Experimental Approaches for Pulp Tissue Regeneration

María Cristina Bucchi Morales
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PhD Thesis
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PhD Thesis presented by María Cristina Bucchi Morales

This doctoral thesis has been carried out under the direction of Dr Cristina Manzanares Céspedes and Dr Josep Maria de Anta i Vinyals
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Abbreviations

- AAE: American Association of Endodontists
- APCs: autologous platelet concentrates
- bFGF: basic fibroblast growth factor
- BMP2: bone morphogenetic protein 2
- BSP: bone sialoprotein
- DMPs: dentine matrix proteins
- DMP1: dentine matrix acidic phosphoprotein 1
- DPSCs: dental pulp stem cells
- DSPP: dentine sialophosphoprotein
- ESE: European Society of Endodontology
- FEA: finite element analysis
- FGF2: fibroblast growth factor type 2
- hAECs: human amnion epithelial cells
- HERS: Hertwig’s epithelial root sheath
- IEE: inner enamel epithelium
- IGF-1: insulin-like growth factor 1
- MEPE: matrix extracellular phosphoglycoprotein
- MTA: mineral trioxide aggregate
- NE: nestin
- OEE: outer enamel epithelium
- PDGF-BB: platelet-derived growth factor BB
- PRF: platelet-rich fibrin
- PRGF: plasma rich in growth factors
- PRP: platelet-rich plasma
- q-PCR: quantitative polymerase chain reaction
- REPs: regenerative endodontic procedures
- SCAPs: stem cells from the apical papilla
- SEM: scanning electron microscope
- SR: stellate reticulum
- TGF- β1: transforming growth factor β-1
- VEGF: vascular endothelial growth factor
Abstract

The aim of this PhD thesis was to study experimental approaches for revitalization of necrotic teeth. Revitalization, also known as regenerative endodontic procedures (REPs), is a relatively new treatment for necrotic teeth which tries to regenerate the dentine-pulp complex instead of obturating the root canal with biologically inert materials (root canal treatment). Until very recently, the most reliable option for the treatment of immature necrotic teeth was apexification followed by root canal treatment. However, endodontically treated teeth remain devitalized throughout the patient's lifetime and therefore defenceless to new caries lesions, as the absence of pulp implies the lack of tooth immune mechanisms. On the contrary, the regeneration of the dentine-pulp complex allows further root development and aims to recover the natural immune and secretory system of the pulp, making teeth more resistant to future lesions or traumatisms.

The therapy was developed to treat necrotic immature teeth (i.e. those that have not completed their root development). Clinically, the outcomes can be considered successful since there is a resolution of the symptomatology, healing of the apical pathosis and further root development in most cases. However, histological analysis has demonstrated that the tissues formed after the therapy are reparative tissues – such as cementum-like tissue – instead of dentine, as well as an unorganized connective tissue, instead of pulp with its characteristic odontoblast layer. Currently, numerous efforts are being made to shed light on the clinical and biological aspects involved in the regeneration of pulp.

Chapter 1: As previously said, evidence shows that no dentine but reparative tissues (cementum-like tissue) are responsible for the root development after regenerative endodontics. As cementum is less hard and less elastic than dentine, the question arises whether a root with apposition of cementum can endure mechanical stress similarly to roots completed by dentine. Thus, one of the objectives of this thesis was to compare the biomechanical performance of cementum- and dentine-reinforced teeth, and therefore to evaluate the biomechanical advantages of dentine regeneration after regenerative endodontics. We developed a finite element model of cementum- and dentine-reinforced teeth and studied the stress distribution after the simulation of biting, trauma and orthodontic movement. The results showed that apposition of hard tissue (whether cementum or dentine) after REPs reduces mechanical stress on...
immature teeth and, more important, that the formation of dentine is advantageous because it, unlike cementum, facilitates an even stress distribution throughout the root. As far as we know, ours was the first study showing the biomechanical advantages of dentine regeneration.

Chapter 2: Odontoblasts are post-mitotic cells that secrete dentine. The isolation and culture of odontoblasts may open numerous possibilities to study this cell type under standardized conditions, shedding light on their roles in dentine formation, immune defence and transmission of external stimuli. We evaluated different protocols of enzymatic treatment to isolate primary odontoblasts from human molars. The results showed that, regardless of the enzymatic solution used, odontoblasts in culture did not remain viable after 24 h. Additionally, we identified increased expression of nestin (NE), bone sialoprotein (BSP) and dentine matrix acidic phosphoprotein 1 (DMP1) in the odontoblast layer compared to pulp fibroblasts. Though primary odontoblasts can still not be cultivated after isolation, characteristic genes were identified to differentiate odontoblasts from pulp fibroblasts.

Chapter 3: We analysed the effects of autologous platelet concentrates (APCs) in the clinical and histological outcomes of the therapy and the different clinical protocols clinically used through systematic reviews. The results indicated that APCs improve the clinical and radiographic outcomes of regenerative endodontics since the teeth treated with APCs achieved significantly better thickening of the dentine walls and root lengthening. However, true regeneration of pulp was not achieved with the addition of platelet concentrates, which only stimulated tissue repair. Additionally, most of the studies did not follow a standard clinical protocol for regenerative endodontic therapy and used irritant and intracanal medicaments that are cytotoxic and affect the differentiation and adherence of the stem cells.

Chapters 4 and 5: As will be mentioned in detail, a small apical foramen acts as a physical barrier that hinders tissue ingrowth into the root canal and therefore reduces the possibility of revitalization of mature teeth. We studied different methods for apical foramen enlargement of mature teeth as a basis to apply it in a further animal study. We analysed manual instrumentation at different working lengths and apicoectomy on extracted human teeth and in situ teeth. We concluded that apicoectomy is not an effective technique for apical foramen enlargement and therefore should not be used for that purpose. Instrumentation
0.5mm beyond the apex resulted in the most effective technique. Later, we performed an animal study and evaluated pulp tissue regeneration/repair in mature teeth and the differentiation of the stem cells from the periapical tissues into odontoblast-like cells by adding preameloblast-conditioned medium. Preameloblast-conditioned medium was applied in pulpectomized ferret canines, whose apical foramina were enlarged using the previously developed method. We observed vascularized connective tissue occupying the apical third of the canal space in 50% of the teeth, showing the potential of revascularization of mature teeth. However, no odontoblast-like cells were observed showing that in vivo odontoblast-like differentiation of stem cells is still not possible with the tested technique.

Chapter 6: Finally, we present here the preliminary data of characterization and odontoblast-like differentiation of amnion epithelial cells. Human amnion epithelial cells (hAECs) express pluripotent stem cell markers and have been proven to differentiate in cells of the three embryologic layers. However, as far as we know, these are the first experiments that have proved the potential of odontoblast-like differentiation of these cells in vitro. To induce the odontoblast-like differentiation, we seeded hAECs over dentine disks treated with EDTA and evaluated the morphological characteristic of cells. We observed that hAECs present a characteristic odontoblast-like morphology, with cytoplasmic processes located in dentinal tubuli, after 48 h. Further studies will be carried out with known concentrations of dentine matrix proteins and qPCR.
Introduction
Section 1: The tooth and its cells
1.1 Tooth embryology

Tooth development is a complex process that involves a mutual and sequential interaction between the neural ectoderm-derived cells (neural crest cells) and cells from the oral epithelium. Neural crest cells give rise to odontoblasts, cementoblasts and pulp cells, while oral ectodermal cells give rise to ameloblasts. During early embryogenesis, cells at the edge of the neural plate (neural crest cells) undergo an epithelial–mesenchymal transition that allows them to separate and migrate within different embryo tissues. One of the homing sites for these ectomesenchymal cells is underneath the dental lamina of the oral epithelium, where they regroup. Later, a reciprocal and complex interaction between the oral epithelium and the underlying ectomesenchyme initiates and regulates all stages of tooth development. Tooth development involves approximately 300 signalling molecules (http://bite-it.helsinki.fi/) and includes four sequential phases: the laminal (initiation), bud, cap and bell stages.

Tooth development initiates with the dental lamina, a thickening of the oral epithelium, at ten separate intervals in each of the upper and lower jaws (Fig. 1a). These separated growths of dental lamina are called the dental placodes. Later, the oral epidermal cells grow down to invade the underlying neural-crest-derived mesenchyme (Fig. 1b), which subsequently condenses around the invaginating epithelium, leading to the formation of the tooth bud (bud stage).
Next, during the cap stage, the epithelial cells continue to expand, adopting a concave form within and around the ectomesenchymal cells (Fig. 1c), and later they differentiate. The cap-shaped epithelial growth of the dental lamina is referred to as the enamel organ. It is composed of the outer enamel epithelium (OEE), the stellate reticulum (SR) – composed of star-shaped cells embedded in an extracellular matrix – and the inner enamel epithelium (IEE) (Fig. 1c-d). The IEE constitutes a single cell layer that surrounds a cluster of condensed ectomesenchymal cells called the dental papilla, which will give rise to dentine and pulp tissue (Fig. 1d-g). The IEE will differentiate into ameloblasts that will form the enamel. A basal membrane separates the enamel organ from the dental papilla, while the dental follicle covers the outside of these two structures and will give rise to periodontal tissues. The enamel organ, dental papilla and dental follicle constitute the dental organ or tooth germ.

The continued growth of epithelial cells generates a bell-shaped enamel organ, thus, giving rise to this phase being called the bell stage (Fig. 1d). During this stage, cells start to secrete dental hard tissues (dentine and enamel) by acquiring
their final phenotype (late bell-stage), which leads the tooth to assume its ultimate shape.

By the cap stage, a group of cells located in the inner enamel epithelia is visible histologically. This structure is called the primary enamel knot and acts as a signalling centre. It promotes the proliferation and folding of the adjacent epithelium. In the bell stage, in a multicuspal tooth, secondary enamel knots appear. They are responsible for cusp formation, so their number corresponds to the number of cusps that will be formed in each tooth. These knots initiate odontoblast differentiation of dental papilla cells that are in contact with the IEE\textsuperscript{6,7}. Thus, it is important to stress that while the first signal of differentiation comes from the mesenchyme during the development of the rest of the ectodermal organs, cell differentiation is induced by the epithelium\textsuperscript{8} during tooth development. Later, at the bell stage, differentiated odontoblasts signal back to IEE cells and induce their differentiation into ameloblasts\textsuperscript{9,10}. At this point of the late bell stage, ameloblasts and odontoblasts, which are separated by the basal membrane, initiate the secretion of enamel and dentine, respectively\textsuperscript{1}.

Odontoblast differentiation is assessed morphologically by the secretion of predentine that causes preodontoblasts to retreat, leaving behind a cell extension (Fig. 2a) from which non-collagenous proteins are secreted. Subsequent to the secretion of predentine, basal membrane degradation allows direct contact between odontoblasts and preameloblasts, which is required for ameloblast differentiation\textsuperscript{9,10}. Thus, terminal ameloblast differentiation is triggered by paracrine signals emanating from predentine-secreting odontoblasts\textsuperscript{1}. Once ameloblasts are differentiated, they start secreting specific enamel matrix proteins\textsuperscript{10}.
Crown formation is followed by the histogenesis of the root. The cervical loop of the enamel organ progresses growing down into the mesenchyme as its stellate reticulum regresses (Fig. 1e). At this location, the IEE and the OEE join, forming Hertwig’s epithelial root sheath (HERS). It migrates apically and extends around the dental pulp and dental follicle until it encloses everything except the basal portion of the pulp, where later it will constitute the apical foramen\textsuperscript{12}. As the HERS progressively encloses the dental pulp, the apical mesenchyme continues to proliferate and differentiate into odontoblasts to form root dentine. HERS is also essential for the differentiation of dental follicle cells to form the periodontal tissues (cementum, periodontal ligament and bone)\textsuperscript{13}. Finally, the dental follicle initiates tooth eruption by regulating both alveolar bone resorption and alveolar bone formation\textsuperscript{14,15}.

Molecular mechanisms of root formation in terms of gene expression and signalling molecules are less studied issues in tooth development\textsuperscript{12}. Therefore, additional research in this area is needed to understand and promote a basic knowledge of root development in immature necrotic teeth.
1.2 The dentine-pulp complex

The dental pulp is a non-mineralized, loose connective tissue, located in a rigid chamber provided by dentine that protects it from the environment\textsuperscript{16}. Mature pulp has four histologically distinguishable layers:

(1) the odontoblastic and sub-odontoblastic Höhl’s cell layer. During the bell stage of tooth development, preodontoblasts undergo a final asymmetrical cell division. This cell division gives rise to two cells: one, in contact with the basal membrane, responds to signals and will differentiate into an odontoblast; and the other daughter cell, not in contact with the basal membrane and named Höhl’s cell, was described as undifferentiated until presumably it is implicated in odontoblast replacement\textsuperscript{17}. While odontoblasts, arranged in a palisade at the pulp–dentine interface, are implicated in primary and secondary dentine secretion, Höhl’s cells would then contribute to reactionary dentine formation after the death of odontoblasts. If both odontoblasts and Höhl’s cells die, pulp stem cells contribute to the formation of reparative dentine\textsuperscript{12}.

(2) a cell-free zone (zone of Weil) rich in capillaries and nerves.

(3) a cell-rich zone where cell density is high. This area is characterized by fibroblast pulp cells or pulpoblasts, pulp stem cells, inflammatory and immune cells and endothelial cells\textsuperscript{12}. Fibroblast-like pulp cells are located mainly in the cell-rich zone. They secrete collagen and non-collagenous proteins and are highly proliferative. Although these cells are involved in the turnover of the tissue, only 1–2\% of pulpoblasts are multipotent stem cells (pulp stem cells [DPSCs])\textsuperscript{12}, as they have self-renewal capability, are progenitors of the osteoblast/odontogenic lineage\textsuperscript{18}, and promote angiogenesis\textsuperscript{19}. They may differentiate into osteoblasts, odontoblast-like cells and other cell types\textsuperscript{20,21}, and are involved in reparative dentine formation\textsuperscript{12,18,22,23}.

(4) Finally, the pulp core contains a large number of vessels and nerves. In the adult, blood and lymphatic vessels, and nervous fibres, mostly unmyelinated, enter the tooth through the apical and accessory foramina.
1.3 Odontoblast characteristics and functions

Odontoblasts are highly differentiated, post-mitotic, long-living cells derived from neural-crest\textsuperscript{24}. As previously indicated, during the bell stage of tooth development, preodontoblasts interact with the IEE and differentiate into predentine secreting cells\textsuperscript{25}. Each differentiated odontoblast includes one single cell process embedded in dentine tubuli\textsuperscript{3,23} (Fig. 2a-b), whereas the cell body stays at the pulp–dentine interface, giving the odontoblast its characteristic morphology (Fig. 2a).

During its lifetime, an odontoblast undergoes three functional phases, or phenotypes: secretory, mature and old\textsuperscript{23} (Fig. 3). During the secretory stage, odontoblasts present a polarized distribution of the organelles that are found near the cell process. This allows rapid secretion of primary dentine, while the nucleus is located at the opposite pole (distal pole). At the time the tooth erupts and reaches the occlusal plane, most organelles of the mature odontoblasts relocate to the distal pole and autophagy vacuoles start to accumulate in the cytoplasm\textsuperscript{26}. Hence, the secretion of secondary dentine is significantly slower, and also the cell number start to decrease\textsuperscript{27}. Maturing odontoblasts gradually flatten, giving rise to old odontoblasts (Fig. 3), which finally form a monolayer at the pulp–dentine interface\textsuperscript{23}. 
Fig. 3. Scheme of the life stages of a primary odontoblast: secretory, mature and old. GC = Golgi complex; JC = junctional complexes; Ly = lysosome; M = mitochondria; N = nucleus; OP = odontoblastic process; PC = primary cilium; RER = rough endoplasmic reticulum; SG = secretory granules. Image reprinted from Couve et al. 2013.

Although odontoblasts are post-mitotic cells (i.e., they are unable to generate new cells), if they undergo necrosis, they may be replaced by odontoblast-like cells generated by pulp stem cell differentiation. As previously stated, these cells synthesize reparative dentine, an atubular tissue with cells trapped within it. This tissue is also called “osteodentine”, because of its similarity to bone matrix surrounding osteocyte lacunae. Therefore, the tissue formed after REPs in an immature necrotic tooth has been described as osteodentine or cementum-like tissue instead of tubular dentine.
Odontoblasts have secretory, sensitive and immune functions, which are described as follows:

1. Dentine-secretory activity

Secretory activity is probably the main and best-known function of odontoblasts. Odontoblasts produce high amounts of an extracellular matrix (predentine) during the whole life of a person. Predentine is composed mainly of type I collagen, but it also has non-collagenous proteins (glycoproteins, proteoglycans and dentine phosphoproteins). The first product secreted by odontoblasts is mantle dentine, which occupies 30–150 μm of the most external zone of the crown dentine. Mantle dentine is an atubular, poorly mineralized and resilient structure\textsuperscript{3,12}. In the root, the atubular, most external dentine is called the Hopewell-Smith layer, or Tomes’ granular layer. The secretion of tubular dentine starts after the secretion of the atubular dentine finishes. Both atubular and tubular dentine generated during tooth development are called primary dentine\textsuperscript{12}, which is produced at a rate of 4–8 μm per day, and allows the bulk of the tooth to be formed in 2–3 years of continuous secretion\textsuperscript{23}. Once the tooth becomes functional and the root is complete, secretion of secondary dentine starts\textsuperscript{31} at a rate of 0.5 μm per day, being responsible for the progressive reduction of the canal space\textsuperscript{3,23}. The structure and composition of the tubular primary and secondary dentine are similar, the only observable difference being a slight change in the direction of the dentinal tubuli\textsuperscript{12,31}.

After predentine secretion is complete, odontoblasts are also responsible for its mineralization. Mineralization starts with odontoblasts uptaking Ca\textsuperscript{2+} and Pi ions at their basal part and releasing them at the apical pole. Moreover, odontoblasts secrete non-collagenous proteins (osteopontin, bone sialoprotein, DMP1, DSPP, osteonectin, MEPE, proteoglycans and others) deposited in the extracellular matrix direct (or inhibit) the nucleation and growth of these minerals that precipitate to form hydroxyapatite crystals\textsuperscript{31}. However, mineralization of peritubular dentine (Fig. 2b) is different from intertubular dentine (Fig. 2b). Peritubular dentine results not from the simple transformation of predentine into dentine but from the passive deposit of serum-derived molecules along the tubular walls, giving rise to an amorphous network of hypermineralized tissue\textsuperscript{31} in which non-collagenous proteins are also accumulated.
Odontoblasts can also produce reactionary dentine against mild or moderate injuries (e.g. caries or abrasion). This tissue has many histological, biochemical and functional similarities to primary and secondary dentine. However, reactionary dentine has its own characteristics that make it more resistant to caries progression, such as fewer and less ordered dentinal tubuli, which have a helicoidal instead of a circular lumen, and also wider hydroxyapatite crystals. Reactionary dentine production is promoted by low amounts of pro-inflammatory cytokines, though it is inhibited by intense inflammation. Thus, the reduction of reactionary dentine production would orient odontoblasts’ metabolism to the production of inflammatory cytokines, inducing a protective immune response.

2. Triggering the pulp immune response

Due to their strategic location, odontoblasts are the first cells to detect the presence of bacteria. The proliferation and metabolic activity of Gram-positive and Gram-negative cariogenic bacteria release bacterial components into dentinal tubuli triggering an immune response. Odontoblasts are able to detect these bacterial components by expressing several bacterial receptors, and they also secrete chemokines that recruit dendritic cells. Animal studies suggest that dendritic cells uptake antigens and migrate to regional lymph nodes, triggering an adaptative immune response. Thus, under a caries lesion, dendritic cell accumulation in the odontoblast layer is the earliest event of dental pulp response to bacteria.

On the other hand, chemokines produced by odontoblasts also control angiogenesis and are involved in the augmentation of capillaries in the pulp under a caries lesion, favouring inflammation.

In addition to the production of pro-inflammatory cytokines, necessary for the inflammatory response, odontoblasts increase their antimicrobial activity by producing beta-defensins, necessary to disrupt the integrity of the bacterial membrane, as well as nitrous oxide, a free radical with antibacterial activity.

3. Sensor for external stimuli

It has been demonstrated that odontoblasts act as sensory cells for both pathological and physiological stimuli. It is hypothesized that they are able to transduce signals to nearby nerve cells, although this mechanism is not well understood. The following membrane receptors are expressed in odontoblasts.
a. L-type Ca\textsuperscript{2+} channels, mechanosensitive K\textsuperscript{+} channels and voltage-gated Na\textsuperscript{+} channels which transduce tooth pain.

b. Several members of the transient receptor potential (TRP) superfamily which play a role in sensory physiology. They detect thermal, mechanical and chemical stimuli.

c. Acid-sensing channels (ACICs) which detect a wide range of pH fluctuations under normal and pathological conditions.

Because of the numerous singularities and functions of odontoblasts through tooth development and after, understanding the odontoblast biology has attracted much interest. Several studies have developed culture models to study and understand odontoblast properties \textit{in vitro}. Among these models are the culture of crown while the odontoblast layer is still attached to the dentine surface\textsuperscript{40,41}, the culture of odontoblasts still connected to the removed pulp tissue\textsuperscript{42}, the culture of odontoblasts that have been scraped from the pulp chamber\textsuperscript{43}, and more recently, by enzymatic detachment from the dentine surface\textsuperscript{44,45}. However, it remains controversial whether it is possible to isolate odontoblasts from human teeth and keep them in culture or whether they are irreversibly damaged by the inevitable disruption of the odontoblast processes due to the isolation procedure. The culture of odontoblasts may open numerous possibilities to study this cell type under standardized conditions, shedding light on their roles in dentin formation, immune defence and transmission of external stimuli, and furthermore to compare them to other related cell types such as pulp fibroblasts or osteoblasts.
Section 2: Regenerative endodontics, a new treatment for necrotic teeth
2.1 REPs of immature necrotic teeth

Root maturation begins after crown formation is complete\textsuperscript{46}, ending 2–3 years after tooth eruption. Therefore, during tooth eruption, the root is still in an immature state\textsuperscript{47} (i.e. short and thin). Additionally, due to the characteristics of the enamel along with the typically deficient cleaning behaviour of children\textsuperscript{48}, the erupting immature tooth is prone to develop caries, which if not treated in a timely manner may lead to the infection and necrosis of the pulp.

When an erupting immature tooth undergoes pulp necrosis, dentine apposition stops and so does the formation of the root. As a consequence, the root's dentine walls remain fragile, thin and short (Fig. 4a), compromising tooth prognosis\textsuperscript{49}.

![Fig. 4. Scheme of an immature tooth (a) and a mature tooth (b). Immature teeth have thin and short dentine walls, while mature teeth have completed their root development and therefore have long and thick dentine walls. This image was taken from Bucchi et al. (2019)\textsuperscript{50}.

Until recently, the standard therapy for immature necrotic teeth was apexification, which aimed to achieve an apical barrier with mineralized tissue or with an apical plug of hydraulic calcium silicate cements, to allow the subsequent obturation of the empty canal space with synthetic root filling material\textsuperscript{51}. Therefore, apexification allows only a small, and often negligible, root development in terms of width and length of dentine walls and does not allow a revascularization of the tooth\textsuperscript{52,53}. Moreover, apexification, achieved with calcium hydroxide (Ca(OH)\textsubscript{2}) or mineral trioxide aggregate (MTA), has other disadvantages. Apexification with
calcium hydroxide needs several patient visits and changes of the canal dressing and makes the tooth more prone to cervical fractures. On the other hand, apexification with MTA involves a long setting time, difficult handling, high cost, and the potential for tooth discoloration.

In this context, regenerative endodontics appeared as a promising therapeutic option for treating immature necrotic teeth. Regenerative endodontics is defined as a “biologically based procedure designed to replace damaged structures, including dentine and root structures, as well as cells of the pulp-dentine complex.” This treatment has been introduced relatively recently. While the first evidence of vital new tissue formation in empty canals and the potential of repair of the pulp tissue was introduced in the second half of the past century, the research on this topic was substantially initiated in the early 2000s. The first case reports were reported by Iwaya et al. in 2001 and by Banchs and Troppe in 2004. Since then, they have been increasing exponentially, with an average annual growth of publications of 40.4% between 2007 and 2017.

Regenerative endodontic therapy starts with the disinfection of the root canal of the immature necrotic tooth (Fig. 5a), mainly with the use of a solution such as sodium hypochlorite and with intracanal medication, ideally avoiding instrumentation. Once the tooth is asymptomatic, the root canal is treated with EDTA to liberate dentine matrix proteins trapped in the dentine matrix. Then, a blood clot, induced by overinstrumentation, invades the canal space. The blood clot (Fig. 5b), which should fill the canal space until 2 mm underneath the cemento–enamel junction, includes stem cells from periapical tissues. After the induction of the blood clot, a collagen sponge is placed over it and the crown access is filled with restorative materials. Finally, after a period of follow-up (usually over 6 months) the resolution of periapical lesion, further root development and a positive response to the vital pulp test are to be expected.
Fig. 5. Scheme of regenerative endodontic therapy. After disinfection of the immature necrotic tooth (A) and when the tooth is asymptomatic, a blood clot is induced by overinstrumentation (B). In general, after a follow-up period, repair of hard and soft tissue is observed (C).

The clinical outcomes of REPs have been reportedly successful\textsuperscript{52,67} with a thickening and lengthening of the dentine walls, closure of the apex, and resolution of periapical lesions\textsuperscript{53,68,69}, as well as, in most cases, an improvement of clinical symptomatology\textsuperscript{30}. The American Association of Endodontists (AAE)\textsuperscript{70} and the European Society of Endodontology (ESE)\textsuperscript{63} have published official guidelines for using regenerative endodontics procedures in immature teeth with the aim of standardizing the clinical protocol of this new treatment\textsuperscript{63}, which is being applied as a first treatment option in many countries.

Unlike the positive clinical outcomes of REPs, the histological analyses are less encouraging since evidence of tissue regeneration has not been observed so far. Most of the animal studies and case reports have described that the generated tissue after this therapy is neither pulp nor dentine\textsuperscript{30,71,72}. In fact, the presence of ectopic tissue like dental cementum and the lack of cells with a distinct odontoblast phenotype are observed in vivo\textsuperscript{73–75}. The tissue responsible for the narrowing of the canal space and lengthening of the root is cementum-like tissue instead of tubular dentine\textsuperscript{30,71}, and the soft tissue formed inside the root canal consists of unorganized connective tissue instead of pulp with its characteristic odontoblast layer.
Many studies focused on the identification of factors influencing regenerative treatment outcomes have been reported during the last decade. The first publications reported a wide variety of clinical protocols in different teeth, regardless of the cause of pulp necrosis and using irrigation solutions and medicaments today known to be harmful for the outcomes of REPs. Currently, it is known that regenerative endodontics outcomes may be affected by the following factors:

- The cause of pulp necrosis and the inflammatory state of the periapical tissues which may affect the survival of stem cells\(^7\). Most cases of failure after REPs is in teeth where the cause of pulp necrosis was trauma\(^7\). When treated with REPs, these teeth present little root development, which has been attributed to trauma damaging Hertwig’s epithelial root sheath (HERS)\(^7\).
- The irrigation solutions and medicaments used during treatment. The use of sodium hypochlorite, chlorhexidine and antibiotic pastes in high concentrations affect the survival of DPSCs and SCAPs\(^7,8\), whereas EDTA and calcium hydroxide have been shown to promote adhesion, migration and differentiation of DPSCs and SCAPs\(^7,8,1\),\(^2\).
- The bacteria and endotoxins remaining in the root canal after treatment. Residual bacteria and their membrane components have also been related to the lack of root development, thus hindering odontoblast-like differentiation of stem cells\(^8\),\(^3\),\(^4\). Therefore, disinfection and detoxification of the root canal are clearly advisable before each regenerative endodontics treatment\(^8\).

As previously stated, although a true regeneration of the pulp/dentine complex has not been achieved, much information about factors influencing the presence of reparative tissues in the root canal and, consequently, REPs outcomes, has been collected. The advances achieved in the last decade as well as the potential benefits of the therapy justify further efforts to improve the current clinical protocol.

To be considered as regenerated, a dental pulp tissue must meet the following requirements\(^8\):  

1. The tissue must be innervated and vascularized.  
2. It must have a cell density and extracellular matrix architecture similar to the natural dental pulp.  
3. It must generate odontoblasts aligned adjacent to the pre-existing dentine and producing new dentine.
Of these requirements, only the first one has been achieved so far. Meanwhile, numerous efforts have been made to regenerate pulp tissue after necrosis. To date, the administration of heterologous\(^87\) and autologous growth factors\(^{81,88}\); the use of exogenous stem cells or the cell-homing approach\(^{89-91}\); and the use of various scaffolds (autologous platelet concentrates\(^{30,92}\), synthetic materials\(^{93}\), collagen and fibrin\(^{91,94}\), among others) have been tested. However, no odontoblast-like cells or new tubular dentine have been detected in humans so far, with the exception of a single case report\(^95\).

Structural differences between reparative tissues and dentine have a critical clinical significance. These are due to two main reasons:

- Reparative tissues (cementum-like tissue/fibrodentine) are, unlike dentine, atubular structures. Consequently, they would not allow the placement of odontoblasts cellular process into the hard tissue. The lack of odontoblasts processes, especially in the newly formed hard tissue attached to coronal third or the root, compromises the immunological and sensory functions of the pulp\(^23\), as well as the secretion of reactionary dentine\(^{37,96}\). Moreover, the atubular reparative tissue cannot be clinically controlled and its end result cannot be predicted\(^97\).

- The fact that root development is completed with atubular cementum-like tissue instead of dentine may have an impact on the biomechanical performance of the tooth when enduring physical forces, such as biting, dentoalveolar trauma or orthodontic movement. Physical strength is a prerequisite for long-term survival of immature teeth after REPs, which entails a high risk of fracture or resorption due to regular mastication, dental trauma or orthodontic interventions\(^96\). As dental cementum has a lower hardness and elasticity in respect to dentine\(^99\), the question arises whether a root with cementum apposition can resist mechanical stress of a similar strength as can roots completed by dentine\(^100\).

2.2 REPs of mature necrotic teeth

Currently, the minimally invasive biologically based therapies for treating affected teeth is gaining ground in dentistry. Very recently, the European Society of Endodontology (ESE) released a position statement advising in specific cases the management of closed apex teeth with a clinical diagnosis of irreversibly pulpitis
can be treated with complete pulpotomy and hydraulic calcium silicate cement\textsuperscript{101}. This treatment aims to maintain pulp vitality and avoid root canal treatment, based on promising clinical outcomes shown in clinical studies\textsuperscript{102}. The conservation of pulp with a clinical diagnosis of irreversible pulpitis in adult patients was unthinkable some years ago and leaves in evidence the current conservative focus of endodontics.

When the pulp is necrotic, regenerative endodontics can offer a biologically based procedure for its treatment. As with immature teeth, mature necrotic teeth are normally submitted to root canal treatment, which consists of obturating the canal with biologically inert materials. At present, conventional endodontic treatment is considered the most reliable treatment for mature teeth, although the failure rate is close to 25\% at 3–5 years\textsuperscript{103}. Root-filled teeth remain susceptible to reinfections and fractures\textsuperscript{104}, as the restoration of a functional dentine–pulp complex cannot be achieved by this treatment. Endodontically treated teeth will remain devitalized throughout the life of the patient, and because of the absence of pulp they lose the ability to trigger an immunological response to new caries lesions\textsuperscript{23,37}.

As previously stated, regenerative endodontics was initially used to treat injured immature teeth, as an attempt to complete the development of the fragile dentine walls and revitalize the tooth. However, the potential benefits of this therapy, such as the recovery of the natural immune system and the achievement of a more structurally resistant tooth\textsuperscript{103} are equally desirable for mature teeth.

Most cases of pulp necrosis in the dental practice affect mature teeth. According to some cross-sectional studies, more than 20\% of mature teeth are endodontically treated\textsuperscript{105,106} and more than 80\% of adult patients had at least one root-filled tooth\textsuperscript{105}. Despite this epidemiological data, only a few studies, mainly case reports\textsuperscript{107} and preliminary clinical trials\textsuperscript{108}, have tested regenerative endodontics in mature teeth\textsuperscript{107,109,110}. However, these studies do show positive outcomes, such as the end of signs and symptoms\textsuperscript{109}, positive response to electric pulp testing\textsuperscript{108,111}, periapical healing\textsuperscript{108–110} and the formation of vascularized connective tissue in the root canal\textsuperscript{71}.

Regenerative endodontics in mature teeth is more challenging compared to its use in immature teeth because of the difficulty of disinfection of the root canal system, as well as the narrow apical pathway for cell migration\textsuperscript{71}, which could affect tooth treatment outcomes.
With respect to the narrower apical pathway for cell migration, recent studies have described that the diameter of the apical foramen does influence the outcome of the therapy\textsuperscript{112}. Although a fully developed root and a narrow apical foramen are desirable characteristics for a good tooth prognosis\textsuperscript{49} when applying regenerative endodontics, a narrow apical foramen may act as a physical barrier by impairing new vital tissue ingrowth\textsuperscript{112}. To overcome this drawback and enhance the probabilities of revascularization, studies applying regenerative endodontics in mature teeth use different methods to enlarge the apical foramen, such as apicoectomy\textsuperscript{113} and instrumentation with different working lengths\textsuperscript{107,111}, among other parameters, although most are poorly described in the literature.
Section 3: Experimental approaches for pulp regeneration
3.1 Autologous platelet concentrates

Autologous platelet concentrates (APCs) are hemocomponents obtained through the centrifugation of a blood sample of the patient, where the most bioactive components of the blood, such as platelets and its growth factors, fibrin, and leukocytes, are collected in a concentrate\textsuperscript{114}. The granules of platelets are rich with procoagulant factors, cytokines, antimicrobial proteins, and growth factors such as platelet-derived growth factor BB (PDGF-BB), transforming growth factor β-1 (TGF-β1), insulin-like growth factor 1 (IGF-1), vascular endothelial growth factor (VEGF), and basic fibroblast growth factor (bFGF)\textsuperscript{115}. These factors may improve tissue vascularization and wound healing\textsuperscript{116} by stimulating the collagen production, triggering angiogenesis\textsuperscript{117}, acting in the reclusion and differentiation of cells and also producing \textsuperscript{118}. One advantage of the use of APCs is that their obtention is simple and consequently can be easily applied in the clinics. Additionally, as they are obtained from the blood of the patient, there is no risk of immunogenic response or disease transmission\textsuperscript{119}.

