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SUCCESS OF PULP REVASCULARIZATION DURING GESTATION IN DOGS: HISTOLOGICAL STUDY

Endodontic

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ABSTRACT

Pregnancy in women is characterized by numerous physiological changes aimed at ensuring the development of the fetus as well as the repair and regeneration of the tissues of the mother. Pulp revascularization is a treatment option for immature teeth with pulp necrosis, an alternative approach to organic bases which, unlike artificial apical barrier techniques, allows the continuation of root development and the recovery of pulp vitality, the success of this treatment relies on the presence of stem cells in the pulp environment, growth factors and certain physiological conditions that are favored during pregnancy.The present study focused on the histological interpretation of the tissues that developed in the root canal and root space of the treated teeth adopting the dog as an animal model to illustrate this experiment, where the objective is to study the success of pulp revascularization in a pregnant dog in comparison with a non-pregnant one. Our results show the presence of primary inflammatory foci, some vascularized tissue, and hard tissue deposition throughout the canal in the pregnant dog, in contrast to the non-pregnant dog where only cementocyte-like cell layer deposition and native bone tissue is observed.

KEYWORDS

Odontostomatological regeneration, pulp revascularization, pregnancy, histology, Stem cells, Dental pulp

INTRODUCTION

Pulpal revascularization is a relatively new technique for the treatment of permanent teeth with an immature apex and infected pulp whose root development has been interrupted. It aims to regenerate the pulp-dentinal complex of necrotic immature teeth by restoring the functional properties. (1, 2, 3, 4).

The principle of the technique is to induce the formation of a blood clot within the previously disinfected canal. This clot will be colonized by stem cells which will participate in the formation of new tissue, in order to create a tissue capable of re-initiating the process of root building, root wall thickening and apex closure (5).

This technique is therefore based on disinfecting the canal, inducing sufficient apical bleeding to fill the canal as much as possible and create the clot that will occupy the entire canal, which is protected by the placement of a tight restoration (6).

In order to optimize revascularization, certain conditions are necessary: The apices must be open: experimental and retrospective studies have shown that revascularization is maximal if the diameter of the apical foramen is greater than 1.1 mm in humans, which corresponds to a file diameter (7). Others speak of a diameter greater than 0.70 mm, that means a file diameter of 70 (8). The root walls must be very thin. The subject must be young: Several studies suggest that the younger the individual, the better the potential for stem cell regeneration and healing capacity (9).

Root canal preparation for revascularization consists of passive chemo mechanical preparation using hand instruments and auxiliary chemicals with antimicrobial properties and low toxicity (10, 11).Revascularization has been introduced as an alternative to apexification since 2001 (12). An increase in tooth wall thickness, closure of the apical foramen and regression of apical periodontitis were observed over a five-month period, demonstrating radiographic success in a case of immature tooth with pulp necrosis (13). Conventional treatment of immature teeth is done by inserting a long-term calcium hydroxide paste in order to induce the formation of a calcified barrier which will also allow subsequent filling of the root canal (14). Substances and antibiotic paste, followed by a final coronal restoration are required. (15)

An alternative technique to the use of calcium hydroxide is to produce an apical MTA barrier, avoiding the periodic exchange of intracanal drugs (16).

However, the disadvantage of both techniques is that they do not allow for continuity of root development, which leads to root weakening and thus increases the risk of fracture (17). Revascularization is a therapeutic option for young permanent teeth with pulp necrosis, as it allows for continuity of root development (18).

Pregnancy is a special period is characterized by a high serum concentration of growth factors (PDGF "Plateled-derived Growth Factor", GH "growth Hormone", BMP "Bone Morphic protein", IGF-1 "insulin like growth factor 1", TGFβ "Transforming Growth Factor-β". (19)

These protein molecules contribute to the differentiation of the embryonic stem cells of the fetus, but also in the repair and regeneration mechanisms of the tissues of the carrier mother (20).

In dentistry, these growth factors are used in the field of tissue engineering to regenerate and restore damaged oral tissues, these engineering techniques consist of culturing pulp stem cells in a suitable culture medium containing a culture carrier, nutrients necessary for cell division and survival, hormones, and selected growth factors to direct the differentiation of these naive stem cells into a specific cell lineage (21).

