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BIOCOMPATIBILITY ASSESSMENT OF CALCIUM-SILICATE BASED ENDODONTIC SEALERS: *IN VITRO* STUDIES

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To My Parents and Brother

"The most beautiful experience we can have is the mysterious –
the fundamental emotion which stands at the cradle of true art and true science."
— Albert Einstein, *Albert Einstein*

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Abstract

Calcium silicate-based materials are used in various endodontic procedures and became popularized with the appearance of MTA in 1993 as a root-end filling material to use in pulp capping, pulpotomy, root perforation repair, apexification, internal resorption or apical barrier formation for teeth with necrotic pulps and open apexes. These numerous clinical applications of MTA are due to its inherent advantages including biocompatibility and regenerative capabilities. Several modifications of MTA have been created along the years to improve some aspects of the physicochemical and biological profile of the original formulation.

Root canal sealers are intended to be contained within the root canal space, but they may extrude through the apical constriction, or eluents from the sealers may come into contact with periradicular tissues. This might cause irritation and delay in wound healing. Moreover, the contact of root canal filling materials with periapical tissues can also affect the normal bone metabolism and regeneration. Regarding this, it is known that angiogenesis is critical in the bone environment for the normal bone remodelling and regenerative events. Accordingly, biological evaluation of endodontic sealers benefit from the biocompatibility profile concerning bone formation and angiogenesis.

In this context, the aim of this study was to compare four calcium silicate-based sealers, namely ProRoot® MTA, MTA Plus™, MTA Fillapex® and Biodentine™ regarding the effects of the sealers' extracts on (i) osteoblastic cell behaviour, by using human bone-marrow derived mesenchymal stem cell cultures and an *ex vivo* osteogenic assay (regeneration of defects created on the parietal bone of neonatal mice), and (ii) endothelial cell behaviour, by using human endothelial cell cultures and an *in vivo* angiogenesis assay (chorioallantoic membrane – CAM - assay). The effects of the sealers' extracts were dependent on the concentration and the time of exposure. Regarding the *in vitro* studies, the toxicity profile was similar for endothelial and mesenchymal stem cells regarding proliferation and functional parameters, however, endothelial cells were more sensitive to the toxic effects observed at the higher extract concentrations. ProRoot MTA and MTA Plus presented the best citocompatibility profile, followed by Biodentine and MTA Fillapex. This pattern of toxicity was also observed in the *ex vivo* regeneration of the rat parietal bone defects. However, on the CAM assay, no significant differences were observed on the angiogenic response elicited by the extracts of the four sealers. Overall, this study corroborates the low toxicity of calcium silicate-based sealers, but ProRoot MTA and MTA Plus presented the best biocompatibility profile.

Resumo

Os materiais à base de silicato de cálcio são utilizados em vários procedimentos endodônticos e tornaram-se conhecidos com o aparecimento do MTA em 1993, como um material de preenchimento radicular para uso em capeamento pulpar, pulpotomia, reparação de perfuração de raiz, apicificação, reabsorção interna ou formação de barreira apical de dentes com necrose pulpar e ápices abertos. Estas inúmeras aplicações clínicas do MTA devem-se às suas vantagens inerentes, incluindo biocompatibilidade e capacidades regenerativas. Várias modificações do MTA foram criadas ao longo dos anos para melhorar alguns aspectos de perfil físico-químico e biológico da formulação original.

Os cimentos endodônticos devem ficar contidos dentro do espaço do canal radicular, no entanto pode haver extrusão através da constrição apical, ou os eluentes dos cimentos podem entrar em contato com os tecidos perirradiculares. Isto pode provocar irritação e atraso na cicatrização. Além disso, o contato dos materiais de preenchimento do canal radicular com os tecidos periapicais também pode afetar o metabolismo e regeneração óssea. Relativamente a este aspecto, sabe-se que a angiogénese é crítica para o ambiente ósseo durante a remodelação óssea normal e os eventos regenerativos.

