of hematoxylin.” Biotechnic & Histochemestry. 2:73-78

Tomes J. (1848) “On the structure of the dental tissues in the order of Rhodntia.” Philosophical Transaction of the Royal Society of London. 140:529-567.

Twort, F.W. (1924). “An improved neutral red, light green double dtaining for animal parasites, microorganisms and tissues”. Journal of State Medicine. 32: 351

Verdenius H.H.W and Alma L. (1958). “A quantitative study of decalcification methods in histology” Journal of Clinical Pathology. 11:229-1958

Weinmann J.P., Sicher H. (1947). “Bone and bone: Fundamentals of bone biology.” St Louis: Mosby

Weinmann J.P., Sicher H. (1955). “Bone and bones: Fundamentals of bone biology”. 2nd edition St Louis: Mobsy

Zerosi C. (1986). “ Manuale di tecnica istologica dentale.” ed Masson 1986