One week after fertilization, the blastocyst has exhausted its nutrient reserves, so its free life can only be short (22). It then differentiates into an embryonic button and a trophectoderm (23). The latter then defines, with the maternal organism through a process of implantation, a structure that will allow it to develop during pregnancy: the placenta. Thus, the placenta is a transitional organ essential for the maintenance of the pregnancy and the development of the fetus (24). The placenta is characterized by an intense endocrine function, with a high capacity for synthesis but with the need for precursors from the fetus or the mother. It also has numerous hormone receptors. It produces steroid hormones, cytokines, glycoproteins, growth factors, and other proteins (25). The metabolism during this period increases by 20% during the third trimester of pregnancy.

The processes of stem cell proliferation and differentiation are enabled by gene transcription activity, which is regulated by transcription factors. These factors modify gene expression and provide information about cell functions.

The prerequisite for differentiation is the existence of an undifferentiated state, maintained by the existence of growth factors, cytokines, intercellular contacts, and extracellular matrix (26).

The differentiation of pulp stem cells into a specific cell lineage is determined mainly by components in their microenvironment, such as growth factors, receptors, signaling molecules, transcription factors and extracellular matrix proteins. (27)

Immunohistochemical staining and in situ hybridization showed the presence of growth factors and their receptors during pulp stem cell differentiation (28). The effect of these molecules on cell differentiation was tested in vitro.

The table below summarizes the different growth factors identified by immunohistochemical markers in the pulp environment.

Table I: Action of growth factors for use in regenerative endodontics (29, 30)

Plateled-derived Growth Factor (PDGF)	• Cell proliferation • Dentinal matrix synthesis Odontoblastic differentiation, Dentinogenesis
Tansforming Growth Factor-β (TGFβ)	• Cell proliferation • Extracellular matrix synthesis Odontoblastic differentiation • Dentinogenesis • Chemotaxis
Bone Morphogenic Proteins (BMPS)	• Odontoblastic differentiation • Dentinogenesis
Vascular Endothelial Growth Factor (VEGF)	• Odontoblastic differentiation • Cell proliferation
Fibroblast Growth Factor (FGF)	• Cell proliferation • Dentinogenesis • Chemotaxis
Insulin-like Growth Factor (IGFS)	• Odontoblastic differentiation • Cell proliferation
Nerve Growth Factor (NGFS)	• Odontoblastic differentiation

Several growth factors that are secreted during pregnancy have been found to contribute to the process of odontostomatal tissue regeneration such as BMPs, PDGF, GH, TGF-β and others.

These factors are produced in relatively higher concentrations during pregnancy than in the normal female state (31).

The mechanism of action of growth factors and the signaling pathways involved in tissue regeneration in dentistry are, until now, poorly explored and clarified.

Among the main growth factors involved in the pulp revascularization process, which are massively produced during pregnancy in women, are PDGF and TGF-β.

In certain pathological conditions, pulp revascularization is interrupted, leading to necrosis.

Researchers have been interested in regenerative techniques to revascularize the tooth, this pulp revascularization process relies on several key points including the presence of stem cells, and biological mediators (hormones and growth factors) to direct the differentiation of pulp stem cells into a specific cell lineage (32).

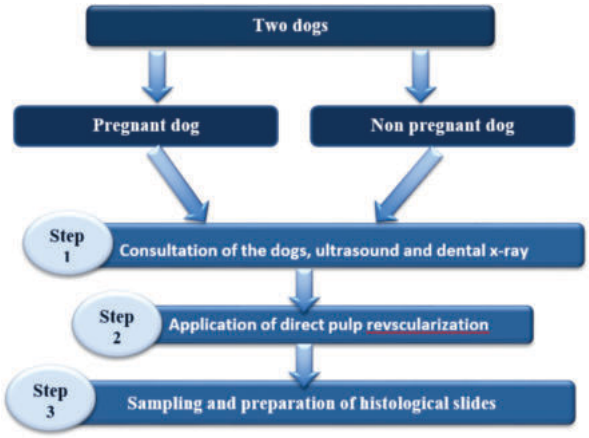
The success of this new therapeutic approach depends on several other physio-chemical parameters, such as the pH, the oxygen content in the pulp environment, the nutrients required for pulp stem cell proliferation, as well as the concentration of biological messengers involved (33).