Deste modo, a avaliação biológica de cimentos endodônticos beneficia do perfil de biocompatibilidade relativo à formação óssea e angiogénese. Neste contexto, o objetivo deste estudo foi comparar quatro cimentos à base de silicato de cálcio, ou seja, ProRoot® MTA, MTA Plus™, MTA Fillapex® e Biodentine™ relativamente aos efeitos dos extratos dos cimentos sobre (i) o comportamento de células osteoblásticas, usando células estaminais mesenquimais derivadas de medula óssea humana e um ensaio *ex vivo* osteogénico (regeneração de defeitos criados no osso parietal de ratos neonatais), e (ii) o comportamento de células endoteliais, através da utilização de culturas de células endoteliais humanas e um ensaio *in vivo* de angiogénese (ensaio da membrana corioalantóide, CAM). Os efeitos dos extractos foram dependentes da concentração e do tempo de exposição. Considerando os estudos *in vitro*, o perfil de toxicidade foi semelhante para células endoteliais e células estaminais mesenquimais em termos de proliferação e parâmetros funcionais, no entanto, observou-se maior sensibilidade das células endoteliais aos efeitos tóxicos das concentrações mais elevadas dos extractos. O ProRoot MTA e o MTA Plus apresentaram o melhor perfil citocompatibilidade, seguido pela Biodentine e pelo MTA Fillapex. Este padrão de toxicidade também foi observado na regeneração *ex vivo* dos defeitos de osso parietal de rato. No entanto, no ensaio CAM, não foram observadas diferenças significativas na resposta angiogénica provocada pelos extratos dos quatro cimentos. Em conclusão, este estudo corrobora a baixa toxicidade dos cimentos à base de silicato de cálcio, mas o ProRoot MTA e o MTA Plus apresentarem o melhor perfil de biocompatibilidade.

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Abbreviation List

AA – Ascorbic acid
ADT – Agar diffusion test
ALP – Alkaline phosphatase
AMTA – Angelus mineral trioxide aggregate
BADGE – Bisphenol-A-diglycidylether
CAM – Chorioallantoic membrane
CH – Calcium hydroxide
CLSM – Confocal laser scanning microscopy
CS – Calcium silicate
FBS – Fetal bovine serum
FDA – Food and Drug Administration
Fungi - Fungizone
GIC – Glass ionomer cement
GMTA – Grey mineral trioxide aggregate
hBMSC – human bone marrow stromal cells
hDPSCs – Human dental pulp stem cells
HIF – hypoxia-inducible factor
hMSCs – Human mesenchymal stem cells
HUVECs – Human umbilical endothelial cells
M199 – Medium 199
MMP-9 – metalloproteinase-9
MTA – Mineral trioxide aggregate
NaOH – Sodium hydroxide
OPC – Ordinary Portland cement
PBS – Phosphate buffer saline
PDL – Periodontal ligament
PEST – Penicillin-streptomycin
PC – Portland cement
RCS – Root canal sealer
RCT – Root canal treatment
SEM – Scanning electron microscopy
VEGF – Vascular endothelial growth factor
WPC – White Portland cement
WMTA – White mineral trioxide aggregate
ZnOE – Zinc oxide eugenol
α-MEM – α-minimal essential medium

I: LITERATUTE OVERVIEW

1.1- Structure of the teeth

A tooth can be promptly divided in two parts: the crown that is the part that is visible in the oral cavity and the root that extends into the bone of the jaw, anchoring the tooth in position. These are composed of the following structures: enamel, dentin, cementum and dental pulp (Figure 1).

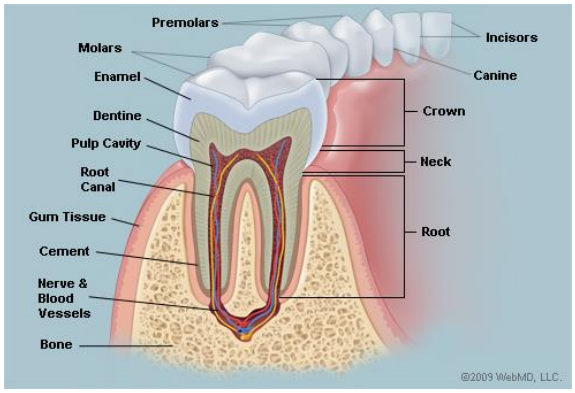


Figure 1- Teeth anatomy.

Enamel is the visible dental tissue of a tooth and the hardest and most highly mineralized substance in the human body. It is composed of 96% of inorganic material (primarily of the mineral hydroxyapatite, which is a crystalline calcium phosphate) and accounts not only for its strength but also for its brittleness [1]; and 4% of water and organic material.

Dentin is the softer material that supports the enamel, decays more rapidly and is subject to severe cavities if not properly treated, but it still acts as a protective layer and supports the crown of the tooth. It forms most of the tooth and is composed of approximately 70% inorganic material in the form of hydroxyapatite crystals, 15-20% of organic matrix consisting of collagen, 1-2% of noncollagenous proteins and the remaining 10-12% of water [2].

When the dentin is fully formed, it's called primary dentin and constitutes the bulk of the tooth, being characterized by the presence of dentinal tubules which normally extend from the dentin-enamel and dentin-cementum junction to the pulp. These tubules may serve entry regions for external irritants into the pulp. These contain dentin liquor, which is a fluid from the pulp tissue filling out the hollows of the dentin, odontoblastic processes, unmyelinated nerve endings and unmineralized and mineralized collagen fibers. Around the tubules there is a dense, highly mineralized tissue with a noncollagenous matrix called peritubular dentin, and between the tubules there is a mineralized collagen tissue called intertubular dentin. Dentinogenesis continues, but at a slower rate, even after the teeth are fully form.