Several types of APCs are obtained according to the preparation method\textsuperscript{119}. The first idea for using blood components for wound healing gave rise to fibrin glues, human plasma derivatives that mimic the final stages of blood coagulation, forming a fibrin clot that acts as a topical biological adhesive, contributing to haemostasis and tissue sealing\textsuperscript{119,120}. However, the method for preparation of fibrin glues is complex and expensive. Later, with the obtention of platelet-rich plasma (PRP), the fibrin sealant properties were combined with the growth factors of platelets, acting as a growth factor delivery system at the site of injury\textsuperscript{119,120} and playing an important role in hard and soft tissue repair. The main disadvantages of this concentrate are that some protocols use bovine thrombin as an anticoagulant for its preparation (and therefore it is delivered as a not-completely autologous preparation), as well as protocol differences in the PRP preparation\textsuperscript{119}. Along with platelets, high concentrations of leukocytes\textsuperscript{121}, known to release pro-inflammatory cytokines, are found in PRP, which may augment the inflammatory response at the application site\textsuperscript{122}. Accordingly, Anitua et al. developed a concentrate named “Plasma Rich in Growth Factors” (PRGF) by eliminating leukocytes from PRP\textsuperscript{122}. Additionally, a so-called second generation of autologous platelet concentrates emerged with platelet-rich fibrin (PRF). PRF may or not include leukocytes and has a standardized preparation protocol. PRF does not need the use of an anticoagulant, thus allowing a natural polymerization
of the fibrin\textsuperscript{114} and generating a mature and flexible fibrin network able to include most of the platelets present in the sample blood\textsuperscript{114,119}.

APCs have been used in several fields of medicine and have been gaining popularity among surgeons because of their positive effects in wound healing. In the field of dentistry, APCs are used in implantology, oral surgery, periodontics and more recently in regenerative endodontics\textsuperscript{71}. The rationale for the use of APCs for the treatment of necrotic teeth is based on the assumption that their high concentration of growth factors represents a potent stimulation for tissue healing obtained through the patient’s own molecules, mimicking the physiological process\textsuperscript{30}, providing a scaffold for tissue ingrowth and also because they can act as bacteriostatic agents\textsuperscript{118}, providing a proper environment for tissue regeneration. Several clinical\textsuperscript{123,124} and animal studies\textsuperscript{71,125} have evaluated the clinical and histological response of adding APCs in the canal space after blood clot formation in regenerative endodontics, and some of them reported a resolution of the symptomatology\textsuperscript{123}, periapical healing\textsuperscript{124} and apical closure\textsuperscript{126}, as well as thickening of the dentine walls and root lengthening\textsuperscript{123,124,126}.

3.2 Amnion epithelial cells

The amnion is a cell layer of cuboidal and columnar epithelial cells, which surrounds the inner surface of the placenta and produces amniotic fluid\textsuperscript{127}. It can be easily detached from the underlying chorion (chorioamniotic membrane)\textsuperscript{128}. The amnion has an epiblastic origin, it is avascular\textsuperscript{127}, and on its maternal surface it remains attached to the basal lamina which is composed of extracellular proteins and dispersed mesenchymal stem cells.

Human amnion epithelial cells (hAECs) express pluripotent stem cell markers and, therefore, have the ability to differentiate into cells of the three embryonic layers\textsuperscript{129,130}. Amnion epithelial cells have been shown to have adipogenic, chondrogenic, osteogenic, myogenic, cardiomyogenic, neurogenic, pancreatic and hepatogenic differentiation potential, among others\textsuperscript{131}. Their odontogenic potential has not yet been tested, although they might result in being very useful for different regenerative therapies.

Among the advantages of using amnion epithelial cells for regenerative endodontics is that they do not elicit an immunogenic response after heterologous transplantation\textsuperscript{130,132}. In addition, hAECs do not show telomerase activity and are non-tumorigenic cells\textsuperscript{127,130,133}. This lack of tumorigenicity is supported by the observation that hAECs display a normal karyotype\textsuperscript{130}. 
In 2011, Marongiu et al. demonstrated that rat amnion epithelial cells had the ability to differentiate into hepatocytes when injected into the hepatic arterial system\textsuperscript{134}. Thus, amnion epithelial cells were able to differentiate into hepatocytes \textit{in vivo} without any need for a prior \textit{in vitro} differentiation process. Subsequent studies have studied the utility of these cells as potential hepatocyte surrogates in patients with liver disease\textsuperscript{135,136}, and several clinical trials are being conducted to study their utility in spastic cerebral palsy, bone fractures, bronchial fistula, primary ovarian insufficiency, among other pathologies (www.clinicaltrials.gov).

In the field of dentistry, the chorioamniotic membrane has been used as an allograft for reconstructive surgery and as a reservoir of cells for periodontal treatment, specifically to stimulate periodontal regeneration\textsuperscript{137,138} since hAECs are able to induce angiogenesis and epithelialization\textsuperscript{139} can reduce inflammation, and possess antimicrobial and antiviral properties\textsuperscript{140}. Amnion grafting has also been used for the treatment of bone defects\textsuperscript{141} and mucositis, as well as preservation of the bone ridge\textsuperscript{142}. However, as said, hAECs have not been used yet in the field of regenerative endodontics, although their properties make them promising for regeneration of pulp tissue.

Multipotent stem cells are present in the canal space after a regenerative endodontic protocol is applied in a tooth\textsuperscript{66,143}. These are mainly multipotent mesenchymal stem cells from the periapical tissues delivered into the canal after overinstrumentation in mature and immature teeth\textsuperscript{66,143}. Dental pulp stem cells have been also added in the canal space after isolation\textsuperscript{110}, and as multipotent stem cells they may differentiate into specialized cells of the tissue from which they are isolated. Although DPSCs and SCAPs are originated from neural crest, the newly formed tissue observed after regenerative endodontics lacks odontoblast-like cells\textsuperscript{144}. Thus, hAECs may represent an interesting option for odontoblast-like differentiation \textit{in vivo}.

3.3 Dentine matrix proteins (DMPs) as inductors of odontogenic differentiation

Similarly to embryonic bone matrix deposition, during the embryonic stage of tooth development, the formation of dentine requires first an organic structure that is later mineralized with hydroxyapatite crystals\textsuperscript{145}. During this process the differentiated odontoblasts secrete bioactive molecules that are released into the extracellular matrix\textsuperscript{145}, after which they are included in the dentine during the mineralization process and remain there for a long time, being liberated in adult life\textsuperscript{146}. These factors are released when the dentine is demineralized, either by pathological processes such as caries\textsuperscript{146}, modifying the immune response and inducing the formation of tertiary dentine, or by the use of dental demineralizing
materials commonly used in dental treatments, such as ethylenediaminetetraacetic acid (EDTA)\textsuperscript{65,81,147}. A recent proteomic analysis of human dentine detected 813 human proteins in dentine extracts and the top three are proteins involved in pulp inflammation and cellular stress response\textsuperscript{147}.

EDTA removes calcium from calcium phosphate crystals, thus demineralizing the surface of the dentine. It also removes the smear layer, leaving a clean dentine surface, with open dentinal tubules\textsuperscript{148}. The use of EDTA as a chelating agent and as a liberator of DMPs has recently been defined as part of the published protocol for the revitalization of immature teeth\textsuperscript{63}. Previous \textit{in vitro} studies have shown that both stem cells of deciduous teeth and stem cells of the dental pulp in contact with dentine treated with EDTA show a higher expression of odontoblast marker genes compared to cells in contact with dentine not treated with EDTA\textsuperscript{149,150}.

In 2017, a method was developed an optimized for the extraction and concentration of dentine matrix proteins (DMPs)\textsuperscript{88}, which allows \textit{in vitro} studies to be performed with known concentrations of DMPs and their effects on stem cells. The liberated growth factors from the dental matrix include transforming growth factor beta (TGF-b)\textsuperscript{146,151}, bone morphogenetic protein 2 (BMP2)\textsuperscript{152}, platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF) and fibroblast growth factor type 2 (FGF2)\textsuperscript{151} and have been shown to stimulate cell migration and proliferation, as well as odontoblastic differentiation, secretion of dentine matrix and formation of tertiary dentine\textsuperscript{146,152}. Additionally, these molecules are effective at very low concentrations, provoking cellular response at the picogram level\textsuperscript{81}.

The success of the odontoblast differentiation of stem cells depends on the presence of appropriate signalling molecules, together with an appropriate stem cell line with odontoblast-like differentiation potential, among other parameters. If the odontogenic differentiation potential of the amnion epithelial cells is found to be stimulated by the biomolecules present in the dentine and released by materials commonly used in clinical practice, these cells (hAECs) would be an interesting and cost-effective therapeutic option to improve the histological results of regenerative endodontic therapy, by promoting the presence of odontoblast-like cells in the regenerated pulp and consequently the secretion of dentine.
Hypotheses and Objectives
1. **Hypothesis:** Deposition of dentine to complete root maturation has biomechanical advantages over reparative tissue (cementum). Dentine allows an even stress distribution along the root.  
   **Objective:** To investigate the distribution of mechanical stress in an immature tooth, a cementum-reinforced tooth and a dentine-reinforced tooth during biting, dental trauma, and orthodontic movement through finite element analysis (FEA).

2. **Hypothesis:** It is possible to isolate and cultivate viable primary odontoblasts. Odontoblasts express different markers than pulp cells.  
   **Objective:** To isolate mature human odontoblasts with three different enzymatic solutions, and to evaluate the viability and integrity of the cells in a cell culture. To compare the expression levels of a selection of genes that play a role during odontoblast-differentiation and dentine formation, in pulp cells and in odontoblasts.

3. **Hypothesis:** Autologous platelet concentrates improve regenerative endodontic clinical outcomes and can regenerate pulp tissue after regenerative endodontics.  
   **Objective:** To evaluate the clinical and histological outcomes of teeth treated with ACPs and regenerative endodontics, through systematic reviews.

4. **Hypothesis:** Apicoectomy is not an effective technique for apical foramen enlargement of mature teeth. On the contrary, instrumentation at the level of 0.5 mm beyond the apex does allow an effective apical foramen enlargement.  
   **Objective:** To evaluate different methods for an effective apical foramen enlargement in extracted and in *in situ* mature human teeth.
5. **Hypothesis**: Revitalization is possible in mature pulpectomized teeth with enlarged apical foramen diameter and treated with a regenerative approach. The addition of preameloblast-conditioned medium to the regenerative endodontic protocol induces odontoblast-like cell differentiation *in vivo*

**Objective**: To evaluate if pulp revascularization is possible in mature teeth of ferrets, with enlarged apical foramen diameter, after the revitalization treatment. If so, to analyse the presence of odontoblast-like cells in teeth treated with preameloblast-conditioned medium.

6. **Hypothesis**: Human amnion epithelial cells cultured onto dentine disks treated with EDTA are able to adhere to dentine and show odontoblast-like morphology with their cellular processes located in the dentinal tubuli.

**Objective**: To evaluate the adherence and the odontoblast-like differentiation of human amnion epithelial cells after being seeded onto dentine disks, through SEM.
Results
Results Chapter 1: Biomechanical performance of immature teeth after REPs
Biomechanical performance of an immature maxillary central incisor after revitalization: a finite element analysis

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Abstract


Aim To investigate the stress distribution in an immature maxillary incisor and the same tooth after simulated revitalization with deposition of tubular dentine or cementum by finite element analysis (FEA).

Methodology A finite element model of a maxillary central incisor was developed on the basis of a µCT scan. The tooth was segmented in two parts: a part that represented a tooth in an immature state and an apical part that represented the tissue formed after revitalization. The apical part was given the mechanical properties of dentine or cementum. The immature tooth and the same tooth reinforced by either dentine or cementum underwent simulation of biting, trauma and orthodontic movement. Von Mises stress values were compared between the scenarios and tooth segments.

Results Maximum stress in the immature incisor developed apically; however, dentine- and cementum-reinforced teeth revealed the greatest stress in the external portion of the root decreasing towards the apex. Greatest mechanical stress was caused by dental trauma perpendicular to the long axis of the root followed by biting and orthodontic movement. Stress peaks were lower in the dentine-reinforced tooth compared with the cementum-reinforced tooth in all scenarios; however, median stress in the immature part was reduced irrespective of dentine or cementum deposition. Dentine reinforcement caused greater stress values in the apical segment due to absorbance of the applied force, whereas stress was not transferred towards deposited cementum.

Conclusions Apposition of simulated hard tissue in a maxillary central incisor after revitalization reduced mechanical stress in the immature tooth. Formation of dentine was advantageous because, unlike cementum, it facilitated an even stress distribution throughout the root resulting in lower stress values.

Keywords: dental cementum, dentine, finite element analysis, mechanical stress, regenerative endodontics.

Introduction

Pulp necrosis in immature teeth frequently occurs after dental trauma, caries or developmental anomalies (Diogenes et al. 2013) and the dental pulp loses function, dentine secretion stops and root development comes to a halt, resulting in thin and short
dentine walls that are prone to fracture (Cvek 1992). Until recently, the standard therapy for such teeth was apicification, where the formation of an apical barrier was induced by calcium hydroxide or the placement of an apical plug with hydraulic calcium silicate cements. However, further root development cannot be expected (Silujjai & Linsuwanont 2017).

Over the last decade, research has increasingly focused on the development of biology-based alternative treatment concepts such as revitalization, which aims for the regeneration of the pulp-dentine complex (Murray et al. 2007) and the promotion of thickening and thickening of dentine walls (Lolato et al. 2016). The clinical outcomes of revitalization procedures have been reported to be successful (Nagy et al. 2014, Lin et al. 2017) with root maturation by dentine wall thickening and thickening of apical plug (Shah et al. 2008, Lolato et al. 2016, Nazzal et al. 2018). However, due to the presence of ectopic tissue such as cementum and the lack of cells with a distinct odontoblast phenotype in vivo (Shimizu et al. 2012, Becerra et al. 2014, Lei et al. 2015), the generated tissue has consequently been described as reparative tissue and not as regenerated pulp tissue (Gomes-Filho et al. 2013, del Fabbro et al. 2015, Saoud et al. 2015). Histological analyses of revitalized teeth demonstrated that apical closure and narrowing of the root canal is mostly based on apposition of cementum instead of tubular dentine (Gomes-Filho et al. 2013, Saoud et al. 2015). Numerous efforts are being made to gain odontoblast-like differentiation of stem cells in vivo, induce further root development by formation of tubular dentine (Zhu et al. 2012, Galler et al. 2016, Widbiller et al. 2018a) and re-establish the functions of the pulp, such as immune response and pain perception (Couve et al. 2013).

This goal might not only be desirable from a biological point of view, but also from biomechanical perspective since physical strength by continued root development is a pre-requisite for long-term survival of immature teeth. In general, teeth with open apices bear a high risk of pulp necrosis and fractures by regular mastication, orthodontic interventions and especially dental trauma (Lam 2016). The risk of pulp necrosis after crown fractures is strongly associated with the stage of root development (Robertson et al. 2000) and depends on the severity of the trauma and whether the pulp is involved (Cavalleri & Zerman 1995). According to Andreassen et al. (2004), pulp necrosis occurred in 22% of intra-alveolar root fractures and, again, the patients’ age and the stage of root development were related to the healing outcome.

Therefore, a progression of root development is highly desirable since mature teeth are less prone to fracture and seem to display a higher mechanical resistance (Cvek 1992, Zhou et al. 2017). However, the type of tissue underlying the process of maturation has not been discussed. As cementum is inferior to tubular dentine in terms of hardness and elasticity (Ho et al. 2010), the question arises whether a root with apposition of cementum can endure mechanical stress similarly to roots completed by dentine (Wang et al. 2010). The biomechanical performance and physical strength of immature teeth and teeth with different regenerative endodontic outcomes have not been evaluated so far. Therefore, this study aimed to investigate the stress distribution in an immature maxillary central incisor, a cementum-reinforced tooth and a dentine-reinforced tooth during biting, dental trauma and orthodontic movement through finite element analysis (FEA).

Materials and methods

Model development

The maxilla of a male patient aged 59 was provided by the Organ Donation Service and Dissection Rooms (SDCSD) of the University of Barcelona (Spain). The study was approved by the Commission of Bioethics of the University of Barcelona and consistent with the Royal Decree 1723/2012. The donor declared his consent to use his body for scientific and teaching purposes in his lifetime. A µCT scan was conducted (Quantum FX microCT, PerkinElmer, Waltham, MA, USA) at 90 kV and 160 mA through FOV 60 mm with an exposure time of 4.5 min (voxel size of 118 × 118 × 118 µm). The acquired images were reconstructed into cross-sectional slices with PerkinElmer software (PerkinElmer) using the Feldkamp algorithm (Fig. 1a).

A complete volume segmentation of the right maxillary central incisor (enamel, dentine and pulp chamber) and its supporting tissues (periodontal ligament, alveolar bone) was carried out using Seg3D software (v. 2.4.3. Center for Integrative Biomedical Computing, Salt Lake City, UT, USA) by application of case-specific thresholds and manual painting techniques. According to templates of immature teeth, the dentine of the mature 3D model (Fig. 1b) was divided into
two portions: (i) the actual tooth in an immature state (IS) with thin walls and lacking root tip at stage 4 based on Cvek’s classification of root development (Cvek 1992) and (ii) intracanal tissue with the root tip. Thus, the core part with the root tip (20% of the root length) represented newly formed tissue, which was assigned with the mechanical parameters (Young modulus and Poisson coefficient) of dentine or cementum (Table 1) to simulate a dentine-reinforced tooth (DR) or a cementum-reinforced tooth (CR). Irregularities caused by segmentation were repaired using refinement and smoothing tools (MeshLab 2016, ISTI-CNR, Rome, Italy).

Finite element analysis (FEA)

A non-linear structural static analysis assuming large deformations was performed to evaluate the tooth in different clinical scenarios using the finite element package ANSYS® 17.1 (Canonsburg, PA, USA) in a Dell Precision™ Workstation T7910 (Hopkinton, MA, USA). Elastic, linear and homogeneous material properties were assumed for enamel (Gialain et al. 2016), dentine (Gloria et al. 2018), cementum (Malek et al. 2003) and bone (Liao et al. 2016) (Table 1), whereas a non-linear hyperelastic material was assumed for the periodontal ligament using a nine parameters Mooney–Rivlin constitutive equation (Qian et al. 2009):

\[
W = C_{10}(I_1 - 3) + C_{01}(I_2 - 3) + C_{11}(I_1 - 3)(I_2 - 3) + C_{20}(I_2 - 3)^2 + C_{30}(I_1 - 3)^3
\]

where \( W \) is the stored deviatoric third-order deformation strain energy function, \( I_1, I_2 \) and \( I_3 \) are the strain invariants, and \( C_{10}, C_{01}, C_{11}, C_{20} \) and \( C_{30} \) are material parameters described in Table 2.

Table 1 Young’s modulus and Poisson coefficient of dental (enamel, dentine and cementum) and support tissues (bone)

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Young’s modulus</th>
<th>Poisson coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enamel</td>
<td>84 100</td>
<td>0.31</td>
</tr>
<tr>
<td>Dentine</td>
<td>18 600</td>
<td>0.31</td>
</tr>
<tr>
<td>Cementum</td>
<td>8200</td>
<td>0.3</td>
</tr>
<tr>
<td>Bone</td>
<td>14 700</td>
<td>0.31</td>
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</tbody>
</table>

Figure 1 Model development. (a) Sagittal view of the µCT scan and the FEA model. (b) Segmentation of a mature tooth (left) into an immature part (middle) and a portion of newly formed tissue in the intracanal and apical region (right).
The complete model was built with an adaptive mesh of about 1.8 million hexahedral elements (Marcé-Nogué et al. 2015). These meshes met the conditions defined by Marcé-Nogué et al. (2016) to create a quasi-ideal mesh (QIM), which allows statistical analysis of the values in the whole mesh.

Loading scenarios

Three typical clinical situations (biting, dental trauma and orthodontic movement) were selected, and force application (Fig. 2a) was simulated for the IS, the DR and the CR model. Briefly, for the biting scenario 240 N were applied in the incisal edge with an angle of 120° with respect to the long axis of the tooth. To simulate a dental trauma from vestibular direction, 300 N force was applied perpendicular to the vestibular face and to simulate orthodontic movement in mesial direction 0.8 N were applied in the middle third of the vestibular crown surface.

Analysis of von Mises stress

The von Mises stress distributions were evaluated for all groups (IS, DR and CR) in the different scenarios. Quantitative measurements of the relative strength of the structure under study were used to summarize the strength of the whole model. The recently proposed quasi-ideal meshes (QIM) and its percentile values were used as a basis for the analysis (Marcé-Nogué et al. 2016), since the use of a QIM facilitates the comparison between models and includes corrections to account for the non-uniformity of the mesh.

The immature part and the apical segment were analysed separately to evaluate stress distribution throughout the model. Results of DR and CR were summarized as medians with quartiles (25–75%) and compared with the immature tooth.

The peak stress for each scenario was defined by the 95% quantile of von Mises stress results in each model situation (IS, DR and CR) as proposed by Walmsley et al. (2013). The 95% quantile was assumed to be a peak value to avoid the high artificial noise produced in the FEA model (Marcé-Nogué et al. 2015).

Results

In general, the highest stress values were observed for dental trauma followed by biting and orthodontic movement (Fig. 2b). The peak stress of the cementum-reinforced tooth was higher compared with the dentine-reinforced tooth in all scenarios. In the biting scenario with a rather axially directed load, root maturation by dentine apposition reduced stress, whereas the peak stress after apposition of cementum was similar to the immature tooth. On the contrary, perpendicular forces applied by trauma or orthodontic movement caused greater peak stress in mature than in immature teeth, especially in the CR tooth (Fig. 2b).

Stress patterns in FEA models of the immature tooth (Fig. 3a–c) revealed the highest von Mises stress at the apical root end where dentine walls are thin. Maximum stress involved the whole thickness of the delicate dentine walls. On the contrary, peak stress in DR and CR teeth was found primarily in the external dentine layer and decreased towards the apex as well as towards the underlying intracanalar dentine, which was barely affected.

Whereas the apical third in the DR tooth revealed an even stress distribution towards the newly formed tissue, a sharp boundary was observed in the CR tooth. Though high stress was present at the apical end of the immature segment, force was barely transferred to the apical part of cementum or towards the intracanalar regions (Fig. 3, s. inserts).

In all three scenarios, median stress in immature segments of both DR and CR teeth was lower than in the immature tooth, whereas greatest reduction was visible for biting (Fig. 4a). The apical segments, however, behaved differently (Fig. 4a–c). Whereas the apical part after dentine deposition absorbed the forces, which led to a stress increase in this part, no force was transferred to the apical part in the cementum-reinforced model, and consequently, stress values decreased.
Discussion

Revitalization constitutes a promising therapy for immature teeth with pulpal necrosis and open apices (Diogenes et al. 2016). Newly generated reparative tissue facilitates root thickening and apexogenesis primarily by deposition of cementum in the apical region and along the root canal walls (Gomes-Filho et al. 2013, del Fabbro et al. 2015, Saoud et al. 2015). However, novel tissue engineering approaches aim to restore the entire pulp-dentine complex and enable progression of root development by dentine formation (Murray et al. 2007, Galler & Widbiller 2017). Besides obvious biological benefits, the numerical analysis performed in this study indicates that deposition of tubular dentine might also have biomechanical advantages over reparative tissue due to a broader stress distribution along the root.

The results of the analysis revealed that the cementum-reinforced tooth distributed mechanical stress disadvantageously compared to the dentine-reinforced tooth. Stress peaks were higher in CR compared with DR in all scenarios and deposited dentine widely absorbed stress throughout the root, whereas cementum did not. Thus, dentine formation after revitalization can considerably reduce mechanical stress. Interestingly, root maturation by dentine reduced peak stress in the biting scenario compared with the immature tooth, which allows the conclusion that maturation of the root improves the mechanical performance when confronted with physiological forces.

Since the introduction of revitalization, it has been assumed that immature teeth reinforced by newly formed hard tissue gain stability and become more resistant to physical impact (Cvek 1992). The classical study of Cvek (1992) showed the decreasing risk of cervical root fractures with maturation, which is in line with the results of this study. As mentioned, root maturation by deposition of dentine or cementum led to a reduction in median stress. The maximum stress of immature teeth was observed throughout the delicate root tip, whereas roots reinforced by mineralized tissue were only compromised in the external regions. Similarly, finite element studies of immature teeth with apical plugs also reported concentrated stress but a wide stress distribution in mature teeth (Talati et al. 2007).

In accordance with previous studies (da Silva et al. 2013), force direction had an impact on stress distribution. When the same force was applied perpendicularly to the tooth, maximum stress values were markedly higher than in axial direction. For example,
Figure 3 Stress distribution pattern of an immature tooth, a dentine- and a cementum-reinforced tooth during simulation of (a) biting, (b) dental trauma and (c) orthodontic movement. Coloured arrowheads mark the point of force application in each scenario. Inserts display a magnified view of boundaries between dentine and cementum and regions, where stress is not evenly distributed.
maximum stress values were considerably lower in the biting scenario when the force was applied with 135° with respect to the tooth long axis compared with 120° (Figure S1). Those observations should be respected before orthodontic force application and restoration of teeth after revitalization. An axially directed load caused lower peak stress in DR compared with CR and IS, which allows the conclusion that the mechanical performance of a dentine-reinforced tooth during biting is better than of a cementum-reinforced or an immature tooth. In contrast, a perpendicular trauma led to increased peak stress in both the CR and the DR tooth, which is most likely due to the leverage effect by the prolonged root. Deposition of dentine, again, caused a lower maximum stress than deposition of cementum and might increase resistance to traumatic injuries.

Although the distribution of stress during orthodontic movement was also more disadvantageous in the CR than in the DR tooth, the low forces applied during the treatment are negligible from a biomechanical perspective. However, the biological implications of orthodontic movement of revitalized teeth must be considered as they are at risk due to inflammation and root resorption (Kindelan et al. 2008).

Finite element analysis is a useful modelling technique in biological sciences that allows to study physical behaviour of tissues (Kupczik 2008). FEA enables a non-invasive, controlled and repeatable simulation of biomechanical processes under different conditions and, thus, has been used in dental research to investigate stress distribution, for example in implantology and orthodontics (Choi et al. 2014, Papageorgiou et al. 2016). However, FEA has never been applied to evaluate the stress distribution of different biological outcomes of revitalization.

In this study, a 3D model of a mature maxillary central incisor was constructed on basis of a µCT scan. Furthermore, a segment consisting of the intracanal and apical region was separated from the whole tooth to simulate the tissue formed after revitalization. Even though mixed tissues or altered forms of dentine (e.g. osteodentine) might appear in vivo, this clear and structural approach allowed for a biomechanical evaluation of the whole spectrum from tissue repair to regeneration.

The mechanical behaviour – defined by the relationship between stress and strains – of the hard tissues in the model (enamel, dentine, cementum and alveolar bone) was assumed to be linear, whilst the periodontal ligament was assumed to be non-linear based on an hyperelastic model for large deformations. Linear models cannot capture the highly non-linear characteristic and large deformations of the periodontal ligament (Qian et al. 2009), which could change the stress distribution of a structure.

Three typical scenarios that adolescents are faced with were simulated: biting, dental trauma and orthodontic movement. A maximum biting force of 240 N was chosen as reported for incisors (Paphangkorakit & Osborn 1997). For dental trauma, an increased frontal load of 300 N was applied, which is one of the most frequent traumatic events in young patients (da Silva et al. 2013, Lam 2016). However, a mesial orthodontic movement of incisors was simulated with the recommended force of 0.8 N (Wu et al. 2018).

As restorative materials generally aim to have mechanical qualities similar to dental hard tissues and restored access cavities were reported to have no
significant effect on stress distribution in this context (Belli et al. 2018), the restoration as weak spot in the model was deliberately avoided. This allowed the focus to remain on the biomechanical impact of the newly formed tissues. Furthermore, this restriction enabled a direct biomechanical comparison of the mature and the immature tooth, which has no canal access by nature, and to describe the biomechanical contribution of newly formed tissue irrespective of access cavity or restoration quality. Additionally, it must be considered that in order to standardize the clinical situations a static model was used for all scenarios. Thus, ‘time’ was not a variable included in the analysis. In contrast to biting and trauma, where the application of the force lasts for a moment, the force applied in an orthodontic treatment endures for a longer period, usually several months. Therefore, the results show the stress distribution of teeth at a specific moment and not the fatigue caused by real orthodontic treatment. Access cavity design and the combinations of various restorative materials, as well as the fatigue caused by orthodontic treatment, will be basis of future investigations.

Fundamental for the observations made in this study is the fact that dentine and cementum differ in their chemical composition, their microstructure and their mechanical qualities (Ho et al. 2010). Whilst dentine is composed mainly by hydroxyapatite and provides a tubular architecture, cementum may encapsulate cells and is made up mainly by collagen fibres and water (Zhang et al. 2014). Dentine itself, which is a hard but elastic tissue (Ho et al. 2010) with high deformation capacity (Poolthong et al. 2001), has the physiological function to bear chewing forces and distribute them equally to minimize stress. In contrast, cementum is part of the periodontal apparatus and not intended to distribute compressive stress but designed to absorb tensile forces from Sharpey’s fibres (Chan & Darendeliler 2006). As the results of this study demonstrate, an even stress distribution from dentine towards cementum is hardly possible. The newly formed intracanal and apical tissue barely absorbed stress when the root was reinforced by cementum, which resulted in higher peak stress. On the contrary, the stress was transferred towards deposited dentine causing higher stress in the apical segment, but overall lower stress in the tooth.

So far, various clinical factors have been shown to determine the outcomes of revitalization procedures. The aetiology of pulp necrosis as well as the degree of apical periodontitis can affect local stem cell sources (Kim et al. 2018). Acute damage to Hertwig’s epithelial root sheath (HERS) by trauma may result in failure in continuation of root development (Nazzal et al. 2018). Additionally, the use of sodium hypochlorite and antibiotic pastes in high concentrations compromised stem cell survival (Ruparel et al. 2012, Martin et al. 2014). However, calcium hydroxide was less harmful and EDTA even promoted adhesion, migration and differentiation of pulp stem cells on dentine (Galler et al. 2016, Tzafas et al. 2019). Accordingly, the European Society of Endodontology (ESE) released a position statement indicating a clinical protocol for revitalization in 2016 (ESE 2016). Furthermore, residual bacteria and endotoxins have recently been related to a lack of root development (Verma et al. 2017) and to prevent cells of entering odontoblast lineage (Vishwanat et al. 2017, Widbiller et al. 2018b). Thus, root canals need to be disinfected adequately to allow for more controlled stem cell fate in regenerative endodontic procedures (Diogenes & Hargraves 2017).

Conclusion

Apposition of hard tissue in an immature maxillary central incisor reduced mechanical stress during finite element simulation. Moreover, apposition of dentine in the immature tooth model provided considerable biomechanical advantages over apposition of cementum during biting, trauma and orthodontic movement. A greater mechanical resistance and a more even stress distribution by deposited dentine might improve the long-term survival of teeth after revitalization, which constitutes the overarching goal in this field of research.

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Conflict of interest

The authors have stated explicitly that there are no conflicts of interest in connection with this article.
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Marcé-Nogué J, Fortuny J, Gil L, Sánchez M (2015) Improving mesh generation in finite element analysis for...


**Supporting Information**

Additional Supporting Information may be found in the online version of this article:

**Figure S1.** Peak von Mises stress, represented by 95% quantiles, in biting scenarios with two different angulations (135° and 120°) with respect to the long axis of the tooth in an immature state (IS), in a dentine-reinforced (DR) and a cementum-reinforced (CR) situation.
Results Chapter 2: Enzymatic isolation and culture of primary odontoblasts
Isolation of primary odontoblasts: Expectations and limitations

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Keywords
cell isolation, dental pulp, enzyme, gene expression, odontoblasts.

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Abstract
The purpose of this study was to evaluate different protocols of enzymatic treatment (collagenase with either protease, trypsin or hyaluronidase) to isolate mature odontoblasts. Primary odontoblasts were obtained from human molars, which was confirmed by histology and scanning electron microscopy. The combination of collagenase with protease appeared most suitable and resulted in higher cell numbers and better integrity of the odontoblast processes, whereas combination with hyaluronidase or trypsin led to truncated processes and detachment of cell patches instead of single cells. However, trypan blue staining after 24 h showed that odontoblasts in culture did not remain viable. Gene expression analysis was possible after mRNA extraction from tissues ex vivo and real-time semi-quantitative PCR revealed increased expression of collagen, nestin, bone sialoprotein and dentin matrix acidic phosphoprotein 1 in the odontoblast layer. Though primary odontoblasts could not be cultivated after isolation, characteristic genes were identified to differentiate odontoblasts from pulp fibroblasts.