Based on a physiological observation, it has been found that some hormonal changes during pregnancy will favor the pulp revascularization mechanism, as mediators for cell and tissue differentiation and proliferation are secreted to a greater extent in the pregnant woman, thus ensuring the development of fetal tissues and cells, but also the repair and regeneration of injured, damaged or even lost tissues of the surrogate mother. (34)The physiological changes experienced during pregnancy will also contribute to this mechanism.

Like all other cells in the body, pulp stem cells require oxygen for their biological functions, including division and differentiation, which is favored during pregnancy, as during this special period the oxygen content increases remarkably, due to the increase in blood flow, which allows the massive conduction of oxygen by the erythrocytes, as well as the growth factors and nutrients necessary for the success of the pulp revascularization therapy. (35, 36, 37)

MATERIAL AND METHOD

The choice of the animal model After the various tests, the dental X-rays carried out as well as the data collected from the literature on all the animals tested (Albinos, cats, dogs and rabbits), it was concluded that the animal model which will be useful for our research work is the Siberian dog (husky), because the root canal treatment will be easily carried out given the simple dental structure of this animal. We then adopted two dogs, one pregnant and one not pregnant.



Induction of pulp necrosis, hence the creation of an access cavity in the molar of both dogs, in order to induce an intentional necrosis of the tooth under study, this can be done by bacterial infiltration of the exposed pulp tissue.

Application of pulp revascularization took place four weeks later and consists of revascularization therapy, and it was proceeded as follows; Anesthesia of the doges, X-rays of the teeth, Clinical and radiological confirmation of the total necrosis of the tooth concerned, Placement of an endodontic operating field (the dam), Disinfection of the root canal with 1% sodium hypochlorite, Induction of intra-canal bleeding by over-instrumentation, with an overtaking of the endodontic files by the length of the treated roots. (Aggression of the periapical region), Placement of a sterile cotton until the clot forms (3 min), Sealing with a bioactive material (Monoxide Tri Aggregates) MTA, and finally a coronal restoration with a glass ionomer cement

Four weeks later, the harvesting is done by anesthetizing the animal under the control of the veterinarian. General anesthesia is supported by local anesthesia of the harvesting site.

For the first time in dogs, we used a piezoelectric device for cutting and harvesting the osteodental specimen, the principle of which is the preservation of the soft tissue. Bone harvesting is particularly atraumatic. The dogs develop less post-operative swelling and therefore heal better.

Fixation The conservation of the samples taken is done in 10% formalin, the aim of which is to immobilize the cellular and tissue constituents, but also to prevent cellular autolysis following the physio-chemical changes of the conservation medium, which are totally different from the physiological conditions of the animal, **Paraffin embedding,** which will allow for thin and regular cuts; **Microtome cuts:** The paraffin block sections are made with a microtome allowing section slices (cuts) of 2 to 5 µm thickness to be made. The sections are collected on glass slides; **Staining:** The stain used in our case is the one using haematoxylin-eosin as a base stain; **Mounting** After being dehydrated, the stained sections are mounted between the slide and coverslip; **Light microscopy,** each sample was analyzed under the light microscope to check for the presence or absence of vital tissue in the canal space.

The histological identification criteria for dentin, cementum, bone and PDL are as follows: **dentin**: presence of dentinal tubules; **cementum**: absence of dentinal tubules and adhesion to dentin, presence of

cementocyte-like cells; **bone**: presence of Havers' canals with evenly distributed sterocyte-like cells; **and PDL**: presence of Sharpey's fibers and fibers connecting the cementum and bone.

The purpose of the histological study of the dental specimen taken is to visualize the tissues, cells or extra- cellular matrix (ECM) after the application of the pulp revascularization procedure, in order to identify the different tissues and cells that have neo-formed after this treatment by determining their location and configuration.

Histology thus allows the description of cellular and tissue morphology in terms of architecture and molecular interactions.

RESULTS

The first sample is taken from the non-pregnant dog two weeks after the endodontic procedure.

The surgical specimen was fixed with 10% formalin (as previously described), the specimens were then processed, embedded, cross-sectioned and stained with haematoxylin and eosin. Each sample was analyzed under a light microscope.

The histological slides were observed under a light microscope at different magnifications, and showed the absence of a fleshy bud**,** cementoblast-like cells on the surface of the cementum, the presence of cement cells and intracanal bone tissue.

According to the histological examination of the present study, three types of tissue were mainly present in the canal space: cementum, bone tissue, and desmodental tissue.