Cementum is a specialized bone like substance covering the root of a tooth with a yellowish coloration. It is softer than enamel and slightly softer than dentin, being thickest at the root apex. It is approximately composed of 45% inorganic material (mainly hydroxyapatite), 33% organic material (mainly collagen) and 22% water. Cementum is excreted by cementoblasts within the root, which develop from undifferentiated mesenchymal cells in the connective tissue of the dental follicle. Its principal role is to serve as a medium by which the periodontal ligaments can attach to the tooth for stability. Cementum is acellular at the cementoenamel junction (⅔ of the root) and cellular (also more permeable) at the root apex (⅓ of the root apex) [2].

The dental pulp is the soft central part of the tooth which consists of a richly vascularized and highly innervated connective tissue. It is surrounded by dentin, has a form that mimics the outer contour of the various teeth and is in communication with periodontium and the rest of the body through the apical foramen and accessory canals, from where the blood vessels and nerves enter. Other characteristic element is the presence of dentin-forming cells called odontoblasts, which form a continuous and tightly packed sheet (odontoblastic layer) of regularly aligned and polarized cells, located at the periphery of the pulp with cytoplasmic processes extending into the tubules of the predentin and dentin. These are post-mitotic cells apparently incapable of further cell division and their rate of repopulation is extremely slow under physiological conditions [2].

The subodontoblastic region is characterized by a cell-free zone and a cell-rich zone. The last differ from odontoblasts since they have a bipolar, sometimes multipolar arrangement. They resemble fibroblasts of the central pulp and are speculated to have specific functions, being considered preodontoblasts for being capable of proliferation and differentiation into new odontoblasts. In the bulk of the pulp tissue, three main types of cells are present:

- inactive mesenchymal cells (when stimulated undergo cell division with the progeny developing into odontoblasts);
- fibroblasts (most numerous and responsible for extracellular matrix and collagen production and turnover);
- fibrocytes (that probably play a role in the maintenance of collagen fibres);
- other cellular elements belong to the vascular and neural system and occasionally appear inflammatory cells such as lymphocytes, plasma cells and macrophages.

The extracellular matrix of the pulp has a mucoid consistency organized as a heterogeneous colloid with soluble and insoluble components which serves as a matrix in which cells, fibres, and blood vessels are embedded. There are two main types of fibres in the pulp: collagen fibres, broadly present in the intercellular matrix; and elastic fibres, confined to the walls of larger blood vessels.

Blood vessels the size of arterioles branch off the dental artery and enter the pulp through the apical foramen and possibly through accessory canals, spread in the tissue, diminish in size, and finally become capillaries which provide the odontoblasts and other cells of the pulp with and adequate supply of nutrients.

The innervation of the pulp comprises afferent nerves which conduct sensory impulses (myelinated A-fibres surrounded by Schwann cells that enter the pulp with blood vessels) and autonomic nerves (unmyelinated C-fibres that enclose singly or in groups by Schwann cells, that enter in the pulp with sensory fibres) that are mainly involved in neurogenic modulation of the blood flow, but also in the transmission of pain. Nonetheless, the physiological need for the vast number of nerves in the coronal pulp is not immediately understood [2].

The dentin and the pulp of the tooth form the endodontium, as both develop from the dental papilla. Although the dentin mineralizes and the pulp remains a soft tissue, they maintain an intimate structural and functional relationship throughout the life of the tooth. The pulp contains all the cells of the endodontium and only cellular extensions, odontoblast processes, and nerve endings are found in the dentin. Thus, tissue reactions in the dentin are dependent to a great extent on the activity of cells in the pulp and pulpal reactions may be significantly modified by tissue changes in the dentin [2].

1.2- Structure of the supporting structures of the teeth

1.2.1- Alveolar process

The alveolar process is that thickened ridge of bone of the jaws containing the tooth sockets (dental alveoli). The alveolar bone or process is divided into the alveolar bone proper (bundle bone) which provides tooth support and the supporting alveolar bone (cortical bone and trabecular bone) (Figure 2). Microscopically, both the alveolar bone proper and the supporting alveolar bone have the same components: fibres, cells, intercellular substances, nerves, blood vessels, and lymphatics.