Introduction
Odontoblasts are neural-crest-derived cells which line the pulp chamber in mature teeth and feature a highly differentiated, postmitotic state (1). During the bell stage of tooth development, pre-odontoblasts in the peripheral dental papilla interact with the inner enamel epithelium and gradually reduce their rate of mitosis (2). They finally undergo a last and asymmetric cell division that gives rise to post-mitotic daughter cells which elongate, polarise and, as functional odontoblasts, start to form pre-dentin (3). Their sister cells remain progenitors and reside in the subodontoblast layer (Hoehl’s layer) or migrate into the pulp core (4).

Interestingly, each differentiated odontoblast leaves one cell process embedded in the mineralised dentin matrix. Most cell organelles assemble near the process at the proximal pole, which allows for rapid secretion of primary dentin (4–8 μm per day), while the nucleus is located at the distal side (5). At the time the tooth erupts and reaches the occlusal plane, most organelles of the mature odontoblasts relocate to the distal pole and autophagy vacuoles start to accumulate. Hence, the secretion of secondary dentin is significantly slower (0.5 μm per day), and also the number of cells decreases (5,6). Maturing odontoblasts gradually flatten and finally form the typical monolayer at the pulp-dentin-interface (5).

Differentiated odontoblasts feature sensory functions and transduce thermal, mechanical or chemical signals (7). Upon stimulation, they start to form tertiary dentin in an act of defense (8). Furthermore, odontoblasts recognise invading pathogens by various types of receptors and are capable of regulating the innate immune system (9). They play an important and strategic role as the first cells to encounter external stimuli, regardless of whether these are pathogens, dental materials or endogenous proteins released from the dentin matrix.

Consequently, this specific cell type has attracted considerable interest and several culture models have been developed to investigate the behaviour of odontoblasts in vitro. In some studies, extracted teeth were split open, the pulp tissue was removed and the odontoblast layer...
was kept in culture while still attached to the dentin surface (10,11). In other studies, odontoblasts still connected to the removed pulp tissue were cultured and used for experiments (12,13). Further attempts have been made to collect individual odontoblasts from the pulp chamber walls by scraping (13,14), by dissection from removed pulp tissue (15), and more recently by enzymatic detachment from the dentin surface (16,17).

The isolation and culture of odontoblasts may open numerous possibilities to study this cell type under standardised conditions, shedding light on their roles in dentin formation, immune defense and transmission of external stimuli, and to furthermore compare them to other related cell types such as pulp fibroblasts or osteoblasts.

Despite interesting strategies and promising reports in the literature, it remains controversial whether it is possible to isolate intact odontoblasts from human teeth and keep them in culture. Existing studies leave substantial doubts whether isolated cells remain viable in vitro or whether they are irreversibly damaged by the inevitable disruption of the odontoblast processes. Thus, we hypothesised that it is not possible to maintain viable odontoblasts in culture. The objective of the study was two-fold: (i) to assess different enzymatic protocols for isolation and subsequent cultivation of odontoblasts from freshly extracted teeth, and (ii) to compare the gene expression profile of odontoblasts with cells from the pulp core in order to fathom their specific characteristics.

Materials and methods

Isolation of odontoblasts

Third molars were collected from patients aged 19–24 years after obtaining informed consent and with the approval by the Ethics Committee (Faculty of Medicine, University of Regensburg, Regensburg, Germany).

Teeth were collected immediately after extraction and disinfected with a paper towel soaked with 70% ethanol. Until arrival in the laboratory, samples were kept in αMEM (Gibco® MEM Alpha 1 ×, Thermo Fisher Scientific, Waltham, MA, USA) supplemented by 10% FBS and 1% antibiotics (Gibco® Penicillin-Streptomycin, Thermo Fisher Scientific), and penicillin-streptomycin (Penicillin-Streptomycin, Thermo Fisher Scientific), which had been centrifuged at 160 × g for 6 min with the open pulp chamber facing the bottom of the tube to separate remaining cells from the hard tissue. The cell sediment was resuspended in 1 mL MEM with 10% FBS and 1% antibiotics (Gibco® 10 000 U mL⁻¹ Penicillin-Streptomycin, Thermo Fisher Scientific), and transferred into five glass chamber slides (8-well), 200 μL each (Nunc™ Lab-Tek™ Chamber Slide System, Thermo Fisher Scientific, Waltham, MA, USA), which had been coated with 200 μL of 0.1 mg mL⁻¹ poly-L-lysine (Poly-L-lysine solution, Sigma-Aldrich, St. Louis, MO, USA). Isolated cells were incubated for up to 72 h at 37°C and 5% CO₂. Odontoblasts were identified based on their characteristic morphology and stained with 0.4% trypan blue (Trypan Blue solution, Sigma-Aldrich, St. Louis, MO, USA) to evaluate their viability. Images were taken with an inverted microscope (Axio Vert.A1, Carl Zeiss Microscopy GmbH, Jena, Germany).

RNA-extraction from odontoblast layer and pulp

At least two wisdom teeth of five 19- to 24-year-old patients were collected immediately after extraction and kept in saline. After separation of the crown and root as

Table 1 Three enzyme combinations referred to as α, β and γ

<table>
<thead>
<tr>
<th>Group</th>
<th>Enzyme 1</th>
<th>Enzyme 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>α</td>
<td>3 mg mL⁻¹ collagenase†</td>
<td>0.25 mg mL⁻¹ protease‡</td>
</tr>
<tr>
<td>β</td>
<td>2 mg mL⁻¹ collagenase†</td>
<td>1 mg mL⁻¹ trypsin§</td>
</tr>
<tr>
<td>γ</td>
<td>1 mg mL⁻¹ collagenase†</td>
<td>0.1 mg mL⁻¹ hyaluronidase¶</td>
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</tbody>
</table>

†Collagenase from Clostridium histolyticum Type IA, Sigma-Aldrich, St. Louis, MO, USA; ‡Protease from bovine pancreas Type I, Sigma-Aldrich, St. Louis, MO, USA; §Trypsin from bovine pancreas Type I, Sigma-Aldrich, St. Louis, MO, USA; ¶Hyaluronidase from bovine testes Type I-S, Sigma-Aldrich, St. Louis, MO, USA.
described above, the coronal pulp tissue was gently removed with tweezers and placed into 600 μL lysis buffer (Buffer RLT, Qiagen, Hilden, Germany). Likewise, the pulp chamber was filled with lysis buffer to capacity (10–15 μL) and incubated for 30 s to bring odontoblast RNA into solution. mRNA from the coronal pulp tissue and from the coronal odontoblast layer were isolated separately using the RNeasy Mini Kit (Qiagen, Hilden, Germany) and pooled for each donor, respectively. After spectrophotometric quantification (NanoDrop® 2000, Thermo Fisher Scientific, Waltham, MA, USA), 250 ng of nucleic acids were transcribed into cDNA (OMniscript RT Kit, Qiagen, Hilden, Germany) using oligo-dT primers (Qiagen, Hilden, Germany).

Gene expression

To compare expression of selected genes in odontoblasts and pulp cells of the same donor, real-time semi-quantitative PCR (RT-qPCR) was conducted. Primers were designed and synthesised (Eurofins MWG Synthesis, Ebersberg, Germany) for a selection of genes involved in odontoblast-differentiation and mineralisation: collagen type I α1 (COL1A1), alkaline phosphatase (ALP), dentin sialophosphoprotein (DSPP), dentin matrix acidic phosphoprotein 1 (DMP1), bone sialoprotein (BSP), osteopontin (OPN), osteocalcin (OCN), osterix (OSX), nestin (NES), runt-related transcription factor 2 (RUNX2), msh homobox 2 (MSX2), distal-less homeobox 5 (DLX5) and 40S ribosomal protein S18 (RPS18) as housekeeping gene (Table 2).

Polymerase chain reaction was carried out with 20 pmol of each primer, 5 μL cDNA diluted 1 in 5 and qPCR master mix with SYBR® Green in a final volume of 20 μL (Applied Biosystems™ SYBR® Select Master Mix, Thermo Fisher Scientific, Waltham, MA, USA). Following an initial denaturation of the samples at 95°C for 10 min, 40 cycles of alternating denaturation (95°C for 15 s) and annealing (60°C for 1 min) were performed with the StepOnePlus™ Real-time PCR system (Applied Biosystems™, Thermo Fisher Scientific, Waltham, MA, USA).

The gene expression in odontoblasts was quantified by the comparative Ct method (2−ΔΔCt). Firstly, Ct values of all genes expressed in odontoblasts as well as in pulp cells were normalised by the housekeeping gene (RPS18). Furthermore, odontoblast gene expression was calculated relative to pulp cells (fold change) as described previously (18). Median values with 25–75% percentiles summarised results of all donors (n = 5).

Histology and scanning electron microscopy (SEM)

Histological staining and SEM were performed to evaluate the success of enzymatic isolation and RNA-extraction. Crowns before removal of pulp tissue, after removal of pulp tissue and after respective protocols to detach odontoblast cells were fixed in 4% buffered paraformaldehyde solution for up to 3 days at 4°C. After a wash in phosphate buffered saline (Instamed 9.55 g L−1 PBS Dulbecco w/o Ca²⁺ and Mg²⁺, Biochrom AG, Berlin, Germany), samples were demineralised in Tris-EDTA buffer (TE buffer with ethylenediaminetetraacetic acid disodium salt 2-hydrate, AppliChem GmbH, Darmstadt, Germany) for 28 days while the solution was changed daily. Samples were dehydrated by ethanol series, embedded in paraffin, sectioned at 6 μm thickness and mounted to glass slides (Fisher-brand™ Superfrost™ Plus Microscope Slides, Thermo Fisher Scientific, Waltham, MA, USA). After dewaxing in xylene and rehydration, sections were stained with hematoxylin and eosin (H&E), and with Masson’s trichrome (Trichrome Stain [Masson] Kit, Sigma-Aldrich, St. Louis, MO, USA; ethylenediaminetetraacetic acid disodium salt 2-hydrate, Applied GmbH, Darmstadt, Germany) for 28 days to visualise cells and extracellular matrix.

Table 2 Primer details for RT-qPCR

<table>
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<th>Gene</th>
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<td>RUNX2</td>
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Isolation of Primary Odontoblasts

M. Widbiller et al.
Additionally, the pulp chamber walls of crowns before and after treatment were inspected by scanning electron microscopy. Samples were fixed with 2.5% glutaraldehyde in 0.1 mol L\(^{-1}\) Sorensen’s phosphate buffer for 30 min and images were taken on a FEI Quanta 400 environmental scanning electron microscope with a field emitter (FEI Europe B.V., Eindhoven, The Netherlands), which was operated at low-vacuum scanning electron microscopy (LVSEM) imaging mode.

Statistical analysis

Data were treated non-parametrically, and results were analysed pairwise using the Mann–Whitney U-test on an \(\alpha = 0.05\) level of significance (GraphPad Prism 7; GraphPad Software, La Jolla, CA, USA). Statistically significant differences between gene expression in odontoblasts and pulp tissue were indicated by asterisks.

Results

**Isolation of odontoblasts**

Histological and SEM imaging of the teeth before (Fig. 1 a,b) and after (Figs 1 c,d and 2 a) removal of pulp tissue revealed an intact odontoblast layer closely attached to dentin prior to enzymatic treatment. Adhering to the odontoblasts, subodontoblast connective tissue of up to 20 \(\mu\)m in thickness was present. However, SEM imaging was conducted from the perspective of the pulp on the odontoblast layer. The individual odontoblast cell bodies could be differentiated by their round swell in the layer caused by their distal poles (s. arrowhead in Fig. 2 a).

Enzymatic treatment and centrifugation lead to a partial removal of the odontoblast layer, where cell-free zones alternated with remaining odontoblasts on the dentin surface, with similar results for all tested enzyme combinations (Fig. 1 e-g). The odontoblast layer was partially disrupted, and odontoblast processes appeared pulled away from the dentinal tubules after centrifugation (Fig. 2 b-g). Depending on the extent of extracellular matrix degradation, single odontoblasts were recognised in the disrupted layer by their processes (s. arrowheads in Fig. 2 c,f) or their distal poles becoming apparent within the cell complex (Fig. 2 d,e).

After treatment with enzyme combination \(x\) (Figs 1e and 2 b, c), the extracellular matrix appeared degraded and the predentin predominantly dissolved. Among the tested combinations, \(x\) yielded the most favourable results regarding the integrity of cell processes (Fig. 3a,b). On the contrary, enzyme combination \(\beta\) barely affected the extracellular matrix (Figs 1f and 2d,e), hence no individual cells but mostly cell clusters were visible in culture (Fig. 3c,d). Enzymes in test group \(\gamma\) broke down mainly the extracellular structures but did not dissolve predentin (Figs 1g and 2l,g), which generally led to single cells but short processes (Fig. 3e,f). Regardless of different enzymatic digestions, trypan blue staining after 24 h showed mostly non-viable cells for all test groups (Fig. 3b,d,f).

**Gene expression**

After the RNA-isolation procedure, a complete lysis of odontoblast and subodontoblast tissue was evident in histologic sections (Fig. 1h,i) as well as SEM images (Fig. 2h,i). Superficial dentin was decalcified, collagen fibers were exposed on the surface and no cellular structures were observed.

A comparative gene expression analysis between the odontoblast layer and the removed pulp showed that most genes (OPN, OCN, RUNX2, MSX2, DLX5, ALP, OSX and DSPP) were similarly expressed without statistical difference between both tissues (Fig. 4). NES and COL1A1 were upregulated 3.5- and 5-fold in the odontoblast layer compared to pulp tissue with a statistical significance for NES \(P = 0.0079\). The most prominent differences were noticed for DMP1 and BSP, which were significantly increased in odontoblasts by 5- and 77-fold, respectively \(P = 0.0079\). Age-related trends were not observed among cell donors in this study.

**Discussion**

An *in vitro* culture of primary odontoblasts would offer a promising model system to investigate numerous aspects of odontoblast activity. The obtained results reveal serious limitations regarding the isolation and especially the cultivation of single odontoblasts from human teeth. However, the possibility to selectively extract RNA from odontoblasts and pulp cells allows for comparative gene expression analysis.

In this study, teeth were split at the cemento-enamel junction and the pulp tissue was removed from the crown. Histologic and SEM imaging confirmed that odontoblasts and the adjacent subodontoblastic cell layer remained attached to the inner dentin surface. With scraping and mechanical disruption alone, no intact cells could be isolated. Thus, three different combinations of enzymes were chosen in order to detach the cells from the pulp chamber walls. As collagen is the major structural component of dental pulp, collagenase type IA was selected as a constituent of all tested enzyme combinations. Protease type I \(\alpha\) exhibits an unspecific mode of action. Trypsin type I \(\beta\) cleaves peptide bonds of basic amino acids (arginine, lysine) at the C-terminal side (19); as it dissolves adhesive contacts, it is commonly used to remove cells
from culture dishes and was selected for this study to singularise and detach odontoblasts. Hyaluronan is a component of the extracellular matrix and hyaluronidase has been used previously to dissociate odontoblasts from rat teeth (17). Thus, it was selected for combination c.

In order to minimise events of cell death, both the concentrations as well as the incubation periods were optimised for all mixtures a to c in preliminary experiments. Short incubation times and low enzymatic activity did not sufficiently break up the extracellular matrix and allow cell detachment from dentin. However, too long incubation or higher concentrations visibly harmed the cells. All parameters for odontoblast isolation were tested thoroughly and cytocompatibility of enzyme mixtures was also evaluated with pulp fibroblasts. Furthermore, centrifugation of the tooth crowns after enzymatic treatment considerably increased the quantity of isolated cells and proved to be crucial to harvest a satisfactory number of odontoblasts. Histologic analysis of tooth crowns after centrifugation showed detachment of the cell layer on 40% to 60% of the dentin surface.

With enzyme combination x, collagenase and protease degraded the extracellular matrix and predentin efficiently, and single odontoblast cell bodies were observed in culture dishes. The combination β, supposedly gentler due to a lower concentration of collagenase combined with trypsin, did not sufficiently degrade the extracellular matrix but apparently attacked the interface between cells and dentin. Thus, clusters of cells were observed where cell processes appeared truncated. Collagenase...
and hyaluronidase (γ) led to degradation of the matrix and single odontoblast cell bodies were visible in cell culture. The predentin surface was moderately eroded compared to collagenase/protease (α) and the odontoblast processes were exposed over a shorter distance as reported recently (16). Most isolated cells in culture were scattered and showed short processes.

Generally, the degradation of extracellular matrix was crucial to expose odontoblasts and allow the separation of cell bodies. This could be achieved by collagenase in combination with protease or hyaluronidase. Type I protease widely exposed the odontoblast processes through erosion of the less mineralised predentin, which allowed isolation of cells with longer processes. Despite a gentle isolation procedure and optimised conditions for cell attachment by poly-L-lysine-coated culture surfaces the number of isolated odontoblasts was moderate.

Light microscopy showed that odontoblasts were isolated by enzymatic treatment in all test groups. Despite slight differences in quality and quantity, cells were generally not viable for more than 24 h in culture. Trypan blue staining, a well-accepted dye exclusion test, was selected to evaluate cell viability (20). Viable cells with intact membranes have the ability to exclude the dye, whereas coloration of the cell body indicates penetration of the dye and thus substantial damage. Considering the
Figure 3 Isolated cells under an inverted light microscope (odontoblast processes indicated by arrowheads). (a) Cells isolated by collagenase and protease (a) showed comparatively long processes. (b) Their cytoplasm was stained with trypan blue after 24 h incubation. (c) Treatment with collagenase and trypsin (b) led to detachment of large cell clusters, (d) stained with trypane blue as well. (e) Digestion with hyaluronidase and collagenase (c) enabled the separation of numerous single cells from dentin, but (f) cellular processes were truncated and membranes were not intact after 24 h in culture. Scale bars: 10 μm.

Figure 4 Gene expression in odontoblasts compared to pulp cells. Median values and 25–75% percentiles were calculated from five experiments with cellular material from five different donors (n = 5). Statistically significant differences were found for NES, DMP1 and BSP and indicated by asterisks (P = 0.0079).
mechanical and enzymatical strain the cells experienced during isolation, this parameter appeared to be crucial. However, further assessment of the isolated cells by e.g. MTT assay, which has been used in a previous study (16), was not considered as appropriate or necessary. The MTT assay is based on the enzymatic conversion of the MTT dye by cellular enzymes and thus quantifies their metabolic activity. As the cell metabolism after isolation of primary cells is highly disturbed, enzyme remnants might cause false positive results and a reasonable positive control (successfully isolated odontoblasts) is hardly possible, a MTT assay did not appear expedient (21).

Membrane wounds in eukaryotic cells are normally able to reseal within a few seconds. Small lesions (< 100 nm) such as porous defects are closed by caveolar endocytosis or shedding of ESCRT (endosomal sorting complex required for transport) vesicles (22). If membranes are damaged to a larger degree (> 100 nm), calcium-influx leads to caveola-mediated wound constriction and endocytosis of the damaged membrane region, which will be degraded intracellularly and likely be shed as vesicles (23). Here, odontoblast processes appeared torn and cells were not able to exclude the dye after 24 h. The damage seemed too grave to be cured by the internal repair mechanisms. Though cultivation of viable odontoblasts was generally not successful over a time period of 24 h, it might still be possible that single cells survive the isolation procedure and allow for patch-clamp electrophysiological techniques, immunostaining or in situ mRNA hybridisation to be applied within a few hours after isolation (16,17).

A comparison of gene expression in the dentin-adhering odontoblast layer and the pulp core should shed light on characteristic features of mature human odontoblasts. Therefore, genes that play a role during odontoblast differentiation (DLX5, RUNX2, MSX2, OSX) and matrix mineralisation (OPN, OCN, ALP, DSPP, NES, COL1A1, DMP1, BSP) were selected. After pulp removal, odontoblasts and a subodontoblastic cell layer remained attached to dentin. The teeth selected for this study showed completed root formation and were without carious lesions. Thus, the odontoblast cells were fully differentiated and undamaged. Histologic imaging also revealed a layer of mature odontoblasts, and up- or downregulation of typical transcription factors that play a role during odontoblast differentiation (DLX5, RUNX2, MSX2, OSX) was not observed compared to the core pulp.

However expression profiles largely resembled one another, three genes were significantly higher represented in the odontoblast layer: nestin, dentin matrix acidic phosphoprotein 1 and bone sialoprotein. Nestin is an intermediary filament, which is typically found in neural-crest-derived cells and regarded as a specific marker for functional odontoblasts of young permanent teeth (24). During dentin formation, odontoblasts secrete collagen that provides a template for mineral deposition. Here, the slightly elevated level of COL1A1 in odontoblasts compared to the core pulp was less an indicator for active mineralisation, but rather accumulates during the baseline production of extracellular matrix (25). Likewise, a continuous collagen expression has been observed in rat odontoblasts, which then increased during active dentin formation (26). Furthermore, acidic proteins like dentin matrix acidic phosphoprotein 1 or bone sialoprotein play an important role in this process as they initiate nucleation and guide crystal formation during dentinogenesis (27). Beyond that, Dmp1 acts as a signalling molecule and promotes odontoblast differentiation of mesenchymal precursor cells (28). Both DMP1 and BSP were significantly higher expressed in odontoblasts compared to pulp tissue. In accordance with our findings, these genes have been described as characteristic in vivo-markers of mature bovine odontoblasts in a comparative transcriptionome analysis with pulp tissue by Simon et al. (1). To further support these findings, it is necessary to investigate the expression of Col1a1, Dmp1 and Bsp on the protein level by e.g. immunohistochemistry and prove the protein expression in the odontoblast layer.

However, DSPP is also a widely accepted odontoblast marker and upregulated during dentin secretion (16,29). After cleavage of the precursor dentin sialophosphoprotein (Dsp), the resulting dentin phosphoprotein (Dpp) and dentin sialoprotein (Dsp) play a role during mineralisation and are finally embedded in the dentin matrix. Interestingly, DSPP expression was similar in both tissue compartments. This might be due to the fact that most of the crown construction is finished after primary dentinogenesis and the odontoblasts resided in an inactive state. A downregulation of DSPP in mature odontoblasts was previously described for bovine samples (1), and microarray analysis of human teeth after a similar isolation protocol reported unaltered expression between pulp tissue and odontoblasts (30). Thus, DSPP seems to be primarily expressed in odontoblasts or odontoblast-like cells during differentiation or progressive mineralisation (24,31). Likewise, further mineralisation-associated genes (ALP, OPC and OCN) were not upregulated in mature odontoblasts as they are not in a state of active mineralisation. However, the expression of OCN in odontoblasts was reported differentially in the literature and might be species-specific (1,26).

Though it must be pointed out that gene expression of primary odontoblasts in another developmental status or of mesenchymal stem cells undergoing odontoblast differentiation might likely differ, nestin, bone sialoprotein and dentin matrix acidic phosphoprotein 1 seem to be robust markers for mature primary odontoblasts in vivo.
Isolation of Primary Odontoblasts

Conclusion

Single odontoblasts can be isolated from human teeth, but not kept vital for further in vitro experiments. However, the extraction of odontoblast RNA provides a feasible and promising way to analyze cellular characteristics e.g. in organ cultures. Successful in vitro cultivation of whole teeth has been described in the literature and odontoblasts attached to dentin can be kept alive in their natural environment (11,32). Thus, extraction of RNA or other cellular components allows for various experimental designs in a translational set-up.

Conflict of interest

The authors deny any conflicts of interest related to this study.

Authorship declaration

MW, KMG and WB designed the study. MW, CB, KMG analyzed the data and wrote the manuscript. GS was involved in sample preparation and reviewed the manuscript. All authors read the manuscript and are in agreement with it.

References

Results Chapter 3: Systematic reviews of APCs and clinical protocols for REP
Platelet concentrates for revitalization of immature necrotic teeth: a systematic review of the clinical studies

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Platelet concentrates for revitalization of immature necrotic teeth: a systematic review of the clinical studies

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Abstract
This systematic review aimed at determining the effectiveness of autologous platelet concentrates (APC) in the treatment of immature necrotic teeth. An electronic search was performed on MEDLINE, Embase, Scopus, Scielo, Lilacs, CENTRAL. Comparative clinical studies were included, in which APC was tested for pulp regeneration and radicular development. Selected articles underwent risk-of-bias assessment. Clinical and radiographic outcomes were considered. Three randomized parallel studies and one split-mouth case series were included. One study had low risk of bias and three studies had high risk. A total of 61 immature necrotic teeth were treated in 56 patients. Follow-up ranged between 12 and 18 months. All studies used platelet-rich plasma (PRP) in the test group, and one also used platelet-rich fibrin (PRF). After treatment, all teeth of control and experimental groups remained asymptomatic for the entire study duration. Only one study reported response to cold and electric pulp test, showing not significantly better outcomes for the test group. Similarly, periapical healing and apical closure were improved in the group treated with APC although statistical significance was not achieved \((P = 0.08)\) and \((P = 0.06)\), respectively, probably due to the limited sample size. The teeth treated with PRP achieved significantly better thickening of the dentin walls \((P = 0.01)\), and root lengthening \((P = 0.001)\) than control teeth. Despite the potential effectiveness of APC in promoting root development of necrotic immature teeth, scarce evidence exists regarding this subject. In the studies evaluated in this review, platelet concentrates showed promising results that warrant further investigation.

Keywords
Endodontic regeneration, immature teeth, necrotic pulp, necrotic teeth, platelet-rich plasma

Introduction
The treatment of immature necrotic teeth is today a great clinical challenge. The lack of apex closure and the low thickness of the dentin walls make difficult and unpredictable the endodontic treatment of the tooth. Traditionally, the treatment for these teeth have been the apexification, achieved with calcium hydroxide \((\text{Ca(OH)}_2)\) or, more recently, with mineral trioxide aggregate (MTA). \((\text{Ca(OH)}_2)\) induces the formation of an apical barrier, but requires multiple patient visits, a treatment time of 5–20 months and renewal of the intracanal dressing on several occasions [1]. Furthermore, the apical closure is unpredictable [2], and there is a susceptibility of cervical root fracture after prolonged exposure to \((\text{Ca(OH)}_2)\) [3]. On the other hand, MTA provides an effective artificial apical barrier. It seems more biocompatible than \((\text{Ca(OH)}_2)\), does not get resorbed nor weakens the root canal dentin and may allow apexification completion in a single session. However, this material is extremely difficult to handle, it has traces of some toxic elements on its composition, may cause tooth discoloration and also has a high economic cost, which makes difficult its widespread use [4]. Moreover, treatment of immature permanent teeth with \((\text{Ca(OH)}_2)\) or with MTA allows only small, and often negligible, root development in terms of width and length of the dentin walls [5].

In this scenario, regenerative endodontics (RE) appears as an interesting option for the treatment of necrotic immature teeth with open apices and low thickness of the dentin walls. RE is defined as a “biologically based procedure designed to replace damaged structures, including dentin and root structures, as well as cells of the pulp-dentin complex” [6]. The concept of RE is relatively new. While the first ideas were introduced in the early 1970s [7], the therapy was substantially initiated in the early 2000s and most of the clinical studies on this topic have been published in the last few years [8]. Moreover, RE using platelet concentrates is a newer and less-understood concept.

Three requirements are needed for successfully achieving the revascularization of a tooth adopting a regenerative approach [9]:

- An appropriate source of stem cells that are present in the apical papilla [10] and in the periapical tissues of an immature tooth [11]
- Soluble mediators like growth factors that promote the migration, proliferation and differentiation of cells. The growth factors are normally secreted by platelets and other cells present in the blood clot [8]

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A scaffold that forms a three-dimensional environment regulates and provides support to cells' growth and differentiation. The scaffold is naturally provided by the intracanal blood clot, the dentin walls and the fibrin mesh promoted by platelets in the coagulum [5, 8].

Autologous platelet concentrates (APCs) are blood-derivate products with above-baseline concentration of platelets, and consequently, an increased number of platelet-derived growth factors (PDGF) [12]. The growth factors, once delivered into the root canal system, may induce the differentiation of the stem cells. The stem cells from the apical papilla have odontogenic differentiation potential and they are considered the source of odontoblasts, which are responsible for the development of root dentin [13]. Additionally, platelet concentrates stimulate collagen production, promote migration of cells to the surgical site and enhance the vascular ingrowth, thereby speeding up the healing process [9]. All these biological characteristics of platelet concentrates may increase the possibility of pulp revascularization and increase root thickness and length, due to ingrowth of dentin due to the deposition of dentine by newly differentiated odontoblasts.

The most known platelet concentrates that can be prepared with commercially available systems currently used in clinical setting are platelet-rich plasma (PRP), plasma rich in growth factors (PRGF) and platelet-rich fibrin (PRF). PRP is characterized by the presence of leukocytes, and a high platelet concentration (up to 5–8 times the baseline value). It is prepared from anticoagulated blood undergoing a double centrifugation step and requires an activator before use [12]. PRGF is characterized by the absence of leukocytes and a modest increase in platelet concentration (2–3 times the baseline value). It is prepared from anticoagulated blood undergoing a single centrifugation step, and requires an activator before use [14]. PRF is characterized by the presence of most platelets and leukocytes in a dense fibrin matrix that does not require an activator before use. It is prepared from non-anticoagulated blood undergoing a single centrifugation step [15].

Despite the potential effectiveness of platelet concentrates in promoting root development of necrotic immature teeth, there is still scarce literature regarding this subject. The present review is aimed at systematically identifying relevant and possibly well-designed studies available and describes their outcomes.

Main objective: To determine the effectiveness of platelet concentrates in the treatment of immature necrotic teeth, through a systematic review of the literature.

Specific objectives: To determine, through a systematic review of the literature: (a) if the immature necrotic teeth treated with platelet concentrates may achieve radicular development in terms of apex closure, root thickening and thickening of the root canal walls; (b) if the immature necrotic teeth treated with platelet concentrates remain asymptomatic through time and have a positive response to cold and electric pulp test; and (c) in the presence of a periapical lesion, if the treatment with platelet concentrates may resolve the condition, achieving satisfactory regeneration of the apical defect in the absence of signs and symptoms.

Materials and methods

A systematic literature review was carried out following a standard protocol.

Search strategy

An electronic search was performed on the following databases: MEDLINE, ScienceDirect, Scopus, SciELO, Lilacs, Cochrane Central Register of Controlled Trials (CENTRAL). The last search was performed on November 2015. The search terms used were “regenerative endodontics”, “pulp regeneration”, “platelet concentrate”, “platelet-rich plasma”, “platelet-rich fibrin”, “platelet gel”, “growth factors”, “tooth revitalization”, “immature necrotic teeth”, “apexification” and “apexogenesis”. The search terms were used alone or in combination by means of the Boolean operators OR and AND. Furthermore, a manual search of issues from 2000 up to the last issue available on November 2015, including the “Early view” (or equivalent) section, was undertaken on the following journals: Australian Endodontic Journal, Dental Traumatology, International Endodontic Journal, Journal of Endodontics, Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology and Endodontology. The reference list of the retrieved reviews and of the included studies was also searched for possible additional eligible studies not identified by the electronic search.

Inclusion criteria

For being included, studies had to have a comparative design and report clinical results of regenerative procedures in patients having one or more immature necrotic teeth, in which APC was used for pulp regeneration and radicular development.

The search was limited to clinical studies involving human subjects. Restrictions were not placed regarding the language or publication date. Both prospective and retrospective studies were included. Studies with at least three subjects per group were included. The studies had to provide clear and adequate information on agents/medicaments used in regenerative procedures, in addition to the type of platelet concentrate used. Studies that used the platelet concentrates as apical barriers for avoiding the extrusion of material (MTA) into the periradicular tissues were excluded, since they do not report results regarding the development of the immature teeth.

Publications that did not meet the above inclusion criteria and those that were not dealing with original clinical cases (e.g. reviews, technical reports) were excluded. Multiple publications of the same pool of patients were also excluded. When papers from the same group of authors, with very similar databases of patients, materials, methods and outcomes, were identified, the authors were contacted for clarifying whether the pool of patients was indeed the same. In case of multiple publications relative to consecutive phases of the same study or to enlargements of the original sample size, only the most recent data (those with the longer follow-up and the larger sample size) were considered.

Selection of the studies

Two reviewers independently screened the titles and the abstracts of the articles initially retrieved through the electronic search. The concordance between reviewers was assessed by means of the Cohen’s Kappa coefficient. In case of disagreement, a joint decision was taken by discussion with a third reviewer. The full texts of all studies of possible relevance were independently assessed by the same two reviewers to check whether they met all inclusion criteria. For articles excluded at this stage, the reason for exclusion was recorded.

Data extraction

Data were extracted by two reviewers independently. The main variables extracted from each included study were the following: study design, study setting (university, hospital, private practice), number of operators involved, sample size, patients gender and age, proportion of smokers, type of platelet concentrate used, tooth type and location and follow-up duration.
Primary outcomes: presence of symptoms, response to cold and electric pulp test, radiographic healing of the periapical lesion, quality of life of patients assessed by means of questionnaires or interviews, occurrence and type of any complication.

Secondary outcomes: Radicular development in terms of apex closure, root lengthening and thickening of the root canal walls, evaluated radiographically. In order to normalize the possible different scores adopted to evaluate the results (usually scored as excellent, good, satisfactory and unsatisfactory), and to perform a proper statistical analysis, the outcomes were dichotomized. Cases scored as excellent and good were aggregated.