The experimental period for this first harvest for the non-pregnant dog was only two weeks (from endodontic procedure to harvest); therefore, maturation of the tissue generated in the pulp space appeared incomplete.

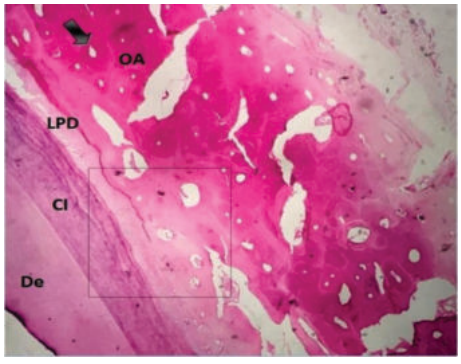


Figure 1: Cross section of the apical third of the root canal of the molar of the non-pregnant dog (magnification x 40). This figure shows the presence of alveolar bone "OA" consisting of osteons (black circles), periodontal ligament (desmodont) "LPD", cementum (CI: intracanal cementum) and dentin "De", it should also be noted that there is no vascularised tissue and no inflammatory reaction, hence no sign of regeneration or partial pulp survival.

- *The presence of the Alveolar Bone "AB". The black circles delineate osteons which constitute the structural unit of the alveolar bone, they are uniform and characterized by the presence of Havers' canals (indicated by the black arrow) which provide passage for capillaries and nerve fibres.* ***(Figure 1)***
- *Presence of the periodontal ligament or "PDL" The periodontal ligament primarily performs a supporting function by attaching the tooth to the surrounding alveolar bone. This function is mainly performed by the periodontal ligament fibers that form a strong fibrous union between the root cementum and the alveolar bone, which are collagenous fibres known as Sharpey fibers.* ***(figure 1 and 2)***
- *The presence of root canal cementum "C" which is a mineralized tissue surrounding the root dentin from the cement-enamel junction to the apex, it is the cellular cementum with intrinsic fibers.* ***(figure 1 and 2)***
- *The presence of dentin characterized by Dentinal canaliculi or tubules, which are cylindrical cavities dug into the thickness of the mineralized dentin.* ***(figure 1 and 2)***No vascularized tissue or partial pulp survival.

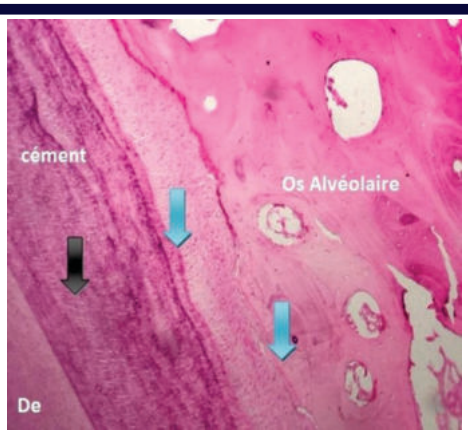


Figure 2: *A magnified view of the area framed in figure 1 (magnification x100)*

The black arrow shows collagen fibers in the cementum tissue, these intrinsic fibers are cementoblastic, a cellular tissue comprising type I collagen fibers synthesized by cementoblasts, fully mineralized, and without any particular orientation.

The blue arrows show the sharpey fibers that characterize the alveolar ligament, which form a junction between the cementum and the alveolar bone.

The main results obtained in the non-pregnant dog: Absence of fleshy buds**,** Presence of cementum cells and bone tissue in the root canal space**,** no signs of regeneration or inflammatory reaction following the endodontic treatment applied, Absence of pulp survivalThe second sample was taken from the pregnant dog (Donna) and is taken three weeks after the endodontic procedure.

The surgical specimen is fixed in 10% formalin and processed in the same way as the first specimen. (Fixation, paraffin embedding, microtome cuts, staining, mounting, and observation of the histological slides under the light microscope).

The difference with the first sample is the plane of the cut with the microtome, this time we proceeded with longitudinal cuts, to be able to study the tissues present all along the pulp canal.

We also removed a healthy tooth (control), which had not undergone any intervention, so that a comparison could be made between the normal tissue present in the canal and the changes observed following pulp revascularization on the previously necrotic and disinfected tooth.

The same criteria (as above) were used for the identification of dentin, cementum, bone and the periodontal ligament histologically.