Risk of bias analysis

The following methodological parameters were also recorded: for randomized studies: the random sequence generation method and allocation concealment; for all comparative studies: blinding of outcome assessment, clear definition of inclusion and exclusion criteria, clear definition of outcomes assessment and success criteria, completeness of the outcome data reported and explanation for dropouts/withdrawal (when applicable), selective reporting, recall rate (it was assumed adequate if dropout <20%), sample size calculation.

The methodological quality of the selected studies was evaluated independently and in duplicate by two reviewers, according to the above methodological parameters. All the criteria were assessed as adequate, unclear or inadequate. The authors of the included studies were contacted for providing clarifications or missing information as needed. Studies were considered at low risk of bias if two-third or more of the parameters were judged as adequate, and were considered at high risk of bias if less than two-third of the parameters were judged as adequate.

Statistical analysis

Descriptive statistics of the included studies was performed by summarizing the total number of cases treated with each surgical approach, the percentage of successful cases and of post-treatment adverse events. For studies reporting comparison between cases treated with APC and cases treated without APC, the estimate of the effects of APC adjunct was planned to be expressed as odds ratio (OR) together with 95% confidence intervals. The statistical evaluation considered both the treatment site and the patient as the analysis unit, when possible. Comparison among studies was performed by meta-analysis, if applicable, combining ORs through a fixed-effects model (Mantel-Haenszel method). When meta-analysis was not feasible, Fisher’s exact test was used for statistical comparison between test and control groups for the main outcome variables.

Results

The flow chart summarizing the screening process is presented in Figure 1. The electronic search yielded a total of 292 articles. Twelve additional articles were found by manual searching and no ongoing controlled clinical study was found in clinical trials online registries. After a first screening of the titles and abstracts, a total of 21 articles reporting results of studies on patients with immature necrotic teeth undergoing regenerative procedures in combination with the use of APCs were found [16–36]. Seventeen of them were excluded for being case reports or non-comparative case series [16–27, 32–36]. No further article was excluded after reading the full text. Finally, four prospective studies were included in this review, all of them having a comparative design [28–31].
Characteristics of the included articles

One study was carried out in Turkey [30] and three were carried out in India [28, 29, 31]. All studies were performed within institutional settings and received ethical committee approval. No article reported dropouts and the duration of follow-up was 12 months in two articles [28, 29] and 18 months in two articles [30, 31].

All studies used blood clot in the control groups. PRP was used in the treatment groups in all studies, and PRF was used in the treatment group of one study [31]. No comparative study was found using PRGF for the treatment of immature necrotic teeth.

One pilot study had a split-mouth design [28] and three a parallel group design [29–31].

Only one article reported a standardized methodology for taking the pre-surgical and control radiography, thus reducing the distortion and magnification [29].

The risk of bias summary of included articles is described in Figure 2. One study was judged at low risk of bias [30] and three at high risk of bias [28, 29, 31].

Characteristics of the participants

The participants in three studies were reported to be 26 males and 15 females, between 7 and 23 years old [28–30], whereas in one study the 15 participants had unknown age and gender [31]. The ages of the participants of the control and experimental groups were similar. All teeth were permanent non-vital immature incisors and premolars (Table I). A total of 61 immature necrotic teeth were treated; 28 of them were treated with the adjunct of PRP, 5 with the adjunct of PRF and 28 with blood clot alone, as control group [28–31] (Table III).

The pulp necrosis aetiology was caries or traumatism. Fifty-five teeth were associated with periapical radiolucent lesion and six were not (Table I).

No study reported information regarding the proportion of smokers and only one article provided information about pretreatment signs and symptoms, and tooth mobility [30] (Table I). Three articles reported that patients treated were healthy [29–31] and one did not report this information [28].

Age range and gender of patients, number and type of tooth treated, pretreatment signs and symptoms, and treatment information of the included studies are summarized in Table I.

Clinical protocol

In general, the clinical protocol of the included articles is described as the removal of the necrotic pulp, irrigation with NaOCl with minimal or no mechanical instrumentation of the dentin walls (Table I). After that, medication of the canal with a triple antibiotic paste is applied (Table I) [28–31]. In subsequent control visits, and after disappearance of tooth symptoms, the platelet concentrate is injected in the root canal, where it tends to polymerize assuming a gel form. The platelet concentrate is used alone [30], in conjunction with collagen sponge [31] or in combination with collagen sponge and blood clot [28, 29]. MTA is then placed into the root canal over the platelet concentrate or blood clot [30]. The protocol used for producing the PRP concentrate was very similar among articles. All of them used the same anticoagulant, with two centrifugation steps and equal revolution for minutes on each step, but with some differences in the activator used (Table II).

Clinical and radiographic findings

The clinical and radiographic outcomes of the treatment and control groups of the included articles are summarized in Table III.

All four articles provided data regarding post-treatment signs and symptoms. All immature necrotic teeth, treated with PRP, PRF or blood clot, remained asymptomatic with complete resolution of signs and symptoms after 12 or 18 months (Table III). The response to sensitivity pulp test after treatment was assessed by only one study: 50% of the teeth treated with PRP displayed a positive response to cold and electrical stimulation, against 20% in the control group, although such difference was not statistically significant [30] (Table III).

In all articles the root development was assessed radiographically [28–31]. Three articles measured apical closure, thickness of the dentin walls and root lengthening [28, 29, 31] and one article measured apical closure and percentage of increased root area [30] that can be seen as a means to estimate the thickness of the dentin walls (>15% was considered as excellent, 5–15% was considered as good and <5% was considered as satisfactory). The teeth treated with platelet concentrates achieved better apical closure, thickening of the dentin walls and root lengthening than teeth of the control group, all these differences being statistically significant except for the former, which was borderline (Table III). Bezgin et al. [30] reported that the time required for achieving complete apical closure was similar between groups (the PRP group required a mean of 8.1 months for complete apical closure, compared with 9 months in the BC group).

Regarding healing of the periapical lesion, no significant differences were found between teeth treated with platelet concentrates and teeth of the control groups (P = 0.08) (Table III). Enlargement of the periapical lesion was reported in only one tooth of the control group [30]. Additionally, Bezgin et al. [30] reported that healing time between the PRP and control group was similar, although complete healing occurred slightly sooner in the PRP group (mean, 6.4 months) than in the BC group (mean, 6.8 months).
<table>
<thead>
<tr>
<th>Authors (Ref.)</th>
<th>Study design</th>
<th>Patients gender (age range)</th>
<th>Aetiology of pulp necrosis (n)</th>
<th>No. of teeth</th>
<th>Tooth type (n)</th>
<th>Pretreatment signs and symptoms</th>
<th>Treatment of the root</th>
<th>Intracanal medication</th>
</tr>
</thead>
<tbody>
<tr>
<td>JadHAV et al. (28)</td>
<td>Case series</td>
<td>Two males, one female (10–23 years old)</td>
<td>Trauma (4)</td>
<td>6</td>
<td>Upper central incisors (6)</td>
<td>Sensitive to percussion and/or palpation</td>
<td>PRP + collagen sponge + BC (3)</td>
<td>Minimal</td>
</tr>
<tr>
<td>JadHAV et al. (29)</td>
<td>RCT Parallel groups</td>
<td>Fourteen males, six females (15–23 years old)</td>
<td>NR (20)</td>
<td>20</td>
<td>Central incisors (20)</td>
<td>NR</td>
<td>PRP + collagen sponge + BC (10)</td>
<td>Minimal</td>
</tr>
<tr>
<td>Bezgin et al. (30)</td>
<td>RCT Parallel groups</td>
<td>Ten males, eight females (7–12 years)</td>
<td>Caries (6)</td>
<td>20</td>
<td>Premolars (6)</td>
<td>Sensitive (16)</td>
<td>PRP + collagen (5)</td>
<td>No</td>
</tr>
<tr>
<td>Narang et al. (31)</td>
<td>RCT Parallel groups</td>
<td>15 patients (&lt;20 years)</td>
<td>NR (15)</td>
<td>15</td>
<td>NR</td>
<td>Sensitive (16)</td>
<td>PRP + collagen (5)</td>
<td>Minimal</td>
</tr>
</tbody>
</table>

NR: not reported; PRP: platelet-rich plasma; PRF: platelet-rich fibrin; BC: blood clot; NaOCl: sodium hypochlorite; TAP: triple antibiotic paste (metronidazole, ciprofloxacin, minocycline); RCT: randomized clinical trial; EDTA: ethylenediamine-tetra-acetate; CHX: chlorhexidine.
antimicrobial effects against several oral microorganisms, which could be relevant in the control of post-treatment reinfection. The post-operative period in fact is particularly at risk since tissues involved are still fragile, poorly vascularized and more susceptible to bacterial challenge [55–58].

The rational basis for using platelet-rich preparations for the regeneration of immature necrotic teeth pulp rests on the assumption that the high concentration of endogenous growth factors, among which is the VEGF (which may trigger angiogenesis improving tissue revascularization), allows for a potent enhancement of the regeneration process stimulating periapical healing, apical closure, lateral dentin wall thickening and root lengthening, in a safe and physiological manner through patient-derived molecules. Platelet concentrates might therefore represent an advantageous option if compared to alternative treatments constituted by various substances like Ca(OH)₂ or MTA, which on the one hand promote apical barrier formation, but on the other hand have been associated with a number of concerns and issues.

One of the first considerations emerging from this review is the current paucity of studies with high evidence level. Of the 21 clinical studies retrieved, 17 were case reports or small-sized case series and only four were comparative studies, all having very low sample size. The relatively recent introduction of platelet concentrates in RE is probably one of the causes for such overall poor evidence level of studies on this topic. Although case report studies were not specifically analysed in this review, all of them reported a beneficial effect of the use of platelet concentrates for the treatment of immature necrotic teeth. Such an effect was substantially confirmed by the recent four comparative studies included for qualitative and quantitative analyses. One of them was a pilot study based on a series of three split-mouth cases [28] in which the experimental treatment was assigned randomly, like in the other three studies with a parallel design [29, 30, 31]. Given the nature of the treatment test, no allocation concealment was possible, at least in two studies [29, 30], where the patients receiving platelet concentrates knew which group they were assigned to, since they were submitted to blood drawing. In the third study with parallel design, two of the four groups were treated with different platelet concentrates (PRP and PRF). Thus, allocation concealment was possible, but it was not reported, neither patients of these groups were blinded to treatment. Owing to different operative protocols, also the blinding of the clinician was not feasible in all these studies. It is not possible, however, to determine if and how such lack of blinding could have affected the outcomes.

The reduced sample size of all included articles may compromise the validity of the outcomes. This is necessary in order to conduct proper randomized controlled clinical trials, to increase...
Table III. Clinical and radiographic outcomes of treatment and control groups, after 12 months of treatment.

<table>
<thead>
<tr>
<th>Authors</th>
<th>No. of° teeth (group)</th>
<th>Follow-up</th>
<th>Presence of symptoms (n)</th>
<th>Response to cold and electric pulp Test (n)</th>
<th>Radiographic regression of the periapical lesion</th>
<th>Apex closure</th>
<th>Root lengthening</th>
<th>Thickening of the dentinal walls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jadhav et al. (28)</td>
<td>3 (PRP + collagen sponge + blood clot)</td>
<td>12 months</td>
<td>No (3)</td>
<td>NR (6)</td>
<td>Excellent + Good</td>
<td>Excellent + Good</td>
<td>Satisf.</td>
<td>Excellent + Good</td>
</tr>
<tr>
<td>Jadhav et al. (29)</td>
<td>10 (PRP + collagen sponge + blood clot)</td>
<td>12 months</td>
<td>No (10)</td>
<td>NR (20)</td>
<td>Excellent + Good</td>
<td>Excellent + Good</td>
<td>Satisf.</td>
<td>Excellent + Good</td>
</tr>
<tr>
<td>Bezgin et al. (30)</td>
<td>10 (PRP)</td>
<td>18 months</td>
<td>No (10)</td>
<td>Positive (5)</td>
<td>Excellent + Good</td>
<td>Excellent + Good</td>
<td>Satisf.</td>
<td>Excellent + Good</td>
</tr>
<tr>
<td></td>
<td>10 (Blood clot)</td>
<td></td>
<td>No (10)</td>
<td>Positive (2)</td>
<td>Excellent + Good</td>
<td>Excellent + Good</td>
<td>Satisf.</td>
<td>Excellent + Good</td>
</tr>
<tr>
<td>Narang et al (31)</td>
<td>5 (PRP + Collagen)</td>
<td>18 months</td>
<td>No (5)</td>
<td>NR (15)</td>
<td>Excellent + Good</td>
<td>Excellent + Good</td>
<td>Satisf.</td>
<td>Excellent + Good</td>
</tr>
<tr>
<td></td>
<td>5 (PRF)</td>
<td></td>
<td>No (5)</td>
<td>5</td>
<td>Excellent + Good</td>
<td>Excellent + Good</td>
<td>Satisf.</td>
<td>Excellent + Good</td>
</tr>
<tr>
<td></td>
<td>5 (BC)</td>
<td></td>
<td>No (5)</td>
<td>3</td>
<td>Excellent + Good</td>
<td>Excellent + Good</td>
<td>Satisf.</td>
<td>Excellent + Good</td>
</tr>
<tr>
<td>P-Value (Fisher exact test)</td>
<td></td>
<td></td>
<td></td>
<td>0.14</td>
<td>0.08</td>
<td>0.06</td>
<td>0.01</td>
<td>0.001</td>
</tr>
</tbody>
</table>

PRP: platelet-rich plasma; PRF: platelet-rich fibrin; NR: not reported.
the number of participants. Additionally, the use of a standardized methodology for taking the radiographs was reported by only one study. It is imperative to use a reproducible methodology, using individual bite-wings, in order to minimize the magnification and/or distortion of the radiograph, which directly alter the measurement of tooth lengthening and thickness of the dentin walls.

In three studies PRP was combined with a collagen sponge acting as intracanal scaffold [28, 29, 31], while in the study by Bezgin et al. only PRP was used [30]. The results of the latter study suggest that, at least in such a small and protected environment as the root canal, the fibrin network formed during PRP activation may represent a proper scaffold for supporting tissue growth in endodontic regeneration. A recent review suggested that PRF could represent an even better substrate for endodontic regeneration, due to its stronger fibrin mesh compared to PRP [59]. In fact, due to the absence of anticoagulant, polymerization of the fibrin filaments in PRF during coagulation occurs in a more natural manner and is characterized by the formation of tetrameric junctions, greater homogeneous three-dimensional organization and thicker filaments with respect to other platelet concentrates [60]. In some of the case reports retrieved, PRF was used, obtaining satisfactory results [16–18, 20]. Only one study [31] compared the use of PRP versus the use of PRF, reporting that the latter “has huge potential to accelerate the growth characteristics in immature necrotic permanent teeth as compared to PRP and blood clot”. Such a conclusion was based on groups composed of only five samples and clearly needs to be confirmed. On the other hand, no comparative clinical study has been published to date, reporting the use of PRGF, which only was tested in an in vitro study [53] and in a case series [33]. Such type of platelet-rich product is characterized by the absence of leukocytes, which causes a reduced pro-inflammatory action, which might be useful in predictably controlling the occurrence of symptoms in the post-operative period. Indeed, it would be interesting to investigate by means of properly designed randomized studies whether different types of platelet concentrates may have different effects on endodontic tissue regeneration.

According to some researchers [54] “regenerative therapy outcomes may vary between teeth exhibiting partial necrosis (i.e., teeth with some vital tissue in the apical portion of the canal) and those exhibiting full necrosis (i.e., teeth in which the pulp has been completely lost)”. Others [55] suggested that the type of pulp regenerative might vary according to such various clinical situations. If pulp presents partial necrosis, the residual pulp tissue in theory might recover after disinfection, contributing to regeneration of the lost portion of the pulp; hence, the prognosis for such teeth is good. Conversely, in the presence of complete loss of the pulp tissue, which requires de novo synthesis of the pulp, prognosis is poor, and a specific regenerative procedure may be needed [54, 55]. In the included studies, all teeth were non-vital, and the positive outcomes achieved in the experimental groups suggest that platelet-rich preparations may indeed represent a valuable tool for the recovery of such necrotic teeth.

It is important to note that none of the included articles report histological outcomes, mainly because histological analysis requires extraction of the tooth, which is not allowed for ethical concerns. For this reason, it was not possible to assess the nature of the neoformed tissue responsible for the thickening and lengthening of the root in the included studies. In the literature, only some controlled animal studies [56–61] and a few case reports [62–64] presented histological outcomes of endodontic regenerative procedures with platelet concentrates. The histological results of the animal studies show that neoformed intracanal tissue is mainly cementum-like and bone-like instead of pulp-like [56, 58, 61]. Conversely, in only one of the three clinical case reports the presence of intracanal pulp-like connective tissue was detected. In such study PRP alone was injected in the root canal during regenerative endodontic treatment [62]. In another study, loose connective tissue like immature pulp containing fibroblasts and mesenchymal cells was observed in the root canal space of an immature human maxillary central incisor diagnosed with irreversible pulpite without apical periodontits 3.5 weeks after revascularization [56]. In the third case report study, dentaloid osteoid tissue (like the animal studies), vital connective tissue and blood vessels formed in immature mandibular molar with apical periodontitis irrespective of the type of scaffold used (PRP or blood clot) [64].

Conclusion

Despite the potential effectiveness of APCs in promoting root development of necrotic immature teeth, there is still scarce evidence regarding this subject. Only four comparative studies satisfied the inclusion criteria of the present systematic review and could be selected. In these studies the use of PRP or PRF showed promising results that warrant further investigation. Last but not least, in addition to biological safety and effectiveness, autologous platelet-rich preparations represent simple and cost-efficient procedures that may have a consistent impact in reducing the economic costs for standard medical treatments in RE.

To better understand and possibly demonstrate the clinical beneficial effect of APC in the treatment of immature necrotic teeth, well-designed clinical studies with solid methodological quality and a follow-up of at least 12 months are necessary. Such clinical studies should have a comparative design where the effect of APC can be assessed against the standard treatment as the blood clotting, or against different types of platelet concentrate. APC should be prepared using standardized and certified technology; the method for obtaining APC must be clearly described as apparently negligible differences in any steps of the procedure may lead to products with critically different features and biological activity.

When planning a comparative clinical study, the sample size should be estimated in advance, in order to have sufficient statistical power to be able to demonstrate the possible superior effect of APC. In addition, an a priori clear definition of the patient’s inclusion criteria and of the outcomes that will be assessed at the baseline and at the recall visits is mandatory. Clinically, apex closure, root lengthening, thickening of the dentine wall and the pulp sensitivity test have to be evaluated; the regression of the lesion has to be monitored on reproducible radiographs. Split-mouth studies should be preferred over parallel design when possible, in order to minimize individual subjects variability; however it is critical to ensure that the bilateral defects are comparable and that the only difference between test and control groups is the use of APC. Any further variation introduced in the protocol may represent confounding variables whose effect on the final result may be difficult if not impossible to be quantified.

Declaration of interest

The authors deny any conflicts of interest related to this study.

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References


Regenerative Endodontics

Autologous Platelet Concentrates for Pulp and Dentin Regeneration: A Literature Review of Animal Studies

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Abstract

Introduction: The purpose of this study was to evaluate the effectiveness of autologous platelet concentrates (APCs) in promoting pulp and dentin regeneration in animal models. Methods: An electronic search was performed on MEDLINE, Embase, Scopus, SciELO, LILACS, and CENTRAL. Animal studies using APC as a root filling material after pulpectomy in mature or immature teeth were included. Articles underwent risk of bias assessment. Histologic evaluation of intracanal neoformed tissue was the primary outcome; root development, root wall thickening, apical closure, and periapical healing in apical periodontitis were the secondary outcomes. Results: Seven articles were included. Platelet-rich plasma (PRP) was used as root filling material during regenerative procedures in the experimental group in either mature or immature teeth. After revascularization with PRP alone or in conjunction with stem cells of a different source, the histologic analyses revealed that, in addition to an odontoblastic cell layer or dentinlike structure, the neoformed intracanal tissues were mainly cementumlike, bonelike, and connective tissues. Conclusions: True regeneration of necrotic pulp may not be achieved with current techniques using PRP, all of which stimulated tissue repair. Benefits of PRP adjunct for pulp tissue regeneration in preclinical studies remain unclear. Further studies with standardized protocols are necessary to assess the actual contribution of PRP in endodontic regenerative therapies. (J Endod 2016;42:250–257)

Key Words

Endodontic regeneration, immature teeth, platelet-rich plasma, pulpectomy

The goal of tissue regeneration is to form a new tissue with the same anatomy and function as the original one (1). Regenerative endodontic procedures rely on tissue engineering and are defined by the American Association of Endodontists as “biologically-based procedures designed to physiologically replace damaged tooth structures including dentin and root structures as well as cells of the pulp-dentin complex” (2). Although several approaches have been used to date, there is still no protocol able to achieve predictable endodontic tissue regeneration (3, 4).

In cases of immature teeth, the necrotic process involving pulp tissue halts further root development and condemns it to a lack of apical closure and reduced thickness of dentinal walls, which compromises the prognosis of the tooth. In the same manner, the pulp necrosis of mature teeth may produce tooth discoloration and infection of the periapical tissues, among other complications. Root canal therapy has been the traditional approach for mature necrotic teeth as well as for immature teeth after an apexitication procedure. However, a vital pulp is critical for the maintenance of tooth homeostasis and longevity (4). In cases of absence of a functional pulp tissue and vascular perfusion, the root canal is not able to support the new tissue formation on its own (5–7). Consequently, current revascularization procedures using blood clot or hemocomponents represent an aid for the management of necrotic teeth.

Regeneration of pulp tissue may be enhanced by the combination of the patient’s own growth factors and bioscaffold. Autologous platelet concentrates (APCs) have recently emerged as a possible tool for enhancing regeneration procedures in the medical field; APCs gained popularity among oral and maxillofacial surgeons as well as in other fields such as orthopedics, plastic surgery, and sports medicine, assuming an important role for increasing the predictability of hard and soft tissue regeneration procedures (8–13). APCs are hemocomponents obtained through the centrifugation of a blood sample of the patient. The basic concept of this technology is to collect the most active components of the blood sample (eg, platelets, fibrin, and in certain cases leukocytes). This process produces a very high-concentration gradient of platelets whose granules are rich with many substances fundamental to promote the healing process including adhesive proteins; procoagulant factors; cytokines and chemokines; antimicrobial proteins; and a number of mitogenic growth factors such as platelet-derived growth factors, transforming growth factor-beta, epidermal growth factors, and vascular endothelial growth factors (14–17), which may trigger angiogenesis and improve tissue vascularization. The APCs can be classified based on the fibrin architecture and cellular content as follows: platelet-rich plasma (PRP) with or without leukocytes (L-PRP and P-PRP, respectively) and platelet-rich fibrin (PRF) with or without leukocytes (L-PRF and P-PRF) (18). L-PRP is characterized by the presence of leukocytes and a high platelet concentration (up to 5–8 times the baseline value). It is prepared from anticoagulated blood undergoing a double centrifugation step and requires an activator before use. P-PRP is characterized by the absence of leukocytes and a modest increase in platelet concentration (2–3 times the baseline value). It is prepared from anticoagulated blood undergoing a single centrifugation step and requires an activator before use (14). L-PRF is characterized by the presence of most platelets and leukocytes in a dense fibrin matrix that does not require an activator before use (19). It is prepared from nonanticoagulated blood undergoing a single centrifugation step. The rational basis for the use of APCs for the treatment of pulpectured teeth rests on the assumption that the high concentration of growth factors represents a potent stimulation for tissue healing obtained through the patient’s own
molecules, mimicking the physiological process. In addition to granule content release, the polymerization of fibrinogen into a fibrin mesh forms a platelet gel or clot that is delivered to the surgical site (14).

Current protocols developed in the context of regenerative endodontic therapy aim at meeting the 3 main ingredients of tissue engineering: scaffold, growth factors, and stem cells. Specifically, fibrin within the blood clot or autologous platelet concentrates may act as a natural scaffold through which stem cells from the apical tissues may embed and repopulate the canal space. Growth factors released from an intracanal blood clot or APCs may modulate such cellular recruitment as well as stem cell proliferation and differentiation (20).

Early animal studies in beagle dogs treated using blood clot observed new tissue formation inside the root canal after revascularization (5, 21–24). In particular, Wang et al (21) in 2010 reported that neoformed intracanal tissues, after blood clot induction, in immature teeth consisted of cementoid and osteoid tissues (that were hypothesized to be responsible for root lengthening and thickening) and periodontal ligament–like tissue. Similar results have been shown in revitalization procedures in mature teeth (25). This suggested that the neoformed intracanal tissues may have little similarity to the healthy pulp tissue and raised the question whether the revascularization procedures with blood components might lead to pulp regeneration or just tissue repair.

The growth factors released by platelet concentrates proved to be effective in inducing angiogenesis and regeneration of different tissues and might therefore represent a useful tool for necrotic pulp treatment (26–28).

The aim of the present systematic review of the literature was to evaluate current preclinical evidence about the effectiveness of APCs in restoring the pulp-dentin complex of a necrotic tooth by promoting pulp and dentin tissue regeneration when they are used after pulpectomy.

**Materials and Methods**

**Search Strategy**

A systematic literature search was performed on the following electronic databases (PubMed, SciELO, LILACS, ScienceDirect, Scopus, and Cochrane Central Register of Controlled Trials) using (platelet OR fibrin) AND (endodont* AND regenerat* OR apex*) as the search string. Once the studies were identified, the search was then restricted to only animal studies in which histologic outcomes were reported. An additional hand search of issues from 2000 up to the last issue available on December 15, 2014, including the “early view” (or equivalent) section was undertaken on the following journals: Australian Endodontic Journal, Dental Traumatology, International Endodontic Journal, Journal of Endodontics, and Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology and Endodontology. The reference lists of the retrieved reviews and the included studies were also searched for possible additional eligible studies not identified by the electronic search. The last electronic search was performed on January 15, 2015. Only articles published in English were considered, and no restrictions regarding publication date were placed.

**Inclusion Criteria**

Animal studies assessing the effectiveness of APCs for stimulating the regeneration of pulp tissue and/or inducing radicular development...
Regenerative Endodontics

TABLE 1. Main Characteristics of the Included Studies

<table>
<thead>
<tr>
<th>Authors</th>
<th>Animal species, n</th>
<th>Tooth type, test/control</th>
<th>Pretreatment state of the root</th>
<th>Treatment of the root</th>
</tr>
</thead>
</table>
| Zhu et al, 2012, 2014 (33, 38) | Beagles, 4        | Upper premolars/ lower premolars | Mature permanent teeth        | cDPSCs, 8A  
|                                |                   |                          |                               | PRP, 8  
|                                |                   |                          |                               | BC + PRP, 8  
|                                |                   |                          |                               | BC + BMA gel, 8  
|                                |                   |                          |                               | BC + PRP + BMA, 8 |
| Gomes-Filho et al, 2013 (25)   | Beagles, 2        | Upper and lower premolars | Mature permanent teeth        | PRP + HA, 8*                          |
| Petrović et al, 2013 (34)      | Monkeys Chlorocebus aethiops, 8 | Lower canines and lower central incisors/  
|                                |                   |                          |                               | Immature permanent teeth            |
| Zhu et al, 2013 (35)           | Beagles, 4        | Lower premolars          | Immature permanent teeth      | DPCs, 10  
|                                |                   |                          |                               | PRP + DPCs, 10 |
| Torabinejad et al, 2014 (36)   | Ferrets, 7        | Upper and lower canines  | Immature permanent teeth      | PRP, 9*  
| Zhang et al, 2014 (37)         | Beagles, 3        | Premolars                | Immature permanent teeth      | PRP, 12C  

BC, blood clot; BMA, bone marrow aspirate; cDPSCs, canine dental pulp stem cells; CH, calcium hydroxide; DPCs, dental pulp cells; HA, hydroxyapatite; MTA, mineral trioxide aggregate; NR, not reported; NaOCl, sodium hypochlorite; PRP, platelet rich plasma; TAP, triple antibiotic paste (metronidazole, ciprofloxacin, minocycline).

Reasons of exclusion from histologic analysis: *1 root cracked during the histologic sectioning procedure; +1 root cracked during the histologic sectioning procedure; 6 roots damaged during sectioning, and 71 root was excluded because it was damaged during sectioning.  

were included. Studies had to have a comparative design in which APC was used as a root filling material and to report any type of findings of endodontic regenerative procedures. Studies treating either mature or immature teeth were included. Any type of platelet concentrate was included, and it could be used alone or in combination with other materials or stem cells of a different source. Publications that did not meet these inclusion criteria and those that were not dealing with animal studies (eg, reviews, clinical cases, and in vitro studies) were excluded.

Selection of the Studies

Titles and abstracts of the initially retrieved articles were screened independently by 2 reviewers (A.L. and M.D.F.) to identify possible eligible studies meeting the inclusion criteria. The concordance between reviewers was assessed by means of the Cohen kappa coefficient. In case of disagreement, a joint decision was reached by discussion with a third reviewer (C.B.M.). When the abstract was not available, the full text was obtained and checked. Publications that did not meet the selection criteria were excluded. Disagreements were resolved by discussion. Full text of all eligible articles was obtained, and the same 2 reviewers independently assessed them to check if they met all inclusion criteria. For excluded articles, reasons for exclusion were reported.

Data Extraction

Relevant data from included articles were extracted and analyzed by 2 independent reviewers (A.L. and C.B.M.). Cases of disagreement were subject to joint evaluation until an agreement was reached.

The primary outcome was histologic evaluation of intracanal tissues. The secondary outcomes were radiologic evaluation of root development, root wall thickening, apical closure in case of immature teeth, and periapical healing in case of periapical periodontitis.

Risk of Bias Analysis

Methodologic quality of the selected studies was also evaluated as part of the data extraction process. The ARRIVE (Animals in Research: Reporting In Vivo Experiments) guidelines for reporting animal experiments in periodontology and implantology (29) and a systematic review on instruments for assessing risk of bias and other methodologic criteria of animal studies (30) were used to identify 10 items suitable for the evaluation of the risk of bias of the studies included in this review.

Quality criteria taken in consideration were as follows:

1. Ethical statement (nature of ethical review permissions and national or institutional guidelines for the care and use of animals)
2. Experimental procedures (precise details of all procedures performed)
3. Experimental animals (details of animal used including species, developmental stage or mean age, type of teeth, and diagnosis)
4. Randomization
5. Allocation concealment
6. Sample size calculation
7. Completeness of information on dropouts
8. Blinding of the evaluator
9. Financial conflict of interest

The evaluation of the methodologic quality of the selected studies was performed by 2 reviewers independently and in duplicate according to the previously described parameters. All the criteria were assessed as adequate, unclear, or inadequate. The authors of the included studies
were contacted for providing clarifications or missing information as needed. Studies were considered at low risk of bias if more than two thirds of the parameters were judged as adequate.

**Results**

The article selection process is presented in Figure 1. The electronic search retrieved 271 articles, whereas the manual search identified 10 additional articles. After the first screening consisting of title and abstract evaluation, 272 articles were excluded: 16 were duplicates, 228 were nonrelated to the topic of this review, and 28 were not dealing with an animal study (15 clinical studies, 8 reviews, 1 letter to the editor, and 4 in vitro studies). Ten full-text articles were assessed for eligibility. After full-text evaluation, 2 articles were excluded: the first one because the endodontic regenerative treatment actually did not involve the use of APC (31) and the second one because it was not possible to obtain translation from Serbian language and the English abstract provided poor information (32). Finally, 7 articles met the inclusion criteria and were included in this review (25, 33–38). The assessed Cohen kappa coefficient value was equal to 0.88, meaning an almost perfect agreement between reviews on the selection of the studies according to the scale of Landis and Koch.

In 2 of the included articles, the same pool of teeth was treated, but different outcomes (radiologic and histologic in 1 study and histochromic and immunohistochemical in the other one) were reported (33, 38).

All articles used PRP as a root filling material after pulpectomy, 3 of them in mature teeth and 4 in immature teeth (25, 33–38) (Table 1).

### Table 1

<table>
<thead>
<tr>
<th>Authors</th>
<th>Type of treatment</th>
<th>Irrigation</th>
<th>Sealing</th>
<th>Follow-up, no. of teeth</th>
</tr>
</thead>
<tbody>
<tr>
<td>BC, 8</td>
<td>Vital pulpectomy</td>
<td>5.25% NaOCl + 17% EDTA + physiological saline</td>
<td>NR</td>
<td>MTA + composite</td>
</tr>
<tr>
<td>BC, 8</td>
<td>Pulpectomy (after pulp disruption and induction of necrosis and apical periodontitis)</td>
<td>2.5% NaOCl + 17% EDTA + sterile saline</td>
<td>TAP</td>
<td>MTA + CH cement + composite</td>
</tr>
<tr>
<td>HA, 8*</td>
<td>Vital pulpectomy</td>
<td>NR</td>
<td>NR</td>
<td>Glass ionomer cement + amalgam</td>
</tr>
<tr>
<td>BC, 10</td>
<td>Pulpectomy (after pulp disruption and induction of necrosis and apical periodontitis)</td>
<td>1.25% NaOCl + sterile saline</td>
<td>TAP</td>
<td>MTA + composite</td>
</tr>
<tr>
<td>BC, 12*</td>
<td>Vital pulpectomy</td>
<td>17% EDTA + sterile saline</td>
<td>NR</td>
<td>MTA</td>
</tr>
<tr>
<td>BC, 17*</td>
<td>Pulpectomy (after pulp disruption and induction of necrosis and apical periodontitis)</td>
<td>3% NaOCl + 0.9% sterile saline</td>
<td>TAP</td>
<td>Glass ionomer cement</td>
</tr>
</tbody>
</table>

### Table 2

<table>
<thead>
<tr>
<th>Authors</th>
<th>Type of concentrate</th>
<th>Manual procedure</th>
<th>PRP Concentration</th>
<th>Anticoagulant</th>
<th>Activator</th>
</tr>
</thead>
<tbody>
<tr>
<td>Petrović et al, 2013 (34)</td>
<td>PRP</td>
<td>1. 1200 rpm × 20 min 2. 2000 rpm × 15 min</td>
<td>NR</td>
<td>Na-citrate</td>
<td>NR</td>
</tr>
<tr>
<td>Zhu et al, 2012, 2014 (33, 38)</td>
<td>PRP</td>
<td>1. 200 g × 10 min 2. 360 g × 15 min</td>
<td>&gt;1,200 × 10⁹/L</td>
<td>Citrate solution</td>
<td>Bovine thrombin + 10% calcium chloride</td>
</tr>
<tr>
<td>Gomes-Filho et al, 2013 (25)</td>
<td>PRP</td>
<td>1. 300 g × 10 min 2. 640 g × 10 min</td>
<td>NR</td>
<td>Citratephosphate-dextrose-adenine-1</td>
<td>Autologous thrombin</td>
</tr>
<tr>
<td>Zhu et al, 2013 (35)</td>
<td>PRP</td>
<td>1. 200 g × 10 min 2. 360 g × 15 min</td>
<td>&gt;1,200 × 10⁹/L</td>
<td>Citrate solution</td>
<td>Bovine thrombin + 10% calcium chloride</td>
</tr>
<tr>
<td>Torabinejad et al, 2014 (36)</td>
<td>PRP</td>
<td>2 steps</td>
<td>NR</td>
<td>NR</td>
<td>Thrombin + 10% calcium chloride</td>
</tr>
<tr>
<td>Zhang et al, 2014 (37)</td>
<td>PRP</td>
<td>1. 100 g × 15 min 2. 3200 rpm × 30 min</td>
<td>800 × 10⁹/L</td>
<td>EDTA-k₂</td>
<td>NR</td>
</tr>
</tbody>
</table>

NR, not reported; PRP, platelet-rich plasma.
The details of the process for obtaining the production of platelet concentrates for any included studies are reported in Table 2. The histologic outcomes of the included animal studies are summarized in Table 3. In addition, radiographic outcomes are detailed in Table 4. Finally, risk of bias assessment of the included studies is reported in Figure 2.

**Characteristics of the Included Studies**

In all studies included in this review, an ethical statement was reported regarding the type of ethical permissions received for conducting animal experiments and the institutional guidelines followed for the care and use of the animals. Precise details of all experimental animal procedures performed were also reported (Table 1). A total of 28 animals were treated: 13 beagle dogs (25, 33, 35, 37), 8 monkeys (34), and 7 ferrets (36).

All animal studies used PRP in the experimental group as root canal filling material. It was used alone (36, 37), in conjunction with canine dental pulp stem cells (33) or dental pulp cells (35), or with bone marrow aspirate gel and blood clot (25) (Table 1). In almost all studies, blood clot was the standard comparative treatment, except in 1 article in which hydroxyapatite (HA) was used as the control (34). The duration of follow-up was 3 months in 6 studies (25, 33, 35–38) and 6 months in 1 study (34).

**Characteristics of the Experimental Units**

In 4 articles in which teeth were depulped (25, 33, 35, 37), for the histologic evaluation, each root canal was considered as an independent unit, in particular 70 experimental root canals received PRP, whereas 44 root canals were treated with blood clot. On the contrary, in the remaining articles (34, 36), the tooth was used as the histologic unit, with a total of 17 teeth treated with PRP, 12 blood clot–treated teeth, and 8 treated with HA. Incisors, canines, and premolars were used. They were permanent teeth with complete or incomplete root formation, and some of them had experimentally induced apical periodontitis (Table 1).

**Root Canal Treatment Protocol**

The protocol for the radicular canal treatment in the included articles is described as one of the following:

1. The disruption of the pulp and induction of experimental periapical periodontitis (25, 35, 37)
2. Pulp removal from immature or mature teeth (33, 36) followed by root canal irrigation with different solutions as described in Table 1

Canal disinfection using triple antibiotic paste was performed in only 3 studies (25, 35, 37). At the subsequent visit, PRP was applied in the root canals, and root canal access was sealed using different composites. In one study (34) the treatment provided to one group of teeth was defined by the authors as high pulpotomy, meaning the removal of the pulp close to the radiographically visible end of the immature root. Hence, in this review such so-called high pulpotomy was considered as pulpectomy. To these teeth gutta-percha was applied along with selected material (PRP + HA or HA alone) and sealing with glass ionomer cement and amalgam (Table 1).

Protocols for preparing PRP are summarized in Table 2; consistent differences among studies were observed. Different authors followed diverse manual procedures (time and speed of the centrifugation steps) and used a different anticoagulant and activator. The final concentration of platelets obtained in PRP was reported in only 3 articles (33, 35, 37).

### Table 1: Histologic Outcomes of the Included Articles

<table>
<thead>
<tr>
<th>Authors</th>
<th>Follow-up (months)</th>
<th>No. of teeth (group)</th>
<th>Defects in periapical zone</th>
<th>Inflammatory reaction of pulp/periapical tissue</th>
<th>Fibrous connective tissue in the canal</th>
<th>Bone-like tissue</th>
<th>Cementum-like tissue</th>
<th>Granulation-like tissue</th>
<th>A</th>
<th>P</th>
<th>A</th>
<th>A</th>
<th>A</th>
<th>A</th>
<th>A</th>
<th>A</th>
<th>A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zhu et al, 2012</td>
<td>3</td>
<td>4</td>
<td>A</td>
<td>P</td>
<td>A</td>
<td>A</td>
<td>P</td>
<td>A</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Gomes-Filho et al, 2013</td>
<td>3</td>
<td>4</td>
<td>A</td>
<td>P</td>
<td>A</td>
<td>A</td>
<td>P</td>
<td>A</td>
<td>5</td>
<td>0</td>
<td>0</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Petrović et al, 2013</td>
<td>3</td>
<td>8</td>
<td>A</td>
<td>P</td>
<td>A</td>
<td>A</td>
<td>P</td>
<td>A</td>
<td>5</td>
<td>0</td>
<td>0</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Zhu et al, 2013</td>
<td>3</td>
<td>10</td>
<td>A</td>
<td>P</td>
<td>A</td>
<td>A</td>
<td>P</td>
<td>A</td>
<td>5</td>
<td>0</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Zhang et al, 2014</td>
<td>3</td>
<td>12</td>
<td>A</td>
<td>P</td>
<td>A</td>
<td>A</td>
<td>P</td>
<td>A</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Torabinejad et al, 2014</td>
<td>3</td>
<td>18</td>
<td>A</td>
<td>P</td>
<td>A</td>
<td>A</td>
<td>P</td>
<td>A</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

A, absence; BC, blood clot; BMA, bone marrow aspirate; cDPSCs, canine dental pulp stem cells; DPCs, dental pulp cells; HA, hydroxyapatite; NR, not reported; P, presence; PRP, platelet-rich plasma.

*Number of roots.
Histologic and Radiographic Results

In all studies, a histologic evaluation of intracanal and periapical tissues was performed (25, 33–37). One study also reported immunohistochemical and histochemical analyses of intracanal tissues (38). Four of the previously described studies also performed radiographic assessment (33, 34, 35, 37). An analytic description of the histologic findings is provided in Table 3, and detailed radiographic outcomes are reported in Table 4.

Risk of Bias Assessment

Only 2 studies (35, 37) were classified as having a low risk of bias, whereas all other included studies presented a high risk of bias based on the parameters evaluated (Fig. 2).

Discussion

In a healthy condition, the root canal is occupied by dental pulp tissue, which is constituted by a well-organized architecture of blood vessels, nerves, and cells, with the main function of producing dentin and maintaining the vitality of the pulp-dentin complex. In particular, among the pulp cells, odontoblasts are specialized to form dentin, the immune cells ensure a quick response against possible microbial contamination and undifferentiated mesenchymal cells, dental pulp stem cells replace the primary odontoblasts depositing the tertiary dentin, and stem cells from the apical papilla (SCAPs) of immature permanent teeth (39) have dentinogenic potential for the production of primary and secondary dentin (40). When the pulp is no longer vital, tooth becomes vulnerable, and the deposition of the dentin stops. Particularly, the treatment of immature necrotic teeth is a great challenge for endodontists. The main difficulty with young teeth having nonvital pulp is adequately cleaning the root canal, paying attention to the fragile and thin dentinal walls, and sealing the open apex properly. Traditional treatments had failed to predictably manage these teeth because there were no adequate therapeutic strategies (40). Root canal obturation using bioactive cements (41) may ensure a clinical success in terms of healing of the periapical lesion (42–44), but the continued root development and strengthening of the root structure may not be warranted (20).

Regenerative endodontics uses the concept of tissue engineering to turn the pathological pulp into a vital functional one by promoting the growth of new vital intracanal tissue, resulting in root development (dentin thickening and root apex closure) (40, 45). Although current regenerative endodontic protocols have reported successful clinical and radiographic outcomes in the treatment of immature necrotic teeth, the biological outcomes are still unpredictable, and a true pulp regeneration may not be achieved (20, 46). Moreover, pulp regeneration of mature teeth has been traditionally considered more challenging than the pulp regeneration of immature teeth, mainly because of the lower amount of stem cells and a narrower apical foramen that may allow revascularization (25). When attempting to regenerate functional pulp/dentin tissue in a root canal, some issues have to be considered. Regenerated pulp must do the following:

A, absence; BC, blood clot; cDPSCs, canine dental pulp stem cells; DPCs, dental pulp cells; HA, hydroxyapatite; NR, not reported; P, presence; PRP, platelet-rich plasma.

Table 4. Radiographic Outcomes of Test and Control Groups of Included Studies

<table>
<thead>
<tr>
<th>Authors</th>
<th>No. of teeth (group)</th>
<th>Follow-up (months)</th>
<th>Periapical healing</th>
<th>Root growth retardation</th>
<th>Root wall thickening</th>
<th>Apical closure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zhu et al, 2012, 2014 (33, 38)</td>
<td>4 (cDPSCs)</td>
<td>3</td>
<td>2</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td></td>
<td>4 (PRP)</td>
<td>3</td>
<td>1</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td></td>
<td>4 (PRP + cDPSCs)</td>
<td>0</td>
<td>4</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td></td>
<td>4 (BC)†</td>
<td>0</td>
<td>4</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td>Petrović et al, 2013 (34)</td>
<td>8 (PRP + HA)</td>
<td>6</td>
<td>8</td>
<td>0</td>
<td>3†</td>
<td>1</td>
</tr>
<tr>
<td>Zhu et al, 2013 (35)</td>
<td>10† (DPCs)</td>
<td>3</td>
<td>8</td>
<td>2</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td></td>
<td>10† (PRP)</td>
<td>10</td>
<td>0</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td></td>
<td>10† (DPCs + PRP)</td>
<td>9</td>
<td>1</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td></td>
<td>10† (BC)†</td>
<td>9</td>
<td>1</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td>Zhang et al, 2014 (37)</td>
<td>18† (PRP)</td>
<td>3</td>
<td>18</td>
<td>0</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td></td>
<td>18† (BC)</td>
<td>18</td>
<td>0</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
</tr>
</tbody>
</table>

*Number of roots.
†Control.
‡One root failed.

Figure 2. Risk of bias summary of the included studies.
Regenerative Endodontics

1. Be vascularized and innervated
2. Have cellular density and extracellular matrix architecture similar to the natural one
3. Produce odontoblasts that must line on the existing dentin layer and produce new dentin upon the existing one (47)

However, pulp regeneration is made difficult by the typical anatomic structure of the tooth. Indeed, pulp tissue is encased by the dentin and has the only blood supply, which is essential for the healing and regenerative processes, coming through the root apical foramen. Because of the highly organized pulp structure and composition, the most challenging problem is the ex novo pulp regeneration from a pulpless tooth and restoration of the ordinary function.

The objective of this review was to assess the effectiveness of APCs in achieving pulp and dentin regeneration. The presence of PRP in the root canal space might be beneficial to the stem cells from the apical papilla or other pulp cells seeded as part of the treatment in order to repopulate the root canal. In fact, stem cell proliferation and differentiation are mainly induced by the growth factors secreted from platelets, whose greater content in APCs may promote a stronger biological activity compared with the blood clot. However, a common feature highlighted among the animal studies included in this review was that the odontoblastic cell layer, dentinlike structure, or new pulplike tissue was rarely detected after revascularization using either PRP alone or in conjunction with pulp stem cells or pulp cells. Indeed, the new intracanal tissue formed was cementumlike tissue, bonelike tissue, and fibrous connective tissue. Only in 1 of the included studies in the present review (37) the regenerated tissue in the canal space was described as pulplike tissue with no difference between the PRP and blood clot groups. However, this tissue could not be considered as true pulp because of the absence of an odontoblast layer. Similar findings emerged from other preclinical studies in which odontoblasts were not identified, even if many pulp tissue elements were histologically detected as fibroblasts, blood vessels, and collagen. Besides unexpected cells or tissues, osteoblasts and cementum were sometimes observed in the root canal (5, 21–24).

Intracanal mineralized tissues were detected both adherent to the dentin wall (dentin-associated mineralized tissue [DAMT]) and forming bony islands in the central region (23). DAMT differs from dentin and bone and although resembling cementum tissue in terms of lack of vasculature and the immunostaining pattern, the organization and maturation of collagen matrix are different from the cementum as well. The pattern of mineralization in DAMT is less uniform than other mineralized tissues such as cementum. Bony islands resemble bone matrix in terms of morphology, collagen organization, and immunoreactivity (24). The cellular source responsible for DAMT and bony island formation is poorly understood. It has been suggested that the SCAPs might differentiate into odontoblasts forming new roots. By contrast, the detection of odontoblast-like cells was reported in vitro models when stem cells were seeded into scaffolds of a different type or in the presence of growth factors (48–53).

To date, few clinical studies on immature human teeth with apical periodontitis performed histologic analysis to investigate the nature of the neofomed intracanal tissues after revascularization/revascularization (54–56). Interestingly, in only 1 such study was the presence of intracanal pulplike connective tissue detected. In this clinical study, PRP alone was injected in the root canal as a regenerative endodontic treatment (54). In another study, loose connective tissue resembling immature pulp and containing fibroblasts and mesenchymal cells was observed in the root canal space of an immature human maxillary central incisor (55). Conversely, in the third histologic study, cementoid/osteoid tissue, vital connective tissue, and blood vessels formed in immature mandibular molars with apical periodontitis irrespective of the type of scaffold used (PRP or blood clot) (56).

Pulp tissue and apical papilla may have been destroyed by the over-instrumentation performed in some articles to contrast the root canal infection, thus impairing ex novo pulp regeneration and root maturation. This could be 1 explanation for the missing detection of the odontoblastic cell layer, dentinlike structure, or new pulplike tissue after revascularization procedures. Another explanation is that when the survival of stem cells located in the apical papilla is compromised because of apical periodontitis, no further odontoblast differentiation occurs, and, therefore, root dentin is no longer deposited. To accomplish regenerative endodontic therapy, it is necessary to stimulate DPSCs or SCAPs to adhere to the inner surface of the root canal dentin although it was reported that the use of some disinfection protocols may prevent the repopulation of stem cells of the dentinal surface, impairing the treatment outcome (57, 58). We have to consider that in the absence of a proper cell population, adjunctive treatments like platelet concentrates may become ineffective because growth factors released by platelets need to interact with target cells to affect their biologic activity.

Nevertheless, after the completion of the healing process, root development (consisting of root wall thickening, root lengthening, and root apex narrowing) occurred presumably through the growth of cementumlike and bonelike tissue. Therefore, clinical success may not correspond to histologic pulp regeneration.

In conclusion, from the histologic results of the studies included in the present review, it seems that there is no current protocol using APCs able to achieve a true regeneration of the necrotic pulp tissue either in immature or mature teeth. In fact, tissue repair seems apparently stimulated. Thus, the root canal may become repopulated with a living tissue that only marginally resembles the original pulp but may not have the same functional activity. Despite this, root maturation may be achieved, and teeth function is not compromised, which represents a success from a clinical standpoint. Thus, the results of clinical studies evaluating pulp regeneration procedures should be considered and compared with preclinical ones. In fact, the latter may not fully reflect the clinical environment, at least for the present topic. The benefit of the additional use of PRP for predictable regeneration of the pulp tissue in preclinical studies is unclear. Further studies with a higher evidence level and a standardized protocol are required to shed light on the actual role of PRP in such clinical applications.

Acknowledgments

The authors deny any conflicts of interest related to this study.

References

Regenerative endodontic therapy: a systematic review of clinical protocols

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Abstract: Aim: The aim of this research was to describe the clinical protocols used for regenerative endodontic therapy through a systematic review of animal and clinical studies. Materials and methods: A systematic review was performed using the MEDLINE, Scopus, Cochrane Library, SciELO, Google Scholar, ScienceDirect and EMBASE databases. Study search and selection was performed by two independent researchers. Animal and clinical studies in which regenerative endodontic therapy was performed on immature necrotic teeth were included. Only prospective studies (with comparative design or case series), with a sample size of 10 or more teeth were included. Results: Twenty-three articles were included in this review. All clinical studies used sodium hypochlorite (NaOCl) as a root canal irrigant, although concentrations varied from 1% to 6%. Chlorhexidine, saline, sodium thiosulfate and hydrogen peroxide were also used in some studies. For intracanal dressing, nine out of eleven studies used a triple antibiotic paste (TAP), although time of application varied from 2 to 6 weeks. Use of EDTA was reported by two clinical studies only. Animal studies used mostly NaOCl for irrigation, at concentrations of 1.25% to 5.25%, and intracanal dressing was mainly a mixture of metronidazole, ciprofloxacin and minocycline, for variable periods of time. Conclusions: Most of the studies did not follow a standard clinical protocol for regenerative endodontic therapy.

Keywords: Regenerative endodontics, immature teeth, clinical protocols

Introduction

Regenerative endodontic therapy (RET) is a new approach for teeth with necrotic pulp and immature roots. RET is defined as a biologically-based procedure designed to replace damaged structures, including dentin and cells of the pulp-dentin complex [1]. RET is possible due to the presence of stem cells in the apical papilla with odontogenic differentiation potential [2]. Moreover, dentin walls provide the scaffolding that supports new tissue formation, as well as growth factors [3], which induce cell proliferation and differentiation.

Before regenerative endodontic therapy came into use, immature necrotic teeth were treated with apexification, which allowed only limited and often negligible root development in terms of the width and length of the dentin walls [4]. Once the pulp undergoes necrosis, the deposition of dentin ceases and so does the root development. The open apex and the low thickness of the dentin walls make endodontic treatment of the tooth difficult and imprecise, thus compromising its prognosis.

Although RET has the three basic requirements for achieving regeneration (the presence of stem cells, scaffolding and growth factors), true pulp and dentin regeneration have not yet been reported. In animal and clinical studies, radiographically observed root development is due to the ingrowth of cementum-like and bone-like tissue rather than dentin, and intracanal tissue consists of connective tissue without an odontoblast layer, rather than pulp tissue [5]. The most encouraging results have been reported
Clinical protocols for regenerative endodontics

with the use of dental pulp stem cells seeded into scaffolds and transplanted subcutaneously in animals; the results are regenerated dentin [6] and odontoblast-like cells with cellular processes extending into the dentin tubules [7].

Most studies do not follow a standard protocol for RET. In general, studies report that the necrotic pulp is removed with minimal or no mechanical instrumentation. Disinfection is achieved with different irrigants and intracanal dressings at variable concentrations left in the canal for varying periods of time. Sodium hypochlorite, chlorhexidine and/or EDTA are commonly used as irrigants, as well as calcium hydroxide and antibiotic pastes as intracanal dressings. When the tooth is asymptomatic, the blood clot is induced through over-instrumentation. The blood invades the root canal carrying stem cells and growth factors. However, the absence of clear clinical protocols could be partly responsible for the lack of true tissue regeneration. It has been shown that some irrigants and intracanal dressings, such as sodium hypochlorite, chlorhexidine and antibiotic pastes, have a detrimental effect on stem cell survival and on the release of growth factors from the dentin walls [3, 8-10]. In contrast, other dressings such as calcium hydroxide or EDTA significantly increase SCAP (stem cells from the apical papilla) survival and proliferation [9-11]. The materials used (i.e. irrigants and intracanal medications), and their toxic concentrations, as well as the lack of use of other materials (i.e. EDTA) could eventually be one of the causes of the absence of true pulp and dentin regeneration and for cases with no root development.

The aim of this research was to describe the clinical protocols used for regenerative endodontic therapy of immature necrotic teeth through a systematic review of animal and clinical studies.

Materials and methods

Search strategy

The electronic search was carried out by two independent researchers. The search was made in the MEDLINE, Scopus, Cochrane Library, SciELO, Google Scholar, ScienceDirect and EMBASE databases and the terms used were ("regenerative endodontic" OR revitalization OR revascularization) AND (tooth OR teeth). The last electronic search was performed on May 9, 2016.

Inclusion and exclusion criteria

We included studies in which regenerative endodontic therapy was performed on immature necrotic teeth. Only prospective animal and clinical studies (with comparative design or case series) with a sample size of 10 or more teeth were included. The studies had to provide clear and adequate information on the clinical procedure and the irrigants/dressings used in regenerative therapy. The search included studies in English, Spanish, French, Portuguese and Italian. No restriction criteria were applied regarding date of publication.

Study selection and data extraction

Selection of the studies was carried out by two independent researchers. In case of disagreement a third reviewer was consulted. The first screening was performed by reading the title and abstract, and articles not complying with inclusion criteria were excluded. The full text of eligible articles was then examined to assess whether they complied with inclusion criteria.

The following information was extracted from clinical studies: patient/tooth characteristics and clinical protocol (age of the patient, teeth, pulp and periapical diagnosis, anesthetic used, mechanical instrumentation, irrigant and its concentration, intracanal dressing and period of time inside the canal, use of any other adjunct, and sealing material), clinical outcomes (pain, response to pulp vitality/sensitivity test, tooth discoloration) and radiographic outcomes (resolution of periapical lesion, root development). Information from animal studies was extracted separately and included: animal species and clinical protocol (teeth, anesthetic used, mechanical instrumentation, irrigant and its concentration, intracanal dressing and period of time inside the canal, use of any other adjunct, and sealing material), information regarding radiographic outcomes (resolution of periapical lesion and root development) and histological outcomes (type of newly formed tissue).

Results

Nine hundred and eighty-four studies were screened in the electronic search, and three
more studies were found through hand searching. Unrelated studies, duplicate studies and articles not complying with inclusion criteria were discarded. Finally, twenty-three articles were included in this review (Figure 1), of which eleven were clinical studies and twelve were animal studies.

Characteristics of clinical studies

Six studies were case series [12-17] and five were pilot clinical studies [18-22]. In all, 222 patients were treated with regenerative endodontic procedures on immature necrotic teeth, of which 160 were associated with periapical lesions. The age of patients was from 6 to 28. All patients were diagnosed with pulp necrosis, except 8 in one study [12], who reported pain when files were introduced into root canals, which could be interpreted as partial pulp necrosis.

Table 1 shows the clinical protocol used for RET. All studies used sodium hypochlorite (NaOCl) as a root canal irrigant, although concentrations varied from 1% to 6%. Chlorhexidine, saline, sodium thiosulfate and hydrogen peroxide were also used in some studies. For intracanal dressing, nine studies used a triple antibiotic paste (TAP), of which the majority used a mixture of metronidazole, ciprofloxacin and minocycline, and the rest used cefaclor, doxycycline, amoxicillin or clindamycin instead of minocycline (Table 1). The time of application varied from 2 to 6 weeks (Table 1). At the second appointment, two studies [13, 19] reported using a local anesthetic with a vasoconstrictor. One of these two studies reported difficulties achieving blood clot formation [13]. At the second appointment NaOCl was the most used irrigant, in variable concentrations. The use of EDTA was reported by two studies only (Table 1). The follow-up period was from 6 months to 3 1/2 years.

Table 2 includes the radiographic outcomes (root development and resolution of periapical lesions) and the clinical outcomes (response to pulp vitality test and discoloration for TAP, MTA or Ca(OH)2) of the clinical studies included in this review. Only one patient out of 222 reported post-operative pain in the treated tooth [19].

Characteristics of animal studies

Twelve animal studies were included in this review. Table 3 shows the protocol for the regenerative endodontic therapy in the selected animal studies. The animals used were dogs [23-32], ferrets [33] and monkeys [34], with a total of 275 teeth and 32 canals treated (Table 3). In most studies, the pulp was removed or was disrupted and left in the canal. The pulp chamber was left exposed to the oral cavity, until formation of a periapical lesion. In the rest of the studies, vital teeth were depulped and RET was applied immediately, without the use of intracanal dressing. All teeth with apical lesions were irrigated with NaOCl at concentrations of 1.25% to 5.25%, and intracanal dressing was mainly a mixture of metronidazole, ciprofloxacin and minocycline, for variable periods of time.

Table 4 shows the radiographic outcomes (root development and resolution of the periapical lesion) and histological outcomes (type of newly
### Table 1. Characteristics, clinical protocol, clinical and radiographic outcomes of the clinical studies

<table>
<thead>
<tr>
<th>Authors</th>
<th>Groups</th>
<th>N of teeth/n of periapical lesion</th>
<th>Intratreatment</th>
<th>Irrigants 1st session (concentrations)</th>
<th>Intracanal dressing (time)</th>
<th>Irrigants 2nd session (concentration)</th>
<th>EDTA (concentration) x Time</th>
<th>Blood clot</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bezgin et al. 2015 [21]</td>
<td>Control (BC)</td>
<td>10/9</td>
<td>No</td>
<td>NaOCl (2.5%) Chlorhexidine (0.12%), Saline</td>
<td>TAP¹ (3 weeks)</td>
<td>Saline</td>
<td>Yes (17%) x NR</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>Experimental (PRP)</td>
<td>10/7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>NR</td>
<td>No</td>
</tr>
<tr>
<td>Jadhav et al. 2012 [18]</td>
<td>Control (BC + Collagen sponge)</td>
<td>10/10</td>
<td>Minimal</td>
<td>NaOCl (2.5%)</td>
<td>TAP¹ (NR)</td>
<td>NR</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>Experimental (BC + PRP + Collagen sponge)</td>
<td>10/10</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>NR</td>
<td>No</td>
</tr>
<tr>
<td>Nagata et al. 2014 [19]</td>
<td>Control (TAP)</td>
<td>12/6</td>
<td>No</td>
<td>NaOCl (6%) Sodium thiosulfate + Chlorhexidine (2%) + Saline</td>
<td>TAP¹ (3 weeks) Calcium hydroxide + Chlorhexidine gel (2%) (3 weeks)</td>
<td>Saline</td>
<td>Yes (17%) x 3 minutes</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>Experimental (Calcium hydroxide)</td>
<td>11/5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>NR</td>
<td>No</td>
</tr>
<tr>
<td>Nagy et al. 2014 [20]</td>
<td>Control (BC)</td>
<td>12/NR</td>
<td>Minimal</td>
<td>NaOCl (2.6%)</td>
<td>TAP¹ (3 weeks)</td>
<td>NaOCl (2.6%) + Saline</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>Experimental (BC + hydrogel + bFGF)</td>
<td>12/NR</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>NR</td>
<td>No</td>
</tr>
<tr>
<td>Narang et al., 2015 [22]</td>
<td>BC</td>
<td>5/5</td>
<td>Minimal</td>
<td>NaOCl (2.5%)</td>
<td>TAP³ (4 weeks)</td>
<td>NaOCl (2.5%)</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>PRP + Collagen</td>
<td>5/5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>PRF</td>
<td>5/5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Chen et al. 2012 [12]</td>
<td>-</td>
<td>20/20</td>
<td>Minimal</td>
<td>NaOCl (5.25%)</td>
<td>TAP³ (in cases of discoloration minocyclin was replaced with cefaclor) (2-6 weeks)</td>
<td>NaOCl (5.25%)</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>18/11</td>
<td>No</td>
<td>NaOCl (5%), Saline</td>
<td>TAP³ (4 weeks)</td>
<td>NaOCl (NR)</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Dabbagh et al. 2012 [13]</td>
<td>-</td>
<td>16/14</td>
<td>No</td>
<td>NaOCl (1%)</td>
<td>TAP³ (4 weeks)</td>
<td>NaOCl (1%)</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Kahler et al. 2014 [14]</td>
<td>-</td>
<td>32/22</td>
<td>Minimal</td>
<td>NaOCl (3%) or Chlorhexidine (NR)</td>
<td>TAP³ (4-6 weeks) (n=10)</td>
<td>NaOCl (NR)</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Mctigue et al. 2013 [15]</td>
<td>-</td>
<td>20/17</td>
<td>No</td>
<td>NaOCl (2.5%), Saline</td>
<td>TAP³ (2 weeks)</td>
<td>Saline</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Shah et al. 2008 [17]</td>
<td>-</td>
<td>14/14</td>
<td>Minimal</td>
<td>NaOCl (2.5%), Hydrogen peroxide</td>
<td>Formocresol (NR)</td>
<td>N/A</td>
<td>No</td>
<td>No</td>
</tr>
</tbody>
</table>

BC: Blood clot. PRP: platelet-rich plasma. PRF: platelet-rich fibrin. bFGF: Basic fibroblast growth factor. TAP¹: Metronidazole, ciprofloxacin and minocycline. TAP²: Metronidazole, ciprofloxacin and cefaclor. TAP³: Metronidazole, ciprofloxacin and doxycycline. TAP⁴: Metronidazole, ciprofloxacin and amoxicillin. TAP⁵: Metronidazole, ciprofloxacin and clindamycin. NR: Not reported. NA: not applicable.
<table>
<thead>
<tr>
<th>Authors</th>
<th>Groups</th>
<th>N of teeth/n of periapical lesion</th>
<th>Apical closure</th>
<th>Root lengthening</th>
<th>Dentin walls thickness</th>
<th>Healing of the periapical lesion</th>
<th>Discoloration for Pulp vitality</th>
<th>Minocycline</th>
<th>MTA</th>
<th>Ca(OH)2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bezgin et al. 2015</td>
<td>Control (BC)</td>
<td>10/9</td>
<td>6</td>
<td>NR</td>
<td>9</td>
<td>8</td>
<td>NA</td>
<td>12</td>
<td>NA</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Experimental (PRP)</td>
<td>10/7</td>
<td>7</td>
<td>NR</td>
<td>9</td>
<td>7</td>
<td>5</td>
<td>NA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Jadhav et al. 2012</td>
<td>Control (BC + Collagen sponge)</td>
<td>10/10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>NR</td>
<td>NA</td>
<td>NA</td>
<td>NR</td>
</tr>
<tr>
<td></td>
<td>Experimental (BC + PRP + Collagen sponge)</td>
<td>10/10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>5</td>
<td>NA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nagata et al. 2014</td>
<td>Control (TAP)</td>
<td>12/6</td>
<td>8</td>
<td>5</td>
<td>5</td>
<td>6</td>
<td>10</td>
<td>NR</td>
<td>NA</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Experimental (Calcium hydroxide)</td>
<td>11/5</td>
<td>6</td>
<td>3</td>
<td>5</td>
<td>4</td>
<td>NA</td>
<td>NR</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Nagy et al. 2014</td>
<td>Control (BC)</td>
<td>12/NR</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>NA</td>
<td>NA</td>
<td>NR</td>
</tr>
<tr>
<td></td>
<td>Experimental (BC + hydrogel + bFGF)</td>
<td>12/NR</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>NA</td>
<td>NA</td>
<td>NR</td>
</tr>
<tr>
<td>Narang et al., 2015</td>
<td>BC</td>
<td>5/5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>NR</td>
<td>NA</td>
<td>NA</td>
<td>NR</td>
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<td></td>
<td>PRP + Collagen</td>
<td>5/5</td>
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<td>5</td>
<td>NA</td>
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<td></td>
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<td>5</td>
<td>NA</td>
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<tr>
<td>Chen et al. 2012</td>
<td>BC</td>
<td>20/20</td>
<td>NR</td>
<td>15</td>
<td>20</td>
<td>20</td>
<td>NA</td>
<td>2</td>
<td></td>
<td>NR</td>
</tr>
<tr>
<td></td>
<td>PRF</td>
<td>5/5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>NA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dabbagh et al. 2012</td>
<td>BC</td>
<td>18/11</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>2</td>
<td>NA</td>
<td></td>
<td>NR</td>
</tr>
<tr>
<td>Kahler et al. 2014</td>
<td>BC</td>
<td>16/14</td>
<td>2 (out of 16)</td>
<td>4 (out of 9)</td>
<td>8 (out of 9)</td>
<td>12</td>
<td>NA</td>
<td>12</td>
<td></td>
<td>5</td>
</tr>
<tr>
<td>Mctigue et al. 2013</td>
<td>BC</td>
<td>32/22</td>
<td>23</td>
<td>21</td>
<td>22</td>
<td>22</td>
<td>21</td>
<td>NA</td>
<td>7</td>
<td>NR</td>
</tr>
<tr>
<td>Shah et al. 2008</td>
<td>BC</td>
<td>20/17</td>
<td>NR</td>
<td>10</td>
<td>9</td>
<td>15</td>
<td>NR</td>
<td>NR</td>
<td></td>
<td>NR</td>
</tr>
<tr>
<td>Bezgin et al. 2015</td>
<td>BC</td>
<td>14/14</td>
<td>NR</td>
<td>10</td>
<td>8</td>
<td>14</td>
<td>NA</td>
<td>NA</td>
<td></td>
<td>NR</td>
</tr>
</tbody>
</table>

formed tissue and presence of inflammatory cells) of the animals studies included in this review. In most studies [23, 24, 27-29, 31, 32] the follow-up period was just 3 months. The others lasted 6 to 7 months [23, 25, 26, 34] and one lasted 12 months [30].