The following are the results of the observations of the histological slides under the light microscope.

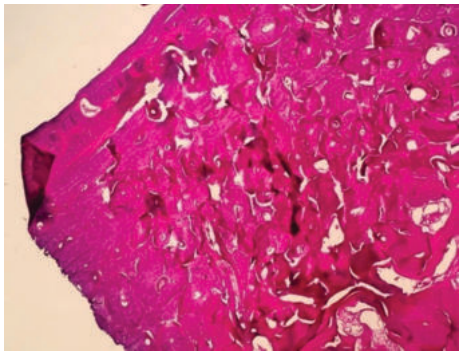


Figure 3: *Presence of neo-formed vascularized tissue at the apical region of the removed tooth.*

This figure shows a cross-section of the apical part of the tooth extracted from the pregnant dog "Donna," from which the formation of a new highly vascularized tissue can be observed. The blue arrows show

newly formed young blood vessels. (40x magnification)

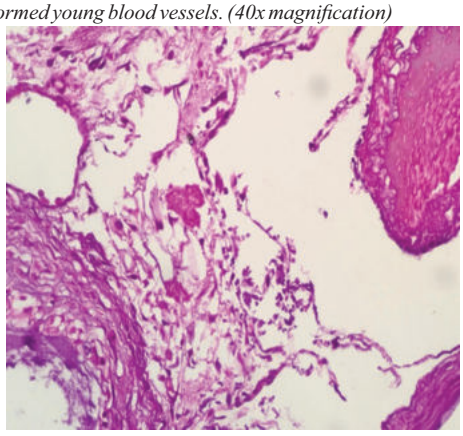


Figure 4: *Intracanal healing with inflammatory infiltration and cementum-like tissue formation in the apical third of the removed tooth root.*

The red arrows show lymphocytic cells surrounded by young, highly vascularized tissue (black arrows show red blood cells) and the white arrow indicates one of the newly formed blood vessels.

The large blue arrow shows the formation of new cement-like tissue (magnification x100)

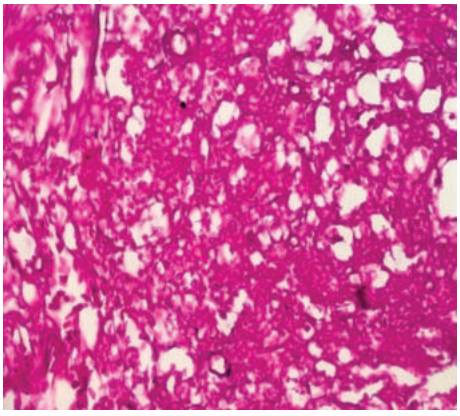


Figure 5: Presence of inflammatory infiltrates in the middle of highly vascularized tissue (X400)

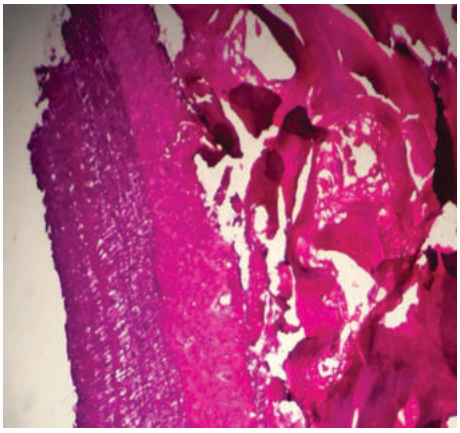


Figure 6: Presentation of the sites of primary inflammation and the different regenerated tissues in the canal.

Intracanal healing with inflammatory sites and numerous lymphocyte infiltrates. External resorption of the apex exists at the site of inflammation.

the periapical tissues are regenerated with loose soft tissue.

Presence of the lamina dura (cribliform lamina) which is the denser region than the alveolar process with perforations that allow the passage of blood vessels and nerves (x 40).