Discussion

In general, the regenerative endodontic treatments reviewed could be considered clinically successful, since 84.14% of the treated teeth (or canals) had some degree of root development, and 79.8% of teeth showed healing of periapical lesions. However, the histological analysis of the newly formed tissue in the animal studies showed that the tissues described as responsible for root development are either cementum-like and/or bone-like, and connective tissue, rather than dentin and pulp. Moreover, one animal study showed that, despite having used dental pulp stem cells in immature necrotic teeth, there was no true dentin or pulp regeneration [32]. On the other hand, other studies reported the most encouraging results with the use of dental pulp stem cells seeded into scaffolds and transplanted subcutaneously in animals; the results are odontoblast-like cells with the cell process extending into the dentine tubules [7], pulp-like tissue and regenerated dentin [6].

The materials used (i.e. irrigants and intracanal medications), and their toxic concentrations as well as the lack of use of other materials (i.e. EDTA) could be partially responsible for the absence of true pulp and dentin regeneration and for cases with no root development. It has been proven that irrigation with 2% chlorhexidine is highly cytotoxic for stem cells [8]. In the same way, survival of stem cells of the apical papilla after irrigation with hypochlorite 6% is greatly reduced. However, hypochlorite at 0.5%, 1.5% and 3% induced the lowest decrease in survival of these cells (37% approx.) and final irrigation with EDTA seemed to revert the negative effect of hypochlorite [11]. Additionally, hypochlorite 6% + EDTA reduced the adhesion of stem cells to dentin walls [35]. All but one of the clinical studies included in this systematic review used a hypochlorite concentration greater than 1.5%, and three used concentrations of 5% or more. Clinical studies that used a concentration of sodium hypochlorite of 5% or more presented mixed results, as did the only two clinical studies that used EDTA (Tables 1 and 2).

The survival of SCAP is also conditioned by intracanal medication. An in vivo study proved that calcium hydroxide was the only medication tested that was associated with SCAP survival at all concentrations, particularly at a concentration of 1 mg/ml [9]. By contrast, antibiotics at concentrations from 1 to 6 mg/ml led to the death of 50% of the cells [9]. Additionally, an in vitro study showed that the release of some growth factors from dentin decreased after the use of TAP or chlorhexidine gel, but increased with the use of calcium hydroxide water-based paste [3]. All but three articles used TAP as intracanal medicament. Only one clinical study in this review compared the use of two different intracanal dressing materials: TAP and calcium hydroxide + chlorhexidine gel (2%). Unfortunately, as calcium hydroxide was not used alone, and chlorhexidine gel (2%) is cytotoxic for stem cells [8], a comparison of clinical outcomes after TAP or calcium hydroxide was not possible.

Disinfection is imperative since apical repair will not occur in the presence of infected tissues. In RET, disinfection depends almost exclusively on irrigants and intracanal medication, since instrumentation should be avoided or minimal in order to preserve the dentin walls. It is necessary to use irrigants at concentrations that are effective as well as non-cytotoxic for stem cells.

This systematic review differs from others reviews of clinical protocols for RET in that it includes animal studies and therefore histological outcomes. However, due to the heterogeneity of the analyzed studies, it was not possible to analyze quantitatively the influence of agents (irrigants and intracanal medications), their concentrations and time for application on the clinical, radiographic and histological outcomes after RET. It is essential to use biocompatible agents in the treatment of immature teeth to
Table 3. Characteristics, protocol, histological and radiographic outcomes of animal studies

<table>
<thead>
<tr>
<th>Authors/Year</th>
<th>N/periapical lesion</th>
<th>Instrumentation</th>
<th>Irrigant 1st session (concentration) (n)</th>
<th>Intracanal dressing (n) (time)</th>
<th>Irrigant 2nd session (concentration) (n)</th>
<th>EDTA (concentration) x time</th>
<th>Blood clot formation</th>
<th>Additive</th>
</tr>
</thead>
<tbody>
<tr>
<td>da Silva et al. 2010 [23]</td>
<td>56 (c)/Yes</td>
<td>Minimal</td>
<td>NaOCl (2.5%) + saline (56)</td>
<td>None (28)</td>
<td>NA (28)</td>
<td>No</td>
<td>Yes</td>
<td>None</td>
</tr>
<tr>
<td>Khademi et al. 2014 [24]</td>
<td>29 (d)/Yes (20), No (9)</td>
<td>No</td>
<td>NaOCl (5.25%) (20), NR (9)</td>
<td>TAP (2 weeks) (28)</td>
<td>NaOCl (5.25%) + saline (20)</td>
<td>No</td>
<td>Yes</td>
<td>None</td>
</tr>
<tr>
<td>Londro et al. 2015 [25]</td>
<td>32 (c)/NR</td>
<td>No</td>
<td>NaOCl (2.5%) (32)</td>
<td>TAP (32) (2 weeks)</td>
<td>NaOCl (2.5%) (32)</td>
<td>Yes (17%) x 3 minutes</td>
<td>NA (9)</td>
<td>NR</td>
</tr>
<tr>
<td>Petrovic et al. 2013 [34]</td>
<td>15 (t)/No</td>
<td>NR</td>
<td>NA</td>
<td>None (28)</td>
<td>NA (28)</td>
<td>No</td>
<td>Yes</td>
<td>None</td>
</tr>
<tr>
<td>Rodriguez-Benitez et al. 2015 [26]</td>
<td>64 (c)/Yes</td>
<td>NR</td>
<td>NaOCl (1.25%) + saline (64)</td>
<td>None (32), TAP (32) (15 days)</td>
<td>NA (32)</td>
<td>Yes (17%) x 1 minute</td>
<td>No (32), Yes (32)</td>
<td>None</td>
</tr>
<tr>
<td>Saoud et al. 2015 [27]</td>
<td>16 (t)/Yes</td>
<td>NR</td>
<td>NaOCl (2.5%) + saline (16)</td>
<td>TAP (16) (3 weeks)</td>
<td>NaOCl (2.5%) + saline (16)</td>
<td>No</td>
<td>No (17%), Yes (31*)</td>
<td>None</td>
</tr>
<tr>
<td>Thibodeau et al. 2007 [28]</td>
<td>41 (c)/Yes</td>
<td>No</td>
<td>TAP (10*) (stay)</td>
<td>TAP (31*) (4 weeks)</td>
<td>TAP (31*) (4 weeks)</td>
<td>Yes (17%) x NR</td>
<td>No (12)</td>
<td>NR</td>
</tr>
<tr>
<td>Torabinejad et al. 2014 [33]</td>
<td>21 (t)/No</td>
<td>No</td>
<td>Saline (21)</td>
<td>None</td>
<td>NA</td>
<td>Yes (17%) x NR</td>
<td>No (9)</td>
<td>None</td>
</tr>
<tr>
<td>Yamauchi et al. 2011 [29]</td>
<td>96 (c)/Yes</td>
<td>No</td>
<td>NaOCl (2.5%) (96)</td>
<td>TAP (96) (2 weeks)</td>
<td>NaOCl (2.5%) + saline (96)</td>
<td>No</td>
<td>Yes (48), Yes (48)</td>
<td>None</td>
</tr>
<tr>
<td>Yoo et al. 2014 [30]</td>
<td>40 (c)/Yes</td>
<td>No</td>
<td>NaOCl (3.5%) (40)</td>
<td>TAP (40) (2 weeks)</td>
<td>NaOCl (3.5%) + saline (40)</td>
<td>No</td>
<td>Yes</td>
<td>NR</td>
</tr>
<tr>
<td>Zhang et al. 2014 [31]</td>
<td>36 (c)/NR</td>
<td>NR</td>
<td>NaOCl (3%) + saline (36)</td>
<td>TAP (36) (4 weeks)</td>
<td>NR</td>
<td>No</td>
<td>Yes (18)</td>
<td>None</td>
</tr>
<tr>
<td>Zhu et al. 2013 [32]</td>
<td>40 (c)/Yes</td>
<td>NR</td>
<td>NaOCl (1.25%) + saline (40)</td>
<td>TAP (40) (2 weeks)</td>
<td>NaOCl (1.25%) y saline (40)</td>
<td>No</td>
<td>Yes</td>
<td>None</td>
</tr>
</tbody>
</table>

(t): teeth; (c): canals. NR: Not reported. TAP: Metronidazole, ciprofloxacin and minocycline. TAP*: Ciprofloxacin, metronidazole and cefixime. TAP**: Ciprofloxacin, metronidazole and cefixime, HA: hydroxyapatite. PRP: platelet-rich plasma. DPSC: dental pulp stem cells. EDTA: Ethylenediaminetetraacetic acid. *Information provided by the author. NA: Not applicable.
## Clinical protocols for regenerative endodontics

### Table 4. Characteristics, protocol, histological and radiographic outcomes of animal studies

<table>
<thead>
<tr>
<th>Authors/Year</th>
<th>N/n periapical lesion</th>
<th>Apical closure</th>
<th>Root lengthening</th>
<th>Dentin walls thickness</th>
<th>Healing of periapical lesion</th>
<th>Connective intra-canal tissue</th>
<th>Inflammation tissue</th>
<th>Mineralized tissue</th>
<th>Cementum-like tissue</th>
<th>Bone-like tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>da Silva et al. 2010 [23]</td>
<td>56 (c)/Yes</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>26</td>
<td>28</td>
<td>10</td>
<td>24</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td>Khademi et al. 2014 [24]</td>
<td>29 (d)/Yes (20), No (9)</td>
<td>13</td>
<td>NR</td>
<td>8</td>
<td>13</td>
<td>14</td>
<td>NR</td>
<td>16</td>
<td>NR</td>
<td>NR</td>
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<tr>
<td>Londero et al. 2015 [25]</td>
<td>32 (c)/NR</td>
<td>NR</td>
<td>24</td>
<td>NR</td>
<td>NA</td>
<td>28</td>
<td>25</td>
<td>26</td>
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<tr>
<td>Petrovic et al. 2013 [34]</td>
<td>15 (t)/No</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>NA</td>
<td>2</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
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</tr>
<tr>
<td>Rodriguez-Benitez et al. 2015 [26]</td>
<td>64 (c)/Yes</td>
<td>8</td>
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<td>12</td>
<td>14</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
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<tr>
<td>Saoud et al. 2015 [27]</td>
<td>16 (t)/Yes</td>
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<td>NR</td>
<td>16</td>
<td>16</td>
<td>16</td>
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<td>16</td>
<td>16</td>
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</tr>
<tr>
<td>Thibodeau et al. 2007 [28]</td>
<td>41 (c)/Yes</td>
<td>5</td>
<td>NR</td>
<td>3</td>
<td>4</td>
<td>2</td>
<td>NR</td>
<td>3</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td>Torabinejad et al. 2015 [33]</td>
<td>21 (t)/No</td>
<td>18</td>
<td>NR</td>
<td>17</td>
<td>12</td>
<td>10</td>
<td>NR</td>
<td>19</td>
<td>NR</td>
<td>NR</td>
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<tr>
<td>Yamauchi et al. 2011 [29]</td>
<td>96 (c)/Yes</td>
<td>NR</td>
<td>NR</td>
<td>37</td>
<td>33</td>
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</tr>
<tr>
<td>Yoo et al. 2014 [30]</td>
<td>40 (c)/Yes</td>
<td>34</td>
<td>NR</td>
<td>37</td>
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<td>15</td>
<td>11</td>
<td>NR</td>
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<tr>
<td>Zhang et al. 2014 [31]</td>
<td>36 (c)/Yes</td>
<td>16</td>
<td>NR</td>
<td>12</td>
<td>18</td>
<td>15</td>
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<td>13</td>
<td>12</td>
<td>0</td>
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<tr>
<td>Zhu et al. 2013 [32]</td>
<td>40 (c)/Yes</td>
<td>NR</td>
<td>NR</td>
<td>28</td>
<td>3</td>
<td>NR</td>
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<td>NR</td>
<td>31</td>
<td>24</td>
</tr>
</tbody>
</table>

(t): teeth, (c): canals. NR: Not reported. NA: Not applicable.
maximize root development as well as to contribute to the regeneration of true dentin and pulp. Recently, a clinical protocol for revitalization procedures was published [36]. According to that clinical protocol and according to in vitro studies [4, 8-11], the medications that seem to be most adequate for therapy are: irrigation with 1.5%-3% sodium hypochlorite, intracanal dressing with calcium hydroxide water-based paste, and 17% EDTA as a chelating agent, used at the first and second appointment [36]. However, the majority of the studies analyzed in this review did not follow this clinical protocol; several studies used sodium hypochlorite in concentrations higher than recommended, and only two studies used calcium hydroxide as intracanal dressing. Finally, EDTA was used in only six studies. Consequently, it is necessary to conduct clinical and animal studies to establish whether the protocol described above is indeed related to better clinical, histological and radiographic outcomes.

Disclosure of conflict of interest

None.

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Clinical protocols for regenerative endodontics


Results Chapter 4: Apical foramen enlargement of mature teeth
Enlargement of the apical foramen of mature teeth by instrumentation and apicoectomy. A study of effectiveness and the formation of dentinal cracks

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Enlargement of the apical foramen of mature teeth by instrumentation and apicoectomy. A study of effectiveness and the formation of dentinal cracks

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ABSTRACT

**Objective:** In the last few years there have been attempts to revascularize mature necrotic teeth instead of performing a standard root canal treatment. Apical foramen enlargement (AFE) would be necessary for regenerative treatments of mature teeth. In the literature, AFE has been made through apicoectomy and instrumentation. However, no standardized methods have been described yet, which may affect the success of the therapy. Our aim was to describe the effectiveness and damage to dental structures of five methods for AFE.

**Methods:** Two hundred and ten human teeth were assigned to one control group (\(n = 10\)) and four treatment groups (\(n = 50\) each): instrumentation was up to file #80 0.5 mm coronal to the apex (I), at apex level (II), 0.5 mm beyond the apex (III) and apicoectomy at 2 and 4 mm from the apex (IV). The apical foramen diameter was measured before and after treatment. The formation of clinically visible fractures (CVF) and microcracks was analysed clinically and with ESEM, respectively. Thirty-two in situ sheep's teeth were also instrumented, to compare damage in in situ and ex vivo teeth.

**Results:** The foramen diameter was augmented by 0.15, 0.47, 0.54 0.06 and 0.32 mm in human teeth of groups I, II, III, apicoectomy at 2 and 4 mm, respectively. CVF were more frequent as the working length was augmented. No statistical differences were found for microcrack formation. In situ teeth showed significantly less damage.

**Conclusions:** Instrumentation at apex level seems to be the most effective and least harmful technique for AFE, while apicoectomy is not a useful method.

Introduction

Traditionally it has been considered that a closed apex is a requirement for a successful root canal treatment. Recently, however, a more conservative and biologically-based approach for the treatment of necrotic teeth has been proposed: regeneration or revitalization of the pulp tissue [1], which would benefit from a wide apical foramen. The absence of an apical constriction, and therefore a wide apical foramen, would allow the ingrowth of blood vessels and nerve tissue, as well as cell migration into the root canal [2]. Previous studies have indicated that the size of the apical foramen is not the all-decisive factor for successful revascularization and ingrowth of new tissue [3]. However, a recent study showed that apical diameter does influence the success of regenerative procedures, since root thickness, length and apical narrowing are greater in teeth with an apical diameter greater than 1 mm [4]. This is according to a study in autotransplanted and replanted teeth, which indicated that revascularization is unpredictable when the apical foramen diameter was smaller than 1 mm [5]. Thus, enlargement of the apical foramen in order to allow revascularization of mature teeth would be necessary in three different clinical procedures: autotransplantation, replantation and regenerative endodontics [3].

According to the literature, enlargement of the apical foramen for the treatment of mature teeth is performed by instrumentation [6–8] or apicoectomy [3]. However, there is no detailed description of the clinical protocol for performing enlargement of the foramen, which may be influencing the success of the therapy. It is likewise unclear whether instrumentation and apicoectomy are effective methods for foramen enlargement, and what effects might be caused to dental structures by instrumentation at apex level with wide files.

Studies on foramen enlargement do not report the length at which the instrumentation or apicoectomy is done [3,8,9]. In cases of foramen enlargement by instrumentation, since the position of the foramen varies depending on several factors [10], it is clinically relevant to establish a working length to effectively enlarge the foramen. Sub-instrumentation (with respect to the foramen) would not enlarge the foramen, and over-instrumentation would affect the periapical tissues, leading to symptoms in the patient [11]. For apicoectomy, the distance at which the root is resected directly affects the final diameter of the enlarged foramen. The radicular canal has a...
conical anatomy in which its diameter diminishes apically. Additionally, it is important to know how many millimetres the enlarged apical foramen will measure after the apicoectomy, and therefore whether this method is effective. An in vivo study performed apicoectomy on mature dog’s teeth, in which the final diameter of the foramina varied from 0.24 to 1.09 mm [3]. However, the size of the foramen before apicoectomy was not measured and therefore it is not possible to evaluate whether the apicoectomy was effective.

The object of our study was to assess the effectiveness and damage to the dental structures of five methods (instrumentation until file K #80 at three different working lengths, and apicoectomy 2 and 4 mm from the apex) used to enlarge the apical foramen of mature human extracted teeth. Additionally, 32 sheep’s teeth, which were set in the alveolar bone were also instrumented in order to compare damage in in situ and ex vivo teeth.

Materials and methods

Human teeth

The project was approved by the Bioethics Commission of the Universitat de Barcelona (IRB 00003099).

The sample size was 210 extracted human teeth, obtained from the dental hospital of Universitat de Barcelona. Only mature, single rooted (incisor and canine), mandibular and maxillary teeth were included. Teeth with external or internal resorption, with filling materials, posts or root fractures and/or dilacerated teeth were excluded.

Teeth that complied with the inclusion criteria were washed in 0.12% chlorhexidine and cleaned manually with a universal curette (Hu-Friedy, Chicago, IL) to remove calculus and remaining periodontal tissue. They were then preserved in saline for 1–3 months, until instrumentation/apicoectomy.

The teeth were randomly assigned to one of the five groups:

- **Group I** (n = 50): working length 0.5 mm coronal to the tooth apex (sub-instrumentation);
- **Group II** (n = 50): working length at the level of the tooth apex;
- **Group III** (n = 50): working length 0.5 mm beyond the tooth apex (over-instrumentation);
- **Group IV** (n = 50): apicoectomy at 2 and 4 mm from the tooth apex;
- **Group V** (n = 10): control teeth, without any treatment.

The heads were frozen for approximately 7 days. Teeth were treated in the mandibles. Crown access was created and the radicular canal was located. Pulp tissue was extracted with a 22 mm carborundum cutting disc (Renfert Dynex, Hilzingen, Germany). The first resection was performed 2 mm from the root apex and the second apicoectomy was performed 4 mm from the root apex.

The maximum diameter of each apical foramen was measured pre-treatment and post-treatment by a single operator, previously trained and calibrated. The calibration process consisted of theoretical training and measurement of the apical foramen of 20 teeth, repeated 10 days later. The intra-observer agreement was calculated using the intra-class correlation coefficient with SPSS software (V. 20.0, Chicago, IL). The measurements were taken under a 10× binocular stereo microscope (Premiere, Manassas, VA) with a crown-gauge-thickness instrument (Patterson Iwanson Spring Caliper, Saint Paul, MN) (accuracy of a tenth of a millimetre), and entered in an Excel document. Any adverse event such as root/apex fracture was recorded.

Fifteen teeth of each treatment group and 10 control teeth (not instrumented/apicectomized) were randomly selected, and were examined and photographed by environmental scanning electron microscope (ESEM) (Quanta 200, FEI Co., Hillsboro, OR). Teeth were coated with silver and gold, and analysed individually under 80× magnification at high vacuum conditions. One observer blindly analysed each image. The examiner was previously calibrated. The calibration process consisted of theoretical training and measurement of the apical foramen of 20 teeth, repeated 10 days later. The intra-observer agreement was calculated using the Cohen’s kappa coefficient with SPPS 20.0 software. To be considered a complete dentinal crack, the fracture had to compromise both interior and exterior walls of the root (Figure 1(A–D)) and its extension on the exterior wall had to attain 0.5 mm or more. The presence/absence of complete dentinal cracks was evaluated and the results were dichotomized (presence/absence of dentinal cracks).

Sheep’s mandibles

Sheep’s teeth set in the alveolar bone were instrumented up to file #80 at the same working lengths as human extracted teeth, in order to evaluate damage to dental structures of instrumentation in non-extracted teeth.

Four heads of 4-year-old sheep were used. The heads were donated by another study, unrelated to the oral cavity. The heads were frozen for approximately 7 days. Teeth were treated in the mandibles. Crown access was created and the radicular canal was located. Pulp tissue was extracted with a K-Flexofile #15 (Dentsply Maillefer, Ballaigues, Switzerland), the teeth were radiographed with a portable digital radiograph system (Dexco ADX6000, Pasadena, CA) and tooth lengths were measured. The incisors (n = 32) were randomly assigned to the experimental groups.

Group a (n = 10): working length 0.5 mm coronal to the tooth apex (sub-instrumentation);
Group b \((n=11)\): working length at the level of the tooth apex;

Group c \((n=11)\): working length 0.5 mm beyond the tooth apex (over-instrumentation).

The root canals were manually instrumented up to file #80 (Dentsply Maillefer, Ballaigues, Switzerland) using the balanced force technique and passive irrigation with 2.5% sodium hypochlorite. Teeth were carefully extracted with forceps and washed with saline. No visible damage to the dental structures was observed after the extraction. Additional cleaning was carried out with a universal curette to gently remove remaining periodontal tissue. Foramen diameters were measured with the same protocol used for human teeth, and any adverse event observed clinically, such as root/apex fracture, was recorded. Subsequently, five teeth of each group, chosen at random, were analysed under ESEM as previously described.

**Statistical analysis**

Human and sheep’s teeth were analysed separately. The collected data were entered into an Excel file and statistical analysis was performed with SPSS software (V. 20.0, Chicago, IL). Descriptive statistics were calculated, including mean and standard deviation. The homogeneity of variance was determined through Kolmogorov–Smirnov test. Pearson Chi-square, Fisher Exact Test and Wilcoxon Signed Rank test were used to evaluate statistical differences. In all statistical analyses a significance level of .05 was used.

**Results**

The intra-class correlation coefficient that determines intra-observer agreement for measurements was 0.96 (almost perfect) and the Cohen’s kappa coefficient was 0.9.

**Human teeth**

The mean pre-treatment diameter of all teeth was 0.41 mm (±0.14), ranging between 0.0 (obliterated) and 0.9 mm. The four groups presented similar pre-treatment diameters of the apical foramen \((p > .05)\). Table 1 shows the frequency of the pre-treatment diameters for each group.

**Group I: sub-instrumentation**

The mean pre-treatment apical foramen diameter was 0.42 mm (±0.15). After instrumentation up to K file #80, only

![Figure 1](image-url)
25 teeth (50%) showed an enlarged apical foramen; the foramen size of the remaining teeth was unchanged (Table 2). Augmentation of the foramen size was only 0.16 mm. The final foramina diameter for this group was 0.57 mm (±0.23). According to the Wilcoxon Signed Rank test, pre-treatment and post-treatment foramen diameters were statistically different. 46.7% of the teeth presented dentinal cracks and 8% presented clinically visible fractures (CVF) (Table 3).

### Group II: working length at apex level

The mean pre-treatment apical foramen diameter was 0.40 mm (±0.17). After instrumentation up to K file #80, 49 teeth (98%) showed an enlarged apical foramen (Table 2). Augmentation of the foramen size was 0.47 mm. The final foramina diameter for this group was 0.87 mm (±0.17). According to the Wilcoxon Signed Rank test, pre-treatment and post-treatment foramen diameters were statistically different. Forty percent of the teeth presented dentinal cracks and 12% presented CVF (Table 3).

### Group III: over-instrumentation

The mean pre-treatment apical foramen diameter was 0.43 mm (±0.11). After instrumentation up to K file #80, all teeth showed an enlarged apical foramen (Table 2). Augmentation of the foramen size of teeth with enlarged foramina was 0.54 mm. The final foramina diameter for this group was 0.97 mm (±0.02). According to the Wilcoxon Signed Rank test, pre-treatment and post-treatment foramen diameters were statistically different. Sixty percent of the teeth presented dentinal cracks and 20% presented CVF (Table 3).

### Group IV: apicoectomy

The mean pre-treatment diameter was 0.4 mm (±0.14). After the apicoectomy 2 mm from the apex the mean apical diameter increased by only 0.07 mm, to a final apical foramen diameter of 0.47 mm (±0.13) According to the Wilcoxon Signed Rank test, pre-treatment and post-treatment diameters were not statistically different. Moreover, it is important to note that only 23 teeth presented increased foramen diameter, and only one tooth achieved an apical diameter of 1 mm (Table 2).

After the 4 mm apicoectomy, the mean apical diameter increased by 0.32 mm from the original apical size, to a final apical foramen diameter of 0.72 mm (±0.21). According to the Wilcoxon Signed Rank test, pre-treatment and post-treatment foramen diameters were statistically different.

---

**Table 1. Number of human teeth with pre-treatment diameter in each category, aleatorized in the treatment groups.**

<table>
<thead>
<tr>
<th>Group</th>
<th>0.0–0.2</th>
<th>0.3–0.4</th>
<th>0.5–0.6</th>
<th>0.7 or more</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sub-instrumented teeth</td>
<td>1</td>
<td>27</td>
<td>20</td>
<td>2</td>
<td>50</td>
</tr>
<tr>
<td>Working length at apex level</td>
<td>4</td>
<td>29</td>
<td>14</td>
<td>3</td>
<td>50</td>
</tr>
<tr>
<td>Over-instrumented teeth</td>
<td>3</td>
<td>30</td>
<td>14</td>
<td>3</td>
<td>50</td>
</tr>
<tr>
<td>Apicoectomy</td>
<td>9</td>
<td>20</td>
<td>19</td>
<td>2</td>
<td>50</td>
</tr>
</tbody>
</table>

Chi² test showed no statistical differences between the groups (p < .001).

**Table 2. Number of teeth with increased, decreased and unvarying apical diameter after treatments.**

<table>
<thead>
<tr>
<th>Group</th>
<th>N teeth with increased diameter from original</th>
<th>N teeth with same diameter as original</th>
<th>N teeth with decreased diameter from original</th>
<th>N teeth with increased diameter after 2 mm apicoectomy</th>
<th>N teeth with same diameter after 2 mm apicoectomy</th>
<th>N teeth with diameter &gt; 1 mm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sub-instrumented teeth</td>
<td>25</td>
<td>25</td>
<td>0</td>
<td>NA</td>
<td>NA</td>
<td>5</td>
</tr>
<tr>
<td>Working length at apex level</td>
<td>49</td>
<td>1</td>
<td>0</td>
<td>NA</td>
<td>NA</td>
<td>16</td>
</tr>
<tr>
<td>Over-instrumented teeth</td>
<td>50</td>
<td>0</td>
<td>0</td>
<td>NA</td>
<td>NA</td>
<td>41</td>
</tr>
<tr>
<td>2 mm apicoectomy</td>
<td>23</td>
<td>17</td>
<td>10</td>
<td>NA</td>
<td>NA</td>
<td>1</td>
</tr>
<tr>
<td>4 mm apicoectomy</td>
<td>43</td>
<td>6</td>
<td>1</td>
<td>42</td>
<td>8</td>
<td>8</td>
</tr>
</tbody>
</table>

Chi² test showed statistical differences between the groups with respect to the increased, equal or decreased diameters (p < .001).

**Table 3. Prevalence of microcracks and clinically visible fractures in human and sheep teeth.**

<table>
<thead>
<tr>
<th>Group</th>
<th>N of teeth with dentinal cracks (%)</th>
<th>Mean of dentinal cracks, of the teeth with microcracks (SD)</th>
<th>N of clinically visible apex fractures (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human sub-instrumented teeth</td>
<td>7 (46.7)</td>
<td>1.71 (0.88)</td>
<td>4 (8%)</td>
</tr>
<tr>
<td>Human teeth, working length at tooth apex level</td>
<td>6 (40%)</td>
<td>1.33 (0.47)</td>
<td>6 (12%)</td>
</tr>
<tr>
<td>Human over-instrumented teeth</td>
<td>9 (60%)</td>
<td>1.44 (0.68)</td>
<td>10 (20%)</td>
</tr>
<tr>
<td>Apectected Human teeth (4mm)</td>
<td>3 (20%)</td>
<td>1.33 (0.47)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Sheep sub-instrumented teeth</td>
<td>0 (0%)</td>
<td>0 (NA)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Sheep teeth, working length at tooth apex level</td>
<td>1 (20%)</td>
<td>2 (NA)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Sheep over-instrumented teeth</td>
<td>0 (0%)</td>
<td>0 (NA)</td>
<td>0 (0%)</td>
</tr>
</tbody>
</table>

Microcracks were analysed by ESEM, with a sample size of 15 human teeth per group and five sheep teeth per groups. Clinically visible fractures were analysed in all teeth (200 human teeth and 32 sheep teeth). NA, not applicable.
Forty-three teeth presented an increased apical diameter and eight achieved an apical diameter of 1 mm (Table 2). Twenty percent of the teeth presented dentinal cracks and no teeth presented CVF (Table 3).

The form of the foramina of apicoectomized teeth was irregular (Figure 2(C,D)), with a maximum and minimum diameter; the over-instrumented teeth by contrast presented a round foramen (Figure 2(B)).

Control teeth had no CVF, and only two presented dentinal cracks (Figure 2(A)). The differences in dentinal cracks and CVF between control and treated teeth were statistically significant ($p < .05$).

![Figure 2](image.png)

**Figure 2.** (A) Control tooth, foramen intact and without the presence of microcracks or clinically visible fractures. (B) Over-instrumented tooth, the foramen has an almost perfect round form and without the presence of dentinal cracks. (C) 4 mm apicectomized tooth, no dentinal crack is observed. (D) 4 mm apicectomy tooth, with microcracks. Black arrows: dentinal cracks.

**Intergroup comparison (human teeth)**

Table 4 shows the frequency of the post-treatment diameters for each group. The differences between the post-treatment apical foramen sizes were statistically significant between treatment groups ($p < .05$). Teeth over-instrumented and instrumented at level apex showed greater foramen diameters. The frequency of teeth with augmented, equal and diminished apical diameter was statistically different between the groups. Teeth over-instrumented, instrumented at apex level and with 4 mm apicoectomy had significantly more teeth with augmented foramen diameter (Table 2).

All groups of instrumented teeth (groups I, II and III) presented CVF. The frequency of CVF was statistically greater in over-instrumented and teeth instrumented at level apex in comparison to sub-instrumented and apicectomized teeth ($p < .05$) (Table 3).

All groups contained teeth with dentinal cracks (Figures 1(A–D) and (D), Table 3). Despite the fact that the presence and number of dentinal cracks was higher in over-instrumented teeth, there were no statistically significant differences between groups (Table 3).

**Sheep’s teeth**

The mean post-treatment apical foramen diameters were $0.67 \text{ mm} \ (\pm 0.61)$, $0.82 \text{ mm} \ (\pm 0.21)$ and $0.88 \text{ mm} \ (\pm 0.18)$ for sub-instrumented, instrumented at apex level and over-instrumented teeth, respectively. These differences were not statistically significant.

No teeth presented CVF after instrumentation. The presence of microcracks, evaluated by ESEM, was observed in only one tooth, instrumented at apex level (Figure 3(A)).
According to the Fisher Exact test these differences were not statistically significant. The rest of the teeth had no microcracks (Figure 3(B)).

**Human ex vivo teeth versus in situ sheep teeth**

No statistically significant differences were found for the prevalence of CVF between human and sheep teeth. The prevalence of dentinal cracks was greater in human teeth \( (p = .01) \).

**Discussion**

The current paradigm shift in endodontics from obturation to pulp regeneration implies that the new therapy would benefit from a wide apical foramen. In 1990, Andreasen and co-workers showed that the revascularization of autotransplanted and replanted teeth was unpredictable if the apical foramen measured less than 1 mm \([5]\). This has been confirmed by recent research in regenerative endodontics, which showed that teeth with narrower apical diameters had significantly lower hard tissue formation compared with teeth with apical diameter greater than 1 mm, indicating that apical diameter is a factor influencing the progress of revascularization \([4]\). As has been shown in this study, the mean pre-treatment apical foramen of these selected anterior mature teeth is just 0.41 mm, so it would be necessary to perform apical foramen enlargement when a regenerative treatment is planned for mature teeth.

The location of the apical foramen varies in response to several factors \([10]\), and the exact location must be known for foramen enlargement to be achieved. An imaging study showed that the mean distance between the apical foramen and the apex was 0.9 mm, but that in 68% of teeth the distance was 0.5 mm shorter or longer than the mean \([10]\), which is a large variance in clinical terms. Cone Beam Computerized Tomography (CBCT) and electronic apex locators have been used to determine the location of the apical foramen. However, the literature shows that several factors affect the precision of electronic apex locators in determining working length \([12]\), and that CBCTs are less reliable than electronic apex locators \([13]\). Thus, it is important to establish a standardized clinical protocol to achieve widening of the apical foramen, since use of inaccurate methods could fail to achieve widening, which would compromise the regenerative procedure. Unnecessary over-instrumentation, on the other hand, could lead to patient discomfort and/or apex fracture.