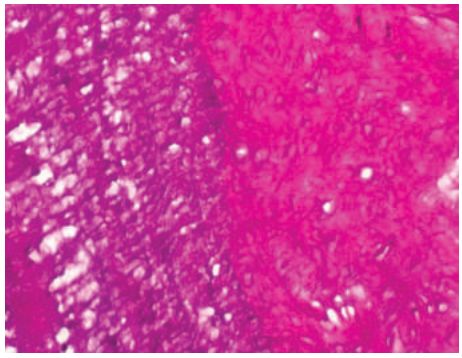


Figure 7: Cement bridge formation at the apex (Grossissx100)

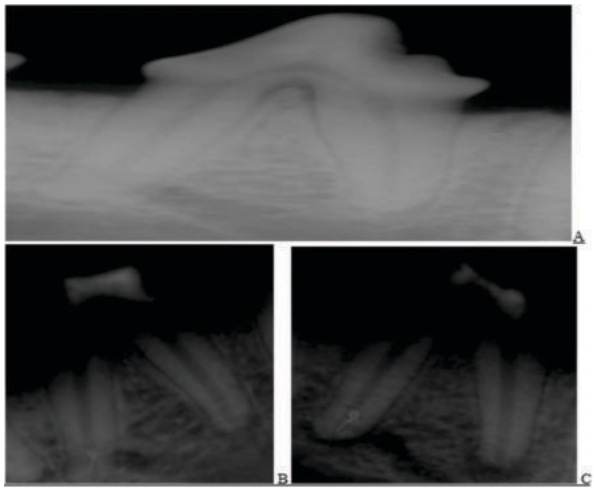


Figure 8 (A) an X-ray of the tooth before treatment, (B) an X-ray of the same tooth just after root canal treatment, and (C) an X-ray taken three weeks after treatment and just before sampling.

The first X-ray (A) shows the normal state of the tooth before the application of revascularization, a normal pulp canal can be seen. Figure (B) shows an X-ray taken a few seconds after root canal treatment where a narrowing of the pulp canal is observed; and for figure (C), it is an X-ray taken just before the removal of the treated tooth, where a small thickening of the canal walls is observed as a result of the deposition of the cementum and the newly formed bone tissues deposited along the canal as shown by the histological results previously described.

The main results obtained:

Presence of primary inflammatory infiltrates; Presence of highly vascularized tissue; Presence of cement cells; Presence of intracanal bone tissue; Wall thickening due to hard tissue deposition along the root canal.

DISCUSSION

Histology is very important in the process of pulp revascularization. Indeed, it will demonstrate almost infallibly that a regenerative process has taken place. This histological analysis involves cutting the tooth into thin strips and therefore extracting it. This is why there are only a few results concerning humans and these are very recent. Indeed, the teeth studied had to be extracted because of root fractures or for orthodontic reasons.

Using the dog as an animal model to illustrate the success of pulpal revascularization during pregnancy, the histological results of the specimen from the pregnant dog show highly vascularized tissue formation around the foci of primary inflammation as shown in **Figure 5 and 6,** in contrast to the dental specimen from the non-pregnant dog which shows almost no weak regenerative response. These results confirm our hypothesis of the success of revascularization in pregnant women, but in the absence of molecular confirmation of the follow-up of pulp stem cells, it is not sufficient to determine more precisely their destiny in the therapeutic process of teeth with necrotic pulp. The technique used is the observation of histological slides under the light microscope, after staining with haematoxylin-eosin, and is not

sufficiently robust, as are other cellular and tissue identification techniques such as immune-histochemical labelling or flow cytometry, to prove that pulp stem cells are indeed the source of newly regenerated tissue. For definitive identification of odontoblasts from cementoblasts, the dentin-specific marker sialo phosphoprotein, which is expressed only by odontoblasts in the canal, should be examined to verify the nature of the hard tissue.

For the first sample from the non-pregnant dog, and based on histological examination, three main types of tissue are present in the canal space: cementum along the dentinal walls causing thickening of the canal walls, bone tissue, and a periodontal ligament-like tissue. The experimental period for this first harvest was only two weeks (from the endodontic procedure to the harvest); therefore, the maturation of the tissue generated in the pulp space seemed incomplete. Loose connective tissue or collagen gel is present in the root canal space. It can be predicted that with a little more time, neofomal tissues (cementum, bone and desmodental) will organize and mature better in the canal space. The absence of inflammatory reactions for this first sample may be due to several factors: the time between the treatment and the sample which was only two weeks, the bleeding which was not sufficient to provide all the elements necessary for the success of this treatment, or the physiological state of the non-pregnant dog. Dental sampling of the pregnant dog (Donna) showed both healing with tissue regeneration and significant amounts of inflammatory infiltrates adjacent to the newly generated tissue. As shown in **Figure 1**, intracanal cementum is present along the dentinal wall of the canal, with the largest amount near the apex and tapering towards the coronal end. There is highly vascularized inflammatory tissue at the apex and in the canal adjacent to the newly regenerated intracanal cementum. Cementoblast-like cells are visible on the surface of the cementum cell. At the level of the canal, and next to the new cementum layer, there are fewer inflammatory cells, while a more condensed extracellular matrix seems to have been produced. External root resorption occurred in the presence of adjacent inflammatory infiltrates. The intracanal cementum generated may be resorbed by the presence of inflammation.