While foramen enlargement has been applied in studies of attempts to regenerate pulp in mature teeth, no details are reported of the length at which instrumentation or apex resection has been performed \([3,8,9]\). As seen in our study, variations of only 0.5 mm in working length produced very different clinical outcomes (98% of foramina were enlarged with working length at apex level, and only 25% in sub-instrumented teeth). Likewise, the distance from the apex at which the resection is performed is a critical factor in the size of the widened foramen. Several studies have used apicoectomy as a method for foramen enlargement of mature teeth \([3,9]\). However, according to our results resection at 2 mm from the apex causes almost no enlargement in

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**Table 4. Number of human teeth with post-treatment diameter in each category.**

<table>
<thead>
<tr>
<th>Group</th>
<th>0.2–0.3 mm (n)</th>
<th>0.4–0.5 mm (n)</th>
<th>0.6–0.7 mm (n)</th>
<th>0.8–0.9 mm (n)</th>
<th>1 or more mm (n)</th>
<th>Total (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sub-instrumented teeth</td>
<td>8</td>
<td>16</td>
<td>15</td>
<td>6</td>
<td>5</td>
<td>50</td>
</tr>
<tr>
<td>Working length at tooth apex level</td>
<td>1</td>
<td>1</td>
<td>8</td>
<td>24</td>
<td>16</td>
<td>50</td>
</tr>
<tr>
<td>Over-instrumented teeth</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>9</td>
<td>41</td>
<td>50</td>
</tr>
<tr>
<td>Apicoectomy at 2 mm from the tooth apex</td>
<td>8</td>
<td>31</td>
<td>10</td>
<td>0</td>
<td>1</td>
<td>50</td>
</tr>
<tr>
<td>Apicoectomy 4 mm from the tooth apex</td>
<td>3</td>
<td>9</td>
<td>13</td>
<td>16</td>
<td>9</td>
<td>50</td>
</tr>
</tbody>
</table>

Chi² test showed statistical association between the type of treatment and the obtained apical foramen diameter \( (p < .001) \).
foramen size, and in 20% of the cases causes a decrease in the foramen diameter, meaning that it is not a useful method for foramen enlargement.

The most effective methods for foramen enlargement are over-instrumentation, instrumentation at apex level and resection of 4 mm of the root; these methods enlarged the foramen by 0.54, 0.45 and 0.32 mm, respectively. However, it should be considered that over-instrumentation and 4 mm resection are invasive methods (20% of the over-instrumented roots presented CVF and 4 mm root resection causes an important loss of dental tissue). Moreover, the fact that over-instrumentation provokes patient pain and discomfort must be considered [14].

An ideal method for foramen enlargement should be both effective and less invasive. Thus, according to the results of our study instrumentation at apex level would be the most suitable method for foramen enlargement. Instrumentation at apex level produces a mean apical diameter size of 0.87 mm. In future investigations would be necessary to establish if files with 0.04 or 0.06 taper achieve better results in terms of greater diameter and fewer fractures and microcracks, or if it is mandatory to use files up to #100 in order to obtain a 1 mm diameter. Instrumentation at apex level causes CVF in 12% of the teeth, however this prevalence probably would be minor if instrumentation is performed in the mouth as it is shown in the in situ sheep teeth, where no CVF were observed.

The working lengths in this study were established with respect to the apex, and considering the fact that the apical foramen could be located 3 mm or more coronal to the apex [15], that the instrumentation is generally asymmetrical [16], and that some apices were fractured (which leads to greater diameters) a 1 mm final foramen diameter was achieved with #80 files in some teeth (Table 2).

The prevalence of complete dentinal cracks in this study was higher than in the results of other investigations [17,18]. This could be explained by differences in methodology, since some studies analysed dentinal cracks in the apical, middle and coronal thirds of the tooth, and not at apex level [17,18]. The apex is a thin structure compared with the apical, medium or coronal thirds of the root, and for this reason is more prone to fracture. Thus, the higher the root third analysed, the lower the prevalence of cracks [17]. Ceyhanli et al. [19] analysed microcracks by micro-CT in the apical 10 mm of roots; the prevalence of microcracks was similar to our findings. Another important reason that could explain the high prevalence of cracks in our study is the use of large files (#80) to widen the foramen. Studies that instrumented teeth only up to file #25 reported considerably fewer dentinal cracks [20].

Finally, a limitation of this study is that the extracted teeth used are more prone to fracture, due to the lack of surrounding tissue support. This could explain why the sheep’s teeth, which were instrumented in their mandibles, presented a significantly lower prevalence of dentinal cracks and no CVF. However, it is important to note that the anatomy and dimension of sheep’s teeth are different from human teeth. As we observed in this study, dentine walls at the apex level of sheep’s teeth had greater dimensions compared to human teeth, which decreases the probability of formation of fractures or microcracks. Studies in non-extracted human teeth (such as cadaveric material) would elucidate whether fracture and microcrack prevalence is lower when teeth are instrumented in situ.

According to the results of this study instrumentation at apex level would be the most suitable method for foramen enlargement. Apicectomy and 0.5 mm sub-instrumentation with respect to the apex are not effective methods. Over-instrumentation by 0.5 mm is an effective but harmful technique.

Disclosure statement
The authors report no conflict of interest

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Objective: Revitalization procedures have been extensively studied during the last decade and offers several advantages over root canal treatment, such as the recovery of the natural immune system. Mature teeth have a small apical foramen diameter (AFD), which could impair the ingrowth of tissue into the root canal. We analysed three methods for apical foramen enlargement by instrumentation in in situ human teeth and evaluated the damage over hard tissues produced by the techniques. Material and methods: Tooth length (TL), defined as the length from the most coronal part of the crown to the point at which the file abandoned the root canal, was calculated. Forty-four in situ teeth were randomized: Group I: instrumentation 0.5mm coronal to TL; Group II: at TL level; Group III: 0.5mm beyond TL. Teeth were instrumented up to K-file #80. The mandibles were scanned in a micro-CT device before and after treatment. Results: Group I: Only 20% of teeth presented an enlarged AFD, with augmentation of 0.09mm. No damage to hard tissues was observed. Group II: 71.4% of the teeth presented an enlarged AFD with augmentation of 0.42mm. 35.7% presented damage to periapical tissues and 7.1% presented microcracks. Group III: 86.7% presented an enlarged AFD with augmentation of 0.43mm. 13.3% presented microcracks and 46.7% presented damage to periapical tissues. All groups presented similar prevalence of teeth with dentine thickness less than 1mm. All mandibular incisors presented areas of thickness less than 1mm. Conclusion: Instrumentation 0.5mm beyond TL is the most effective technique.

Keywords: regenerative endodontics; revitalization; mature teeth; apical foramen enlargement; microcracks.
Introduction

Currently, the most reliable option for the treatment of necrotic or irreversibly inflamed mature teeth is still root canal treatment. However, endodontically treated teeth are susceptible to reinfections and fractures, and the failure rate is close to 25% at 3-5 years. Moreover, endodontically treated teeth will remain devitalized after treatment throughout the patient's lifetime.

Revitalization procedures producing positive clinical outcomes have been extensively studied in recent decades. Teeth revitalization offers several advantages over conventional root canal treatment, such as the recovery of the natural immune system and a structurally more resistant tooth. Regenerative approaches for treatment of pulp necrosis have been mainly studied and applied in immature teeth. One of the reasons for this is the absence of an apical constriction in immature teeth; this would enhance the entrance of cells, growth factors and blood vessels, favoring the neoformation of tissue into the root canal. Mature teeth in contrast have a very small apical foramen diameter, making the achievement of pulp regeneration or repair a greater clinical challenge.

To date it is unclear whether a wide apical foramen would enhance the possibilities of tooth revascularization, in view of the contradictory results reported in the last few years and the relatively low amount of research on this topic. However, previous studies have reported that a larger apical foramen improved regenerative endodontics outcomes, favored a more rapid radiographic repair of apical periodontitis and enhanced root canal disinfection.

Case reports and animal studies published in recent years describe attempts to revascularize mature teeth using a regenerative approach with variable results. These studies used different methods for apical foramen enlargement, thus there are variations in working length and widening of the file, among other parameters. Methods for apical foramen enlargement are poorly described in the literature. Considering that the location of the apical foramen may vary depending on several factors, that it is not possible to determine the exact location of the foramen using the current available technology, and that a variation of only 0.5mm in working length may result in ineffective widening of the apical foramen, there is a need to establish a standardized clinical protocol for foramen widening. Thus, as the major apical foramen is a structure not identifiable by radiography, we propose a standardised methodology to ensure the apical foramen enlargement based on the point at which the file radiographically abandon the root canal.

The objects of the present study were to evaluate the effectiveness of apical foramen enlargement in situ human teeth by instrumentation with different three working lengths, and to compare the damage to hard tissues (dentine microcracks and invasion of periapical bone by over-instrumentation) in each group using micro-CT.

Methodology

The project was approved by the Bioethics Commission of the local university (nº 00003099). Seven wet mandibles containing 4-10 uni-radicular teeth were selected from the collection of the Body Donation Service and Dissection Room of the local university. The sample size was 44 in situ human teeth. Only mature incisors, canines and premolars were included, and in the case of bi-radicular teeth only one canal
per tooth was instrumented. Teeth with obliterated root canal, external or internal resorption, caries, presence of filling materials, posts or root fractures and/or dilacerated teeth were excluded. Throughout the procedure the human mandibles were kept wet in 1% formaldehyde. Before treatment, each mandible/maxilla was scanned in a micro-CT device (Quantum FX mCT, PerkinElmer, Health Sciences, Massachusetts, USA). Briefly, the samples were scanned at 90 kV and 160 mA through FOV 40-60mm, exposure time 4.5 minutes (voxel size of 118 × 118 × 118 µm). The acquired images were reconstructed into cross-sectional slices with PerkinElmer software (PerkinElmer, Health Sciences, Massachusetts, USA) using the Feldkamp method.

Crown access was created under constant irrigation and the pulp chamber was located. Pulp tissue was extracted with a K- Flexofile #15 (Dentsply Maillefer, Ballaigues, Switzerland), the teeth were radiographed with a digital radiograph system (Dexco ADX6000, Pasadena, USA) and tooth length (TL), defined as the length from the most coronal part of the crown to the point at which the file radiographically abandoned the root canal, was calculated. The cone was located perpendicular to the tooth axis and x-ray film.

The teeth were randomly assigned to one of the groups using a randomizer website (researchrandomizer.org):

- Group I (n=15): working length TL - 0.5mm (sub-instrumentation)
- Group II (n = 14): working length to the level of apical foramen
- Group III (n = 15): working length TL + 0.5mm (over-instrumentation)

Teeth were manually instrumented with a 25mm K-file#15 progressing up to K-file#80 (SybronEndo, California, USA), using the balanced force technique under copious irrigation with 2.5% sodium hypochlorite. Files were renewed after instrumentation of 10 teeth. After instrumentation the samples were again scanned with micro-CT, as previously described.

The reconstructed image stacks of the bone blocks before and after canal instrumentation were co-registered using the OsiriX DICOM Viewer v. 9.0 software (Pixmeo DARL, Geneva, Switzerland) for three-dimensional visualization and qualitative analysis of the teeth. Transversal, sagittal and coronal section images of each tooth were screened by a previously calibrated examiner who was blinded to the experimental groups. Screening was intended to identify the presence of dentinal defects (to be considered a complete dentinal crack, the fracture had to compromise both interior and exterior walls of the root), invasion of the periapical bone by the instrumentation (which was defined as the loss of the continuity of the apical cortical bone), dimension of the apical foramen diameter before and after treatment, and post-treatment thickness of the dentine walls. The thickness of the dentine walls was measured 3mm above the apex, and from the inner border to the outer border of the dentine wall. Only the minimum values of wall thickness were recorded. Outcomes were measured twice (7 days between the first and the second measurements). The intra-observer agreement was calculated using Kappa coefficient by means of SPPS 20.0 software.

Statistical analysis
The data collected were entered into an Excel file and statistical analysis was performed by SPSS software (V. 20.0, Chicago, USA). Descriptive statistics were calculated, including means and standard deviation. Data distribution analysis was carried out by means of the Shapiro-Wilk test. Pearson’s Chi-squared, Fisher Exact and Wilcoxon Signed Rank tests were used to evaluate statistical differences. For all statistical analyses a significance level less than 0.05 was used.

Results
The mean pre-treatment apical foramen diameter was 0.24 (±0.09), ranging from 0.1 to 0.34mm (Table 1), with no statistical differences between groups.

Group I sub-instrumented teeth.
Only 20% of teeth presented an enlarged apical foramen diameter. Moreover, the augmentation was only 0.09mm, with a final apical foramen diameter (AFD) of 0.32 (±0.24). According to the Wilcoxon Signed Rank test, pre-treatment and post-treatment foramen diameters were similar (p>0.05) (Table 1). No teeth presented either microcracks or damage to periapical hard tissues. 33% of teeth presented areas of dentine thickness of less than 1mm.

Group II instrumentation at TL level
Most of the teeth (71.4%) presented an enlarged apical foramen diameter. Moreover, the diameter was augmented by 0.42mm, with a final AFD of 0.68mm (±0.38). According to the Wilcoxon Signed Rank test, pre-treatment and post-treatment foramen diameters were statistically different (p<0.05) (Table 1). 35.7% of the samples presented damage to periapical hard tissues (Fig. 1A) and 7.1% of teeth presented microcracks (Fig. 2 A&B). 50% of teeth presented areas of dentine thickness of less than 1mm.

Group III over-instrumentation
Most teeth (86.7%) presented an enlarged apical foramen diameter (Fig. 2 A-B). Moreover, the diameter was augmented by 0.43mm, with a final AFD of 0.68mm (±0.21). According to the Wilcoxon Signed Rank test, pre-treatment and post-treatment foramen diameters were statistically different (p<0.05) (Table 1). 13.3% of teeth presented microcracks and 46.7% presented damage to periapical hard tissues (Fig. 1A). There were two teeth in which the apical foramina were not enlarged due to apical transportation (Fig. 3 A&B). 40% of teeth presented areas of dentine thickness of less than 1mm.

Comparison among groups
Teeth from the over-instrumented group presented a higher percentage of teeth with an enlarged apical foramen compared to Groups I and II (p<0.05). However, they also presented a major prevalence of damage to periapical hard tissues (p<0.05). There were no significant differences between groups in the prevalence of microcracks. Similar prevalence of dentine thickness of less than 1mm was observed between groups (Table 2). However, differences in these parameters were observed when analysed by tooth type. 100% of mandibular incisors presented areas in which the dentine wall presented a thickness of less than 1mm. The second most affected teeth were mandibular premolars, while no maxillary incisors presented wall thickness of less than 1mm (Table 2).
Discussion

The new approach for the treatment of necrotic or irreversibly inflamed pulps attempts to repair/regenerate pulp tissue using the patient’s own potential for angiogenesis and the presence of stem cells and growth factors in the periapical and dental tissues\textsuperscript{18-20}. However, it is necessary for the clinician to provide an appropriate tooth environment to enhance revitalization. Mature teeth present a small apical diameter\textsuperscript{8} which acts as a physical barrier, hindering access by the biological factors to enhance regeneration of the pulp tissue that occupy the entire root canal\textsuperscript{10}. Foramen enlargement is the intentional and mechanical enlargement of the apical foramen\textsuperscript{21}. It has been mainly performed to reduce the bacterial load by excising the infected cementum and dentin in necrotic teeth with apical pathosis\textsuperscript{21} and induce periapical tissue repair\textsuperscript{22}, although for regenerative endodontics also represent an additional benefit, as is to allow the ingrowth of new tissue into the root canal\textsuperscript{7}. Thus, intentional enlargement of the apical foramen may be necessary from a regenerative microbiological viewpoint. Therefore, we aimed to establish a standardized method to achieve apical foramen widening of mature teeth in cadaveric material. Being performed over in situ teeth, this study provides a reliable evidence with clinical application for an effective apical foramen enlargement.

We are not able to identify the exact position of the apical foramen with current technology. Electronic locators and computerized axial tomography are the most promising options, however at present these devices are neither completely precise\textsuperscript{23, 24} nor always available in clinical practice. A previous study\textsuperscript{8} showed that a variation of only 0.5mm in working length alters the effectiveness of apical foramen enlargement procedures, which coincides with our results. Instrumentation 0.5mm short of the apical foramen resulted in widening of the foramen by only 0.09mm, while instrumentation 0.5mm beyond the apical foramen resulted in widening of 0.42 mm. Moreover, it is essential to consider the patient’s discomfort\textsuperscript{21} and the clinical complications that over-instrumentation could cause, and especially the risk of apical extrusion of intracanal bacteria\textsuperscript{25} and debris\textsuperscript{26}.

Instrumentation with large files can result in iatrogenic damage such as dentinal cracks, weakening of the dentine walls, damage to periapical bone tissue and apical transportation\textsuperscript{8, 27}. In this study, two teeth in the over-instrumentation group presented apical transportation, which prevented foramen enlargement. Instrumentation was carried out with wide stainless-steel files (#80), whereas working with nickel-titanium rotary instruments could have improved these outcomes\textsuperscript{28}, as well as diminishing the prevalence of dentinal cracks\textsuperscript{29}. Another complication of instrumentation with wide files (#80) is weakening of the dentine walls. In this study 100% of mandibular incisors and 40.9% of all teeth presented at least one area with dentine wall thickness of less than 1 mm. Although this may represent an issue if the patient needs further prosthetic rehabilitation treatment, the potential of hard tissue formation adhered to the dentine walls after the regenerative endodontic approach\textsuperscript{2, 3} can overcome this disadvantage. Prior analysis of dentine wall thickness before treatment is highly recommended, particularly in mandibular incisors and premolars; this will also allow the possibility of establishing different foramen sizes by tooth type to be assessed.
Instrumentation 0.5mm beyond the apical foramen is the most effective technique for apical foramen enlargement. However, it is important to consider the effect of this technique on periapical bone and weakening of the dentine wall. Study of its effects in vivo is also needed.

References


### Table 1.- Distribution of foramen sizes before and after treatment, by experimental group.

<table>
<thead>
<tr>
<th>Group</th>
<th>Apical foramen diameter before treatment (mm)</th>
<th>Apical foramen diameter after treatment (mm)</th>
<th>Sub-instrumentation (n)</th>
<th>At apex level (n)</th>
<th>Over-instrumentation (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.0-0.2</td>
<td>0.0-0.2</td>
<td>8</td>
<td>8</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>0.21-0.4</td>
<td>0.21-0.4</td>
<td>7</td>
<td>6</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>0.41-0.6</td>
<td>0.41-0.6</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0.61-0.8</td>
<td>0.61-0.8</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>&lt;0.81</td>
<td>&lt;0.81</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0.0-0.2</td>
<td>0.0-0.2</td>
<td>7</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0.21-0.4</td>
<td>0.21-0.4</td>
<td>5</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>0.41-0.6</td>
<td>0.41-0.6</td>
<td>0</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0.61-0.8</td>
<td>0.61-0.8</td>
<td>3</td>
<td>4</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>&lt;0.81</td>
<td>&lt;0.81</td>
<td>0</td>
<td>4</td>
<td>2</td>
</tr>
</tbody>
</table>

Table 2.- Number of teeth with a dentine thickness less than 1mm, distributed by treatment group and by tooth type. *Statistically significant differences.

<table>
<thead>
<tr>
<th>Group</th>
<th>N (%) teeth with dentin walls &lt;1mm</th>
<th>Mean (sd) of the dentine walls thickness (out of walls with thickness less than 1mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sub-instrumentation (group I)</td>
<td>5 (33.3%)</td>
<td>0.53 (0.17)</td>
</tr>
<tr>
<td>At apex level (group II)</td>
<td>7 (50%)</td>
<td>0.72 (0.20)</td>
</tr>
<tr>
<td>Over-instrumentation (group III)</td>
<td>6 (40%)</td>
<td>0.60 (0.19)</td>
</tr>
<tr>
<td>Mandibular premolars (of all groups)</td>
<td>6 (60%)</td>
<td>0.59mm (0.19)</td>
</tr>
<tr>
<td>Maxillary premolars (of all groups)</td>
<td>3 (42.9%)</td>
<td>0.77mm (0.08)</td>
</tr>
<tr>
<td>Mandibular canines (of all groups)</td>
<td>2 (50%)</td>
<td>0.89mm (0.03)</td>
</tr>
<tr>
<td>Maxillary canines (of all groups)</td>
<td>2 (33.3%)</td>
<td>0.76mm (0.16)</td>
</tr>
<tr>
<td>Mandibular incisors (of all groups)</td>
<td>5 (100%) *</td>
<td>0.45mm (0.1)</td>
</tr>
<tr>
<td>Maxillary incisors (of all groups)</td>
<td>0 (0%) *</td>
<td>0</td>
</tr>
</tbody>
</table>
Figures

Figure 1. Sagittal views of over-instrumented teeth (Group III) (A-C). Invasion of the periapical bone is observed (B, black arrow), as well as reduced width of the dentine walls in the apical third of the tooth (B&C white arrows).

Figure 2. Sagittal (A) and transversal (B) views of a tooth instrumented at TL level (Group II). White arrows: microcracks.
Figure 3. Sagittal view of an over-instrumented tooth (Group III) (A & B). Apical transportation is observed (white arrows) which prevented enlargement of the apical foramen (black arrows).
Results Chapter 5:
Revascularization of mature teeth
A Regenerative Endodontic Approach in Mature Ferret Teeth Using Rodent Preameloblast-conditioned Medium

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Abstract. Background: This study evaluated the effectiveness of a regenerative endodontic approach to regenerate the pulp tissue in mature teeth of ferret. The presence of odontoblast-like cells in the newly-formed tissue of teeth treated with or without preameloblast-conditioned medium was evaluated based on morphological criteria. Materials and Methods: Twenty-four canines from six ferrets were treated. The pulp was removed, and the apical foramen was enlarged. After inducing the formation of a blood clot, a collagen sponge with or without preameloblast-conditioned medium was placed underneath the cementoenamel junction. The samples were analyzed at the eighth week of follow-up. Results: Vascularized connective tissue was observed in 50% of teeth, without differences between groups. The tissue occupied the apical third of the root canals. Odontoblast-like cells were not observed in any group. Conclusion: Revitalization of mature teeth is possible, at least in the apical third of the root canal. Further experimental research is needed to produce more reliable outcomes.

This therapy has been studied mainly for treating immature necrotic teeth, as an attempt to complete the development of the fragile dentin walls and revitalize the tooth.

In clinical practice, most cases of pulp necrosis are found in mature teeth. However, studies oriented to regenerate or repair pulp tissue of mature teeth are today scarce, most of them published in recent years (3-7). Currently, the most reliable option for the treatment of necrotic mature teeth is still root canal treatment. However, endodontically treated teeth are susceptible to reinfection and fracture, and the failure rate is close to 25% at 3-5 years (8). Moreover, endodontically treated teeth will remain devitalized throughout the patient’s lifetime and therefore defenseless to new caries lesions, as the absence of pulp implies the lack of tooth defense mechanisms (9, 10). Therefore, teeth revitalization offers several advantages over conventional root canal treatment, such as the recovery of the natural immune system and a structurally more resistant tooth (8, 11, 12).

There are two main limitations to treating permanent mature teeth with a regenerative endodontic approach. One is the small diameter of the apical foramen (13), which acts as a physical barrier hindering new tissue ingrowth into the root canal; and the second is the absence of an apical papilla. Immature permanent teeth present an apical papilla, which is a reservoir for mesenchymal stem cells (14). Stem cells from the apical papilla (SCAPs) have odontoblast-like differentiation potential, and produce dentin in vitro and in vivo, being responsible for root maturation (15). However, a previous study showed that even though the apical papilla is no longer present in mature teeth, an evoked-bleeding technique in these teeth is able to deliver mesenchymal stem cells from the periapical tissues (16). These cells also express mesenchymal stem cell markers, similarly to SCAPs (16).

Although these mesenchymal stem cells have odontogenic potential (15, 17), once in the root canal, they seem to...
differentiate into cementoblast-like cells and osteoblast-like cells, both in mature and immature teeth (18). This might suggest that these cells do not receive the correct signals necessary to promote odontoblast-like differentiation.

During tooth embryogenesis, the inner enamel epithelium and the underlying mesenchyme participate in a mutual interaction by secreting growth factors that induce their differentiation into ameloblasts and odontoblasts, respectively (19). Previous studies have identified 71 factors in the conditioned medium (CM) from preameloblasts (20). These molecules participate in cellular transduction, cell growth, transcription and metabolism and may act as attractants of bioactive signaling molecules required to induce differentiation of mesenchymal stem cells into odontoblast-like cells. An in vitro study has shown that preameloblast-CM induces odontoblast-like differentiation of mesenchymal stem cells (20). Moreover, an in vivo study in immature teeth claimed that newly formed dentin-like and pulp-like tissues were more frequently observed in CM-treated teeth (21).

Taking into account these data, our objectives were the following: Firstly, to evaluate the effectiveness of a regenerative endodontic approach to regenerating the pulp tissue of mature teeth of ferrets after pulpectomy; secondly, to evaluate the presence of odontoblast-like cells in the newly-formed tissue of teeth treated with and without preameloblast-CM; and thirdly, to analyze the suitability of the ferret as an animal model for regenerative endodontics.

**Materials and Methods**

This study was approved by the Ethical Committee for Animal Experimentation of the University of Barcelona, Spain (no. 519116). All animal experiments were conducted using the EU Directive 2010/63/EU for animal experiments and the Guiding Principles in the Care and Use of Animals approved by the Council of the American Physiological Society.

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**Table I. Summary of histological findings found in the intracanal and periapical tissues two months after treatment.**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control group (n=11), n (%)</th>
<th>Experimental group (n=11), n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Presence of newly formed iCT</td>
<td>4 (36.36%)</td>
<td>7 (63.63%)</td>
</tr>
<tr>
<td>Apical third</td>
<td>4 (100%)</td>
<td>7 (100%)</td>
</tr>
<tr>
<td>Medium third</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Coronal third</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Presence of inflammatory infiltrate in the iCT (percentage of teeth with iCT)</td>
<td>2 (50%)</td>
<td>4 (57.14%)</td>
</tr>
<tr>
<td>Presence of inflammatory infiltrate in the periapical tissues</td>
<td>6 (54.54%)</td>
<td>7 (63.63%)</td>
</tr>
<tr>
<td>Debris from biomechanical preparation</td>
<td>9 (81.81%)</td>
<td>6 (54.54%)</td>
</tr>
<tr>
<td>Apical cementum remodeling</td>
<td>8 (72.72%)</td>
<td>9 (81.81%)</td>
</tr>
<tr>
<td>Dentin resorption</td>
<td>0 (0%)</td>
<td>1 (9.4%)</td>
</tr>
<tr>
<td>Presence of intracanal hard tissue (cementum-like tissue)</td>
<td>2 (18.8%)</td>
<td>2 (18.8%)</td>
</tr>
<tr>
<td>Presence of Sharpey’s fibers in the intracanal space</td>
<td>2 (18.8%)</td>
<td>1 (9.4%)</td>
</tr>
</tbody>
</table>

iCT: Intracanal connective tissue.

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**Preameloblast-CM.** Apical bud cells (ABCs) from Sprague–Dawley rats were isolated and cultured as previously described (22). The handling of ABCs was performed inside a laminar flow hood with sterile instruments. Briefly, 20 apical bud tissues of lower incisors of 4-day postnatal rats were collected. The ABCs were enzymatically isolated with type I collagenase (0.62 mg/ml; Sigma, St. Louis, MO, USA) for 30 min, and collected by centrifugation. Cells were cultured in 10 mm Petri dishes in Dulbecco’s modified Eagle’s medium (DMEM; Gibco BRL, Carlsbad, CA, USA) with 10% inactivated fetal bovine serum (FBS) and 1% antibiotics (penicillin/streptomycin) and 2.5 mg/ml ascorbic acid, under 5% carbon dioxide at 37˚C, until confluence. After differential repeated trypsinization (0.25% trypsin/EDTA), cells were seeded onto 6-well plates in keratinocyte-free medium (Gibco BRL) with 0.01 ng/ml epidermal growth factor and 25 μg/ml of bovine pituitary extract until confluence (Invitrogen Corp., Carlsbad, CA, USA). Cells were then washed twice with PBS and cultured with DMEM without FBS. After 24 hours, supernatants were collected and filtered using sterile 0.22-μm diameter pore size membranes, aliquoted and stored at −20℃.

**Ferret model.** Six 4- to 6-month-old male and female ferrets (Mustela putorius furo) were commercially acquired (Isoquimen, Sant Feliu de Codines, Catalunya, Spain) and used in this study. Periapical radiography of the teeth showed complete root development. The four canine teeth from each ferret were treated and randomly assigned to the control (n=12) or the experimental group (n=12); randomization was performed with a coin. In order to study the normal histology of pulp tissue, four untreated molars were also analyzed (negative controls). Ferrets were deprived of food for 12 h before surgical intervention. Dental surgeries were performed at the Biothemium of the University of Barcelona (UEA de Bellvitge B-9900046), in the morning of 6 working days, under general anesthesia (25 mg/kg ketamine and 2 mg/kg xylazine) and analgesia (1 mg/kg meloxicam). The clinical protocol was as follows: Teeth crowns were disinfected with 2% chlorhexidine. Treatment was carried out under local anesthesia (Mepivacaine HCl 3%; Septodont, Lancaster, PA, USA) and endodontic access was performed using a high-speed hand piece under constant irrigation with saline. Pulp was removed with a #15 sterile stainless-steel endodontic hand file (Dentsply Maillefer, Ballaigues, Switzerland) and working length was determined...
using a portable digital X-ray device (iRay D4, Dexcel, Los Angeles, CA, USA). The apical cemental barrier was penetrated with #15 K-file and root canals were prepared 0.5 mm beyond the canal terminus. The root canals were prepared and the apical foramen was enlarged using a #80 K-file (Dentsply Maillefer, Ballaigues, Switzerland) with 2% sodium hypochlorite (23). Root canals were rinsed with 17% EDTA and sterile saline and dried with paper points. A sterile #15 K-file was inserted 2 mm beyond the canal terminus to irrigate the periapical tissues and induce bleeding and the formation of a blood clot inside the root canal. A hemostatic absorbable lyophilized collagen sponge (Hemospon; Techniew, Rio de Janeiro, RJ, Brazil) was placed over the blood clot. The collagen sponge was cut to a size slightly superior to the coronal part of the root canal, and then submerged in DMEM (control group) or preameloblast-CM (experimental group). Teeth were sealed with grey MTA (CHL Medical Solutions, Milan, Lombardy, Italy), flowable resin composite (CHL Medical Solutions) and amalgam (Bestdent, Montebello, Vicenza, Italy).

After completing the surgical procedures, animals were given supportive analgesics for 3 days (1 mg/kg meloxicam). A special soft diet was not needed. Ferrets were given ad libitum access to food and water, kept under a 12-h light/12-h dark cycle with three animals per cage. Eight weeks after surgery, animals were sacrificed using an anesthetic overdose (200 mg/kg pentobarbital).

Jaws and maxillaries including the treated teeth were sectioned and immediately submerged in 10% formalin for 24 hours. Using a diamond burr, a small cavity was created in the crown of the canines to ensure intracanal tissue fixation.

Each mandible/maxilla was scanned in a micro-computed tomographic (CT) device (Quantum FX microCT; PerkinElmer, Waltham, MA, USA). Briefly, the samples were scanned at 90 kV and 160 mA through a 20 mm FOV using an exposure time of 4.5 min. The acquired images were reconstructed into cross-sectional slices using PerkinElmer software (PerkinElmer, Health Sciences) by the Feldkamp method. The reconstructed image stacks of the bone blocks were co-registered using OsiriX DICOM Viewer v. 9.0 software (Pixmeo DARL, Geneva, Switzerland) for three-dimensional visualization.

**Histological processing.** All samples were decalcified using a solution containing 14.28% EDTA and 5% formic acid for approximately 3 weeks. The samples were then processed with a tissue processor (Shandon Citadel 1000; Thermo Scientific, Waltham, MA, USA), dehydrated through ascending gradations of ethanol in the following manner: one cycle of 70% ethanol (8 h), one cycle of 90% ethanol (8 h), three cycles of 100% ethanol (8, 16 and 14 h), three cycles of xylene (5, 5 and 6 h), and two cycles of paraffin (6 and 16 h).

Samples were sectioned using a Leica Jung RM2045 microtome (Leica, Nussloch, Baden-Württemberg, Germany). Longitudinal sections of 5 μm through the root apical foramen were obtained and placed on poly-L-lysine-coated glass slides. Tissues were stained with hematoxylin and eosin (H-E), and toluidine blue, and evaluated under light microscopy (Olympus CH30; Olympus, Tokyo, Japan). Histological analysis was performed by two histologists blinded to the experimental groups. Histological characteristics of the newly formed tissues were performed using morphological criteria and scored according to the histopathological parameters reported by Gomes-Filho et al. (24).

**Statistical analysis.** The categorical data (presence/absence of cementum-like or dentin-like tissues/inflammatory cells, fibroblast, odontoblast-like cells) were analyzed using a Fischer’s exact test, with a significance level of $p<0.05$.

**Results**

Due to the lack of blood clot formation and to fracture of the file in the root canal, one control and one experimental tooth were excluded, respectively. Thus, 11 teeth (from six ferrets) per group were analyzed. No periapical lesions in any teeth were radiographically observed.

**Histological analysis: Control and experimental group.** Newly formed intracanal connective tissue (ICT) was observed in 50% of the teeth, without statistical differences between groups (Table I) ($p>0.05$). Intracanal tissue was characterized as non-organized loose connective tissue with a low to moderate number of blood vessels, localized in the central region of the canal (Figures 1 and 2A and B). However, the ICT only occupied the apical third of the root canal. Cells were mostly fibroblasts (Figure 1B, D and F) and odontoblast-like cells were not observed in any group. Root canals without ICT were observed as being mostly empty and with some chronic inflammatory infiltrate and debris from biomechanical instrumentation (Figure 2C and D).

Acute inflammatory cells in the intracanal tissue were observed in 50% and 57% of control and experimental groups, respectively (Figure 1C, E and F and Figure 2C), without statistical differences between groups ($p>0.05$). In the experimental group, dentin resorption in the apical third of one tooth was observed (Figure 2D).

Cementum-like tissue was observed in four teeth, both attached to the wall canals as well as free in the iCT (Figure 1A-D).

Scarcce inflammatory cells in the periapical area of most teeth of both groups were present (Table I) (Figure 2D). Cementum remodeling at the apical portion of most teeth was also observed (Table I) (Figure 2B).

**Normal histology of mature teeth from ferrets (negative control group).** The normal histology of mature teeth from the ferrets can be seen in Figure 3. Pulp was observed as an organized, mature and vascularized tissue (Figure 3A and B), with odontoblasts arranged in a pseudostratified palisade (Figure 3B), compatible with a mature although young pulp tissue. Dentin was observed with ordered dentinal tubuli and predentin (Figure 3B). The maturation of the root is evident. Teeth presented a small apical foramen and cellular cementum at the apical area (Figure 3A).
Figure 1. Histological appearance with hematoxylin-eosin staining (H-E) of mature teeth treated with a regenerative endodontic approach. The intracanal connective tissue (iCT) occupied the apical third of the root canal of control (A, B) and experimental (C-F) teeth. A large number of fibroblasts (black arrows), inflammatory cells (yellow arrows) and a moderate number of blood vessels (white arrows) were observed (A-D). Intracanal Sharpey’s fibers (green arrows) (B and D) and cementum-like (CE) tissue attached to the inner walls (orange arrows) were also observed. DE: Dentin. *Debris from biomechanical preparation.
observed. Although isolation of teeth with a rubber dam was not possible, the size of ferret canine teeth was adequate to prepare the canals using wide files (#80), irrigation and placement of restorative materials without major complications (Figure 4A-C). The size of the mouth also allowed intraoral radiographs to be taken. The dimension of incisors, however, was too small to perform endodontic experiments (approximately 1 mm width ×2 mm height) (Figure 4B). Periapical radiographs and clinical examination revealed a pronounced curvature of the canine teeth, especially of mandibular teeth. Thus, instrumentation with flexible files is highly recommended. In the micro-CT analysis, canine teeth presented characteristic apical delta, with several accessory canals (Figure 2A and 4D), which may explain the clinical difficulties encountered in reach the working length.

Discussion

In our study, 50% of teeth showed some degree of vascularized connective tissue at the intracanal space. However, it occupied only the apical third of the canal. Moreover, the neo-formed tissue was disorganized and did not have the characteristic layers of mature pulp.

In this study preameloblast-CM was used to simulate the cellular and molecular conditions occurring during tooth embryogenesis between the inner enamel epithelium and the underlying mesenchyme. These tissues participate in a mutual interaction by secreting growth factors that induce their respective differentiation into ameloblasts and odontoblasts (19). Therefore, we hypothesized that growth factors present in the CM from preameloblasts (20) would
Figure 3. Normal histology (H-E) of ferret non-treated molars in longitudinal (A) and transversal (B) views. DE: Dentin; CE: cementum; PT: pulp tissue; AB: alveolar bone; blue arrow: apical foramen; green arrow: odontoblasts arranged in a pseudostratified palisade; white arrow: predentin.

Figure 4. A: Canine crown of 6 mm in height. B: Maxillary incisors, dimension 1×2 mm. C: Canal preparation with file #20 after creating the coronal access of a maxillary canine. D: Three-dimensional reconstruction of the apical third of a ferret canine tooth. Three accessory foramens (black arrows) and one enlarged apical foramen (white arrow) can be observed.
induce the differentiation of mesenchymal stem cells into odontoblast-like cells, as previously reported in in vitro studies (20, 21).

Several attempts at treating immature teeth with a regenerative endodontic approach have been made. With few articles reporting success until date (12), these attempts tried to induce differentiation of SCAPs and dental pulp stem cells into odontoblast-like cells in vivo (18, 25). In mature teeth, the induction of intracanal bleeding attract mesenchymal stem cells from the periapical tissues (16). The differentiation process of stem cells after revascularization procedures always leads to cementoblastic or osteoblastic lineage formation instead of generating odontoblast-like cells. Hence, this leads to the formation of cementum or bone-like tissues instead of dentin (18, 24-26). In this sense, even with the use of preameloblast-CM, the results of our study confirm those previously published. Only periodontal tissues (cementum-like and Sharpey’s fibers) were observed.

The size of the apical foramen seems to be a major concern in pulp repair of mature teeth, since a small apical foramen diameter might be a physical limitation for the ingrowth of new tissue inside the root canal. Although some studies refuted the influence of the apical diameter on clinical outcomes (27), others showed that an apical foramen diameter of <1 mm exhibited a greater increase in root thickness, length, and apical narrowing (28). To date, there is no established standard size of apical foramen required to ensure new tissue ingrowth into the root canal. In the present study, canal preparation was made using a #80 file 0.5 mm beyond canal terminus to ensure the widening of the foramen (13). However, the achieved size might not have been enough to allow new tissue ingrowth, that might explain why only 50% of the teeth presented vital intracanal connective tissue. Therefore, it is necessary to determine the diameter at which the apical foramen should be enlarged in order to arrive at more predictable outcomes when treating mature teeth with a regenerative approach. Moreover, the method for enlargement should also be properly studied since the effect of instrumentation over the apical constriction may also explain the inflammatory response of the periapical tissues, as well as the reaction of the apical cementum, which showed zones of remodeling. In summary it is necessary to establish a standardized clinical protocol for regenerative endodontics in mature teeth, as already exists for immature teeth (23), since the characteristics of mature teeth (a closed apex and the absence of apical papilla) deserve special consideration.

This study confirms the utility of ferrets as a model for regenerative endodontics, as previously proposed (29). Ferrets are easy to handle (2-3 kg), and their mouths and teeth are large enough to perform the clinical procedures for this therapy, although not to perform isolation with a rubber dam. It is also necessary to consider the apical delta and the pronounced curvature of the mandibular canines when planning a treatment.

Conclusion

Revascularization of mature teeth is possible, at least in the apical third of the root canal. However, further research in animal models is needed to create quicker and more reliable regenerative endodontic procedures that can be evaluated in clinical trials.

Conflicts of Interest

The Authors declare that no conflicts of interest exist regarding this study.

Authors’ Contributions

CB, AGS and CMC planned the study; CB, AGS and JMA performed the animal experiments; CB and IVG processed the samples and did the histological analysis; CB researched and wrote the article; CMC, IVG and JMA offered scientific advice and proofread the article. All Authors read and approved the manuscript.

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Results Chapter 6: odontoblast-like differentiation
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Odontoblast-like differentiation potential of human amnion epithelial cells

Objective: To evaluate the odontoblast-like differentiation of human amnion epithelial cells (hAECs) cultured onto dentine disks treated with EDTA. To evaluate if the cells attach to dentine discs and if they are capable of placing their cellular process into the dentinal tubuli.

Materials and Methods

This research project was approved by the Commission of Bioethics of the University of Barcelona and all patients signed the informed consent.

Isolation of hAECs

10 human placentas were collected from caesarean deliveries at the Hospital Sant Joan de Deu (Barcelona) and immediately transported to Laboratory 5307 of the Faculty of Medicine of the University of Barcelona, Bellvitge Campus (Figure 6.a). hAECS were isolated following the protocol of Gramignoli et al. (2016)\(^ {128}\). Briefly, the amnion was detached from the underlying chorion and washed with Ringer solution and PBS to dissolve blood clots. Then, the amnion was placed in a falcon tube with TryPle x10, and left for 30 minutes in an incubator. Cells were centrifuged (300 xg, 10 minutes), the cell suspension was discarded and cells were seeded in T75 Flasks in medium with DMEM, 10% FBS, 1% Pen/strep, 10 ng/ml EGF (3 million cells per flask) (Figure 6.b).

Identification of hAECs

Cell population of passage 1 was characterized to assess the purity of the hAECs. Flow cytometric analysis was conducted using EpCAM and CD49f as positive markers and CD105 and CD44 as negative markers. Briefly, 200,000 cells were collected in each cytometry tube. 4 ul of EpCAM, CD49f, CD44 and CD105 were diluted in 84 ul of staining buffer and analysed in a flow cytometer (FACSCanto, BD Biosciences).

Dentine disks
Sound teeth of patients aged 15–25 years old were collected from the Dental Clinic of the Faculty of Medicine, Universitat de Barcelona. Teeth were kept in 0.5% chloramine and 0.5mm thick dentine disks of the coronal third of the crown were obtained (Figure 6. c and d). Disks were washed with PBS and rinsed with 17% EDTA for 10 (n=6) and 20 (n=6) minutes. Control group were disks without EDTA.

Culture of hAECs on dentine disks and SEM analysis

$1 \times 10^5$ hAECs were seeded over dentine disks in 24-well plates and allowed to adhere in an incubator at 37 °C and 5% CO$_2$. After 48 h, samples were fixed with 2.5% glutaraldehyde in 0.1 M Sørensen’s phosphate buffer for 30 min. Images were taken on a FEI Quanta 400 environmental scanning electron microscope with a field emitter (FEI Europe B.V., Eindhoven, The Netherlands) at high-vacuum conditions. Images were analysed regarding morphological aspects.

**Fig. 6.** Scheme of isolation of hAECs from human amnion (a, b) and 0.5mm-thick dentine disk from mature teeth (c, d). Disks were treated with EDTA for 10 or 20 minutes (d) and hAECs were seeded on the discs for 48 hours (e).

**Preliminary results**
Flow cytometry
The isolated cells were positive for EpCAM and CD49f (>95%) and negative for CD44 and CD105, which confirmed the isolated cells were hAECs.

SEM analysis

Control group (dentine disk not treated with EDTA) presented round cells without or with incipient cytoplasmic processes (Fig. 7 a-c). On the contrary, EDTA groups showed evidence of odontoblast-like morphology with cytoplasmic processes in dentine tubuli (Fig. 7 d-h). Cells adhered to the dentine disks.
Fig. 7. hAECs onto dentine disks not treated (a-c) and treated with 17% EDTA (d-h) after 48 h in culture. Cells of the control group (a-c) show a round morphology without or with incipient cytoplasmic processes. Experimental group (d-h) show an odontoblast-like morphology with cytoplasmic processes located in dentine tubuli.
Discussion
Pulp necrosis in immature teeth frequently occurs after dental trauma, caries or developmental anomalies. After pulp necrosis, dentine secretion stops and so does root development, which compromises tooth prognosis. The classic treatment, apexification, was only capable of inducing an apical closure, while the dentine walls remained thin and short. In 2001, Iwaya reported a case of a necrotic immature premolar in an adolescent in which after disinfecting the tooth, the canal was left empty. Thickening of the canal walls and complete apical closure was confirmed 30 months later, indicating the revascularization potential of young permanent teeth. Iwaya’s report was followed in 2004 by a Banchs and Troppe case report which, besides disinfecting the canal in a necrotic immature tooth, induced a blood clot by mechanical irritation of the apex. The resolution of periapical pathosis and further root development was observed. These case reports reopened the research aimed at regenerating the pulp tissue that was originally initiated more than 50 years before and left forgotten.

Most teeth after REPs, in addition to resolution of signs and symptoms, show the dentine wall thickening and lengthening. However, the newly formed tissue is reparative tissue and not regenerated pulp or dentine. Thus, the presence of ectopic tissue such as cementum and the absence of pulp with a distinctive odontoblast layer has been observed in vivo. Consequently, root development observed after the therapy is mostly based on apposition of cementum-like tissue instead of tubular dentine.

The dental community has discussed whether the lack of dentine regeneration and the development of the root with cementum-like tissue instead of dentine is clinically relevant. The implication for the tooth prognosis of a cementum-reinforced root has not been studied, mainly because the follow-up periods of this new therapy have been short. However, it must be considered that dentine has a complexly ordered structure, while cementum is an amorphous calcified tissue composed mainly of collagen fibres and water. The composition and structure differences of these tissues impact their mechanical properties: dentine is a harder and more elastic tissue than cementum and has a high deformation capacity. Therefore, it is questionable whether a cementum-reinforced tooth can endure mechanical stress originated by regular mastication, orthodontic movement or trauma. To evaluate this, a finite element analysis of a simulated cementum- and dentine-reinforced tooth was performed. The results showed that the formation of dentine is advantageous because, unlike cementum, it facilitates an even stress distribution throughout the root, showing biomechanical
advantages of dentine reinforcement\textsuperscript{50}. Moreover, as expected and in concordance with previous studies\textsuperscript{49,154}, further root development with hard tissue is a desirable outcome since the immature tooth distributed the stress disadvantageously; peak stress concentrated in the most apical part where the walls are considerably thinner\textsuperscript{50}. As far as we know, this was the first study showing the biomechanical advantages of dentine regeneration. The mentioned results justify further efforts to achieve real dentine regeneration after REPs in immature teeth.

Dentine is secreted by odontoblasts from the embryological stage of tooth formation until the cell’s death during a patient’s adult life\textsuperscript{23}. These cells are derived from the neural crest and thus share some characteristics with neurons; they are a static cell population not to be replaced after cell death, and act as sensors for external stimuli\textsuperscript{3,23} recognising invading pathogens in the dentinal tubuli. They also trigger the pulp immune response, acting as a first line of defence against cariogenic bacteria\textsuperscript{3,23,38}. Thus, odontoblasts, as a very specific cell type, have attracted considerable interest, including studies of their behaviour \textit{in vitro} and shedding light on their different roles, especially the dentine secretory function, and consequently with a view towards improving the histological outcomes of regenerative endodontics and vital pulp therapies.

In the literature, a number of methods for odontoblast isolation and their study \textit{in vitro} have been used: organ culture of the crown with the odontoblast layer still attached to dentine\textsuperscript{41}, by scratching the dentine\textsuperscript{43}, by dissecting the pulp tissue\textsuperscript{157} and more recently by enzymatic detachment\textsuperscript{44}. \textit{In vitro} culture of single odontoblasts would open possibilities to investigate numerous characteristics of odontoblast activity in standardized conditions. Therefore, different protocols of enzymatic solutions (collagenase with either protease, trypsin or hyaluronidase) to isolate single primary odontoblasts from sound human molars were evaluated\textsuperscript{11}. We successfully detached the odontoblast layer from the dentine, which was confirmed through histology and scanning electron microscopy. However, the results of our investigation, in concordance with others\textsuperscript{45}, showed that using the current methods it is not possible to keep odontoblasts alive in a cell culture\textsuperscript{11} most probably due to the disruption of the cell membrane of the cytoplasmic process which prevent odontoblasts from attaining homeostasis. Although it is not possible to cultivate odontoblasts for more than 24 hours, in accordance with previous investigations\textsuperscript{158}, we were able to identify three genes that were significantly upregulated in odontoblasts compared to pulp tissue:
nestin (NE), dentin matrix acidic phosphoprotein 1 (DMP1) and bone sialoprotein (BSP). NE is typically found in cells which, like odontoblasts, derive from the neural crest\textsuperscript{159}, while BSP and DMP1 participate in the nucleation and guide crystal formation during dentinogenesis\textsuperscript{160}. Furthermore, DMP1 promotes the odontoblast differentiation of mesenchymal stem cells\textsuperscript{161}. These characteristic upregulated genes differentiate odontoblasts from pulp fibroblasts when comparing both cell types in a patient. It must be taken into account that the expression of these markers, whilst a part of the odontoblast’s molecular profile, does not constitute the singular profile of this cell’s phenotype\textsuperscript{162}. Thus, the identification of the odontoblast-like cell remains a challenge due to the lack of unique molecular markers\textsuperscript{162}.

Since odontoblasts are post-mitotic cells, the development of the root after pulp necrosis and REPs is based on the premise that stem cells from the blood and periapical tissues migrate and differentiate into odontoblast-like cells\textsuperscript{66}, which should be able to secrete a dentine-like structure that would allow the tooth to complete its root development with dentine. However, odontoblast-like differentiation of stem cells is hindered by several factors, such as the infected environment\textsuperscript{83,84,95} and the medicaments\textsuperscript{79,80} used during regenerative endodontics.

In the context of vital pulp therapies, Ricucci \textit{et al.} (2004) claimed that odontoblast-like differentiation and tubular dentine secretion are only produced in animal models with virgin noninfected teeth, while in humans, after pulp exposure and direct pulp capping, a dentine-like structure produced by odontoblast-like cells has never been observed\textsuperscript{96}. Instead, only fibrodentine, an atubular mineralized tissue surrounded by pulp-fibroblasts, is produced\textsuperscript{96}. Moreover, \textit{in vitro} studies show that lipopolysaccharide (LPS), surface components of Gram-positive and Gram-negative bacteria, interfere with odontoblast-like cell differentiation of DPSCs\textsuperscript{84}. Evidence suggests that early inflammatory responses, with low proinflammatory mediators, can lead to regenerative events, whilst chronic inflammation leads to reparative events\textsuperscript{163}. In the context of regenerative endodontics, the inflammatory status of the necrotic pulp after caries and the presence of periapical lesions has been related to the lack of further root development\textsuperscript{83}. Furthermore, almost 80\% of failed teeth after REPs present signs and/or symptoms of persistent infection\textsuperscript{77}.
Natural antimicrobial agents can offer a solution as co-adjuvants for disinfecting the canal without affecting the vitality and odontoblast-like differentiation of stem cells. Autologous platelet concentrates have been proposed as autologous antimicrobial agents since platelets are sentinels of the vascular system, release antimicrobial proteins against bacteria, express a wide range of potential bacterial receptors and may have the ability to internalize bacteria and to release molecules that provide several immune functions. Other benefits can be highlighted beyond the antimicrobial activity of APCs. APCs are biocompatible agents showing positive effects such as favouring stem cell proliferation and triggering angiogenesis, among other advantages for tissue regeneration. Moreover, platelet-rich fibrin, a particular type of APC, also provides a stable scaffold for tissue ingrowth. Several studies have used APCs in the regenerative endodontic therapy, delivering the concentrate into the root canal. Our systematic reviews concluded that although APCs provide improved clinical outcomes such as root lengthening and dentine wall thickening, they fail to provoke the presence of odontoblast-like cells and dentine-like tissue. The bacteriostatic effect of APCs and the medicaments used in the evaluated articles might not be enough to ensure an adequate disinfected and detoxified environment. Furthermore, added to the lack of well-designed studies, the risk of bias assessment of the majority of the evaluated papers was high.

When treating a necrotic infected tooth, one must consider that even after adequate chemomechanical debridement of the root canal, residual antigens have been shown to be present in dentine. Therefore, the medicaments used during REPs should ensure not only a correct disinfection but also a detoxification of the root canal, as recently suggested by Diogenes et al. However, agents used for detoxification and disinfection should at the same time be biocompatible to keep the vitality of stem cells. The systematic review we made during 2016 to evaluate the clinical protocols used during regenerative endodontics reflected that an important number of studies used medicaments with cytotoxic concentrations, although this will most probably change after the publication, also in 2016, of the standardized clinical protocol for REPs of the ESE. Irrigation solutions and medicaments commonly used in dental practice, such as sodium hypochlorite, chlorhexidine and antibiotic pastes, when used at high concentrations, have negative effects on cell survival, proliferation and odontoblast-like differentiation of stem cells and also a negative effect on the release of DMPs. Moreover, a recent case report of a necrotic immature tooth treated with a regenerative approach achieved partial regeneration of dentine in
some tooth areas but bone-like tissue in other tooth areas. The authors attributed the formation of these two types of tissues to their use of 5.25\% sodium hypochlorite and antibiotic paste as disinfectants and intracanal dressing. Therefore, it is possible that certain populations of stem cells may have been exposed to reduced amounts of transforming growth factor beta, which is critical for dentine formation, or have died as a result of the cytotoxic concentrations.

Regenerative endodontics was developed to treat immature teeth, as an attempt to revitalize the tooth and induce further root development, thus improving the prognosis. The regenerative potential of the apical papilla and the absence of an apical constriction ease the formation and entrance of new vital tissue into the root canal. However, the potential benefits of this therapy, such as the recovery of the natural immune system and the achievement of a more structurally resistant tooth, are equally desirable for mature teeth.

Currently only a few studies – mainly case reports and animal studies – reporting the application of REPs in mature teeth have been published. In general, these studies report optimistic outcomes: animal studies describe the revascularization of the root canal and case reports the resolution of symptomatology, the resolution of apical radiolucency and the reestablishment of peri-radicular tissues. Preliminary clinical trials also report favourable clinical and radiographic outcomes, although the follow-up period is limited to one year. Nonetheless, these studies provide preliminary information that requires further investigation. Many of the lessons learned in the study of immature teeth are also applicable to adult mature teeth, even if the latter have characteristics that make regenerative endodontic procedures a greater clinical challenge.

Mature teeth have a narrow apical foramen that may act as a physical barrier to the ingrowth of new tissue. A recent clinical study showed that teeth with wider preoperative diameters (≥1 mm) presented a greater increase in root thickness, length, and apical narrowing. When applying regenerative endodontics, it is necessary for the clinician to provide an appropriate tooth environment to enhance revitalization. Therefore, studies applying REPs in mature teeth enlarge the foramen through different methods, such as apicoectomy and instrumentation at different working lengths. Although it seems necessary for the apical foramen to be enlarged when applying REPs in the clinical practice,
it is not possible to identify the exact position of the apical foramen with current technology. Electronic locators and computerized axial tomography are the most promising options. However, at present these devices are neither completely precise\textsuperscript{173,174} nor always available in clinical practice. Therefore, the working length has a great importance at the moment in enlarging the foramen. We evaluated the effectiveness of standardized methods for apical foramen enlargement in extracted and \textit{in situ} human teeth. Instrumentation at different working lengths and apicoectomy were evaluated, since these methods have been used in the literature to enlarge the apical foramina of mature teeth\textsuperscript{107,113}.

Root resection of 2 mm only enlarges the foramen by 0.07 mm and resection of 4 mm, which represents a considerable loss of dental tissue, by 0.32 mm\textsuperscript{175}. Therefore, we concluded that apicoectomy is not an effective method and should not be used for apical foramen enlargement. Also, variations of only 0.5 mm in working length reduce the effectiveness of apical foramen enlargement. Instrumentation with a #80 file at the root canal terminus based on a periapical radiograph and 0.5 mm beyond that point provoke an enlargement of 0.42 and 0.43 mm, respectively, thus being the most effective methods of the ones tested. However, it is essential to consider the patient’s discomfort and the clinical complications that overinstrumentation could cause\textsuperscript{176} especially the risk of apical extrusion of intracanal bacteria and debris\textsuperscript{177}, when working with wide files at the root apical third. Instrumentation with large files can result in iatrogenic damage such as dentinal cracks\textsuperscript{175}, weakening of the dentine walls, damage to periapical bone tissue and apical transportation. Working with nickel–titanium rotary instruments could reduce the prevalence of apical transportation\textsuperscript{178} and of dentinal cracks\textsuperscript{179}.

To test the effect of REPs in mature teeth, we used an animal model. The pulp tissue of the four mature canines of six ferrets was removed and the apical foramen was enlarged using the previously developed method (instrumentation with a #80 file 0.5 mm beyond canal terminus). The protocol of the European Society of Endodontology\textsuperscript{63} was used with respect to the irrigants. After blood clot induction, the experimental groups had a collagen sponge embedded with preameloblast-conditioned medium. After eight weeks of follow-up, we observed revascularization of the apical third in 50\% of the treated teeth. This result is consistent with a preliminary clinical trial that reported positive response to electric pulp test in half of the mature teeth treated with a regenerative approach\textsuperscript{108}. The lack of vital tissue in the root canal of the rest of the teeth as well as the fact that vital tissue was observed in only the apical third of the revitalized teeth may be
attributed to hard tissue formation after the therapy that might reduce the diameter of the apical foramen. To date, there is no consensus with respect to the final diameter to which the apical foramen should be enlarged to stimulate tissue ingrowth\textsuperscript{112,180}. While a recent review showed better results with a diameter of 0.5–1 mm, experimental studies report higher root thickness, length, and apical narrowing with diameters of >1mm\textsuperscript{112}. The final diameter to which the foramen is enlarged should be decided considering that after regenerative endodontics hard tissue is deposited in the dentine walls, diminishing the size of the foramen and consequently reducing the possibilities of revascularization.

The preameloblast-conditioned medium was used in the experimental groups since during tooth embryogenesis the epithelium-mesenchyme interaction provoked a continuous secretion of growth factors that induced stem cell differentiation into ameloblasts and odontoblasts\textsuperscript{1}. In addition, the molecules present in the conditioned medium constitute part of an adequate pull of bioactive signalling molecules required to induce the differentiation of mesenchymal stem cells into odontoblast-like cells\textsuperscript{181}. Previous studies identified 71 factors in the conditioned medium (CM) from preameloblasts\textsuperscript{182} that participate in cellular transduction, cell growth, transcription and metabolism. Preameloblast-conditioned medium induces odontoblast-like differentiation of mesenchymal stem cells according to an \textit{in vitro} study\textsuperscript{182} and a higher degree of hard tissue formation \textit{in vivo}\textsuperscript{181}. However, the histological analysis showed no evidence of pulp/dentine regeneration in either our control or experimental groups. This lack of regeneration could be caused by the fact that secretion of growth factors in embryogenesis is sequential, acting sequentially at particular moments of the cell differentiation process, while the conditioned medium placed in the root canal may not respect the temporal order.

A more practical approach to inducing odontoblast-like differentiation in the clinical practice is to harness the pull of growth factors produced during tooth embryogenesis and that remain embedded in the dentine matrix\textsuperscript{147}. These growth factors are liberated by EDTA\textsuperscript{81}, a common irrigant in endodontics. The liberated growth factors from the dental matrix include several growth factors\textsuperscript{146,151,152} that play a role in odontoblastic differentiation, secretion of dentine matrix and formation of tertiary dentine\textsuperscript{146,152}. We tested odontoblast-like differentiation of hAECs seeded in dentine disks treated with EDTA and observed odontoblast-like morphology (processes in the dentinal tubuli) in the experimental groups. This preliminary data will be confirmed with a further \textit{in vitro} experiment of hAECs...
cultured with a known concentration of DMPs and evaluated through gene expression (q-PCR). As confirmed in other fields of medicine, hAECs represent a promising cell-type option for regenerative treatments since they can differentiate into the desired cell type once injected in vivo. Other cell types (SCAPs\textsuperscript{80}, DPSCs\textsuperscript{149} and BMSCs\textsuperscript{183}), despite showing odontoblast-like differentiation potential in vitro, differentiate into other cell types that secrete reparative tissue\textsuperscript{125} once they are delivered in the root canal of a necrotic tooth. Therefore, it would be interesting to know the behaviour of hAECs once injected in the root canal in an animal model.

The general trend in medicine is to leave behind the replacement of lost tissue by biologically inert materials and to induce the regeneration of the lost tissue or organ in form and function, which could improve not only the prognosis of the treated organ but also the patient’s quality of life. Regenerative endodontics is in line with this new conservative approach of medicine; however, much remains to be learnt. \textit{In vivo} regeneration of pulp and consequently of dentine remains a challenge, yet the achieved and potential benefits of regenerating the pulp tissue in both mature and immature teeth justify further efforts to improve histological and clinical outcomes.
Conclusions
The aim of this doctoral thesis has been to assess different experimental approaches to improve the clinical and histological outcomes of teeth treated with regenerative endodontics, as well as to evaluate the performance of teeth after the therapy. The main conclusions are the following:

**Chapter 1:** Finite element analysis of the biomechanical performance of immature teeth after REPs

- Apposition of hard tissue (regardless whether cementum or dentine) in immature teeth reduces mechanical stress in finite element simulation.
- Apposition of dentine in the immature tooth model provides biomechanical advantages over apposition of cementum in biting, trauma and orthodontic movement, since the peak stress values of the cementum-reinforced tooth are higher compared to the dentine-reinforced tooth.
- Moreover, an even distribution of stress along the root is observed in dentine-reinforced teeth. Deposited cementum barely absorbed the stress.
- These results in combination with the biological benefits justify the desire and research effort to achieve regeneration of the dental pulp in form and function.

**Chapter 2:** Enzymatic isolation and culture of primary odontoblasts

- Single odontoblasts can be isolated from human teeth. Among the tested enzymatic solutions for isolation, collagenase and protease yielded the most favourable results regarding the integrity of cell processes of the isolated cells.
- Regardless of different enzymatic digestions, after 24 h mostly non-viable odontoblasts were observed. Thus, it is currently not possible to maintain vital odontoblasts *in vitro*.
- Characteristic genes were identified to differentiate odontoblasts from pulp fibroblasts: nestin (NES), dentine matrix acidic phosphoprotein 1
(DMP1), bone sialoprotein (BSP) were significantly upregulated in the odontoblast layer compared to pulp tissue.

Chapter 3: Systematic reviews of APCs and clinical protocols for REPs

- Teeth treated with regenerative endodontics and APCs revealed significantly better thickening of the dentine walls and root lengthening than control teeth.
- Periapical healing and apical closure were improved in the group treated with APCs although with no statistical differences between groups.
- True regeneration of necrotic pulp is not achieved with current techniques using APC, which stimulated tissue repair.
- Consistent differences among studies were observed regarding the protocols for preparing the platelet-rich plasma.
- Scarce evidence exists of the clinical use of APCs for regenerative endodontics as well as the histological and immunohistochemical outcomes, and in general the quality of the studies is low.
- Most of the studies do not follow a standard clinical protocol for regenerative endodontic therapy, and some of them use irrigants/medicaments at cytotoxic concentrations.

Chapter 4: Apical foramen enlargement of mature teeth

- Apicoectomy is not effective in apical foramen widening.
- Significant differences exist regarding apical foramen enlargement when working length during instrumentation varies by only 0.5 mm.
- As expected, instrumentation up to file #80 0.5 mm beyond the apex is the most effective technique for apical foramen enlargement, which achieved a final apical diameter of 0.68 mm. However, it also produces a high prevalence of dentine microcracks, canal transportation and damage to the alveolar bone. The use of flexible nickel–titanium files and the
medication of the patient during and after treatment may overcome these drawbacks.

Chapter 5: Revascularization of mature teeth

- Revitalization of mature teeth is possible, at least in the apical third of the root canal.
- As in immature teeth, no regeneration of pulp tissue was observed after treatment.
- It is necessary to improve the protocol and the knowledge of the biological aspects of revitalization in mature teeth, since the outcomes at this stage are not predictable.

Chapter 6: Odontoblast-like differentiation

- Human amnion epithelial cells attach and show an odontoblast-like morphology when grown onto dentine disks treated with EDTA, suggesting that they are able to differentiate to odontoblast cells.
Future perspectives
The results obtained from the finite element analysis of teeth after REPs showed that dentine regeneration after the therapy is a desirable outcome, not only for the obvious biological advantages, such as the recovery of the pulp’s natural defensive mechanisms, but also because it improves the biomechanical performance when the tooth faces forces, thus improving its prognosis. Therefore, further efforts are justified to achieve dentine regeneration *in vivo* after regenerative endodontic procedures.

Future directions to achieve and evaluate regeneration *in vivo* can be summarised as follows:

- To identify unique molecular markers of the odontoblast-like cell phenotype. As said, no unique markers of the odontoblast phenotype exist; therefore, the diagnosis of pulp odontoblast-like cells in the regenerated tissue is commonly based on a morphological assessment or the use of markers such as nestin, DMP-1, DSP and others that whilst they are upregulated in odontoblasts, are also expressed in other cell types.

- To identify all factors that may be hindering odontoblast-like differentiation and dentine secretion *in vivo*. To date, evidence suggests that the cause of pulp necrosis (trauma or caries), the medicaments used during the therapy and the remaining bacteria after treatment all have an effect on treatment outcomes. Therefore, biocompatible and effective medicaments that ensure disinfection and detoxification without affecting stem cell differentiation potential or the release of growth factors should be fully identified.

- hAECs have shown promising regenerative outcomes in the treatment of liver diseases and are currently being tested in clinical trials for a variety of diseases. In the field of regenerative endodontics, hAECs could lead to the desired differentiation *in vivo*. Our preliminary results in dentine disks
treated with EDTA showed cells with odontoblast morphology. We will perform further *in vitro* analyses, such as by cultivating hAECs with a known concentration of dentine matrix proteins and evaluating odontoblast-like differentiation through q-PCR and the mineralization capacity of these cells. If the results turn out to be positive, a future direction will be to test hAECs in an animal study.

- To study the use of this promising new therapy for mature teeth. Currently mature necrotic teeth are treated by the conventional root canal treatment, which does not repair the weakened tooth structure. Evidence has shown the delivery of stem cells from the periapical tissues into the root canal, as well as positive clinical outcomes after regenerative treatment that justify further research in this field. It is necessary to develop a clear and standardized clinical protocol for regenerative endodontics in mature teeth and to perform clinical trials with longer follow-up periods to establish its reliability as a clinical therapy. Furthermore, we need to identify how other factors, such as patient age or cause of pulp necrosis, play a role in treatment outcomes. Future research should also identify the minimum diameter at which the apical foramen of mature teeth should be enlarged to obtain predictable outcomes, considering the further secretion of hard tissue in the root canal after treatment.
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