It is possible that when the pulp tissue was disturbed by the endodontic instrumentation, the irritated odontoblasts rapidly deposited new (tertiary) dentin before undergoing necrosis as a result of the intentional pulp infection. Subsequently, the generated intracanal cementum was deposited on the tertiary dentin. Overall, hard tissue deposits appear to respond to infection and inflammation and are most likely part of the defense mechanism. Bone tissue is scattered in the canal space in both study cases, we call this structure "intracanal bone tissue". Some bone cells were difficult to differentiate from intracanal cementoblasts. Histologically, the intracanal bone tissue appears to have more cells trapped within the hard tissue than in the intracanal cementoblast tissue. The formation of hard tissue bridges within the root canals was observed in the second sample from the pregnant dog.

In summary, and comparing the two results; the first corresponding to the dental sample taken from the non- pregnant dog shows the presence of cementocyte cells at the level of the canal next to bone tissue as shown in **figures** 1, however it does not show any inflammatory phenomena, nor vascularized tissue; In contrast, the second sample taken a week later from the pregnant dog showed foci of primary inflammation and highly vascularized tissue next to cementum cells and intracanal bone tissue.

In the present study, we confirmed that the neo-formed tissue in the pregnant dog is a calcified cementoblastic tissue on the canal walls, which means the migration of stem cells from the peri-apex to the canal space, with the creation of a vascular bridge ensuring the vitality of the formed tissue. This type of cementary formation is similar to the embryonic formation of a dentin-cement complex.

CONCLUSION

Recently, it has been shown that it is possible to treat a permanent tooth with an immature apex and an infected pulp whose root development is interrupted, in order to heal and promote the growth of new essential tissue in the pulp canal. However, a question that intrigues clinicians is what type of tissue is actually generated in the root canal space after the application of pulp revascularization. This approach is based on inducing bleeding in the previously disinfected canal, and, after that, a bioactive biomaterial was used to cap the intra-canal tissue, thus allowing vital tissue to regenerate in the canal space. The induced bleeding favors the migration of pulp stem cells towards the canal and presents a source of growth factors that will direct the differentiation of these stem cells into a specific cell line, after triggering a cascade of inflammatory reactions that will be the origin of the regeneration process.

Pulpal revascularization is considered a better option for treating immature teeth with non-vital pulp and even in cases of severe periapical infection. The advantage of this approach is twofold: it takes only 2-3 visits in a few weeks, and it allows for gains in root thickness and potentially length. Overall, this approach outweighs conventional calcium hydroxyl apexification, which requires several visits over a period of up to several months. The success of pulp revascularisation relies on two essential elements, namely, the presence of stem cells and growth factors to direct the differentiation of these same cells towards a well-defined lineage that will subsequently lead to the formation of new homo or heterogeneous tissue structures. Cell differentiation, growth and survival require the presence of several elements such as sufficient oxygen to ensure its metabolism, nutrients provided by the induced blood clot, a well aseptic support which the previously disinfected pulp canal.

Pregnancy is a very special period, characterized by physiological modifications that will contribute to the success of the revascularization treatment, as there is a hypersecretion of hormones and growth factors by the placenta and the various other endocrine glands (reference), an increase in the stock of stem cells, and a high demand for oxygen to ensure the proper course of fetal development. We took advantage of all of these modifications to apply pulp revascularization on teeth with artificially necrotic pulp, and the histological results obtained validate our hypothesis.

Indeed, and concerning the pregnant dog, we observed the presence of primary inflammatory foci (wich may over time lead to complete regeneration of the previously destoyred tissue), some vascularized tissue, and the desposition of hard tissue along the entire length of the canal, contrary to the non-pregnant dog, where we only observed the deposition of a cementocyte-type cell layer, and bone tissue.

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