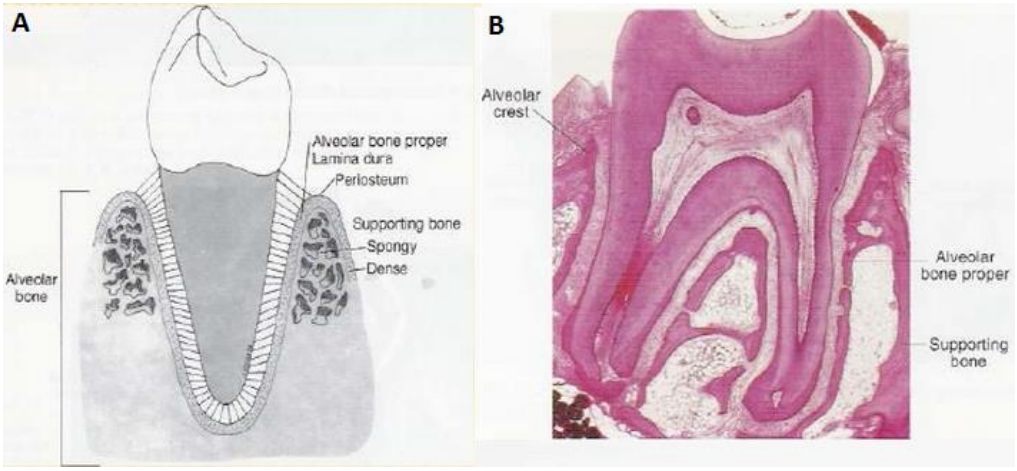


Figure 2- (A) Scheme of alveolar bone; (B) Histological section of a tooth with the alveolar bone structures highlighted.

The cortical plate and alveolar bone meet at the alveolar crest (usually 1.5 to 2 mm below the level of the cementoenamel junction on the tooth it surrounds). Alveolar bone comprises inner and outer components and it is perforated by many foramina, which transmit nerves and vessels, thus it sometimes is referred to as cribriform plate. Radiographically, alveolar bone also is referred to as the lamina dura because of an increased radiopacity which is due to the presence of thick bone without trabeculations that x-rays must penetrate and not to increased mineral content [3].

Embedded within the bundle bone are the extrinsic collagen fibre bundles of the periodontal ligament (PDL), which, as in cellular cementum are mineralized only at their periphery. Bundle bone thus provides attachment for the PDL fibre bundles that insert into it. Histologically, bundle bone generally is described as containing less intrinsic collagen fibrils than lamellar bone, but in some cases the alveolar bone can be made up almost completely of bundle bone. This is a simplistic description, however, because the tooth constantly is making minor movements, and therefore the bone of the socket wall constantly must adapt to many forms of stress. Thus practically all histologic forms of bone can be observed lining the alveolus, even in the same field in the same section. This considerable variation reflects the functional plasticity of alveolar bone [3].

The cortical bone or cortical plate consists of surface layers of lamellar bone supported by compact haversian system bone of variable thickness. The cortical plate is generally thinner in the maxilla and thickest on the buccal aspect of mandibular premolar and molars. The

trabecular bone or spongy bone occupying the central part of the alveolar bone also consists of bone disposed in lamellae. Yellow marrow, rich in adipose cells, generally fills the intratrabecular spaces, although sometimes there also can be some red or hematopoietic marrow. Trabecular bone is absent in the region of the anterior teeth, and in this case, the cortical plate and alveolar bone are fused together [3].

The mineral content of alveolar bone is mostly calcium hydroxyapatite, which is similar to that found in higher percentages in both enamel and dentin, but is most similar to the levels in cementum (50%). Like all bone, mature alveolar bone is by weight 60% mineralized or inorganic material, 25% organic material, and 15% water. The minerals of potassium, manganese, magnesium, silica, iron, zinc, selenium, boron, phosphorus, sulfur, chromium, and others are also present but in smaller amounts. It is important to note that alveolar bone is more easily remodeled than cementum, thus allowing orthodontic tooth movement. When viewing a stained histological section, the remodeled alveolar bone shows arrest lines and reversal lines, as does all bone tissue.

1.2.2- Bone cells

There are mainly four different cell types in the bone tissue that can be classified by:

- Embryologic origin: cells from osteoblastic lineage (osteoblasts, osteocytes and bone lining cells) derived from mesenchymal stem cells (MSC) and osteoclasts derived from bone marrow hematopoietic cells (Figure 3);
- Activity: bone forming cells and bone resorbing cells
- Location: osteoblasts, osteoclasts and bone lining cells are found along the bone surface while osteocytes can only be found in the interior, entrapped by the mineralized extracellular matrix. The development and differentiation of osteoblasts and osteoclasts are controlled by growth factors and cytokines produced in the bone marrow microenvironment and by adhesion molecules which mediate cell-cell and cell-matrix interactions [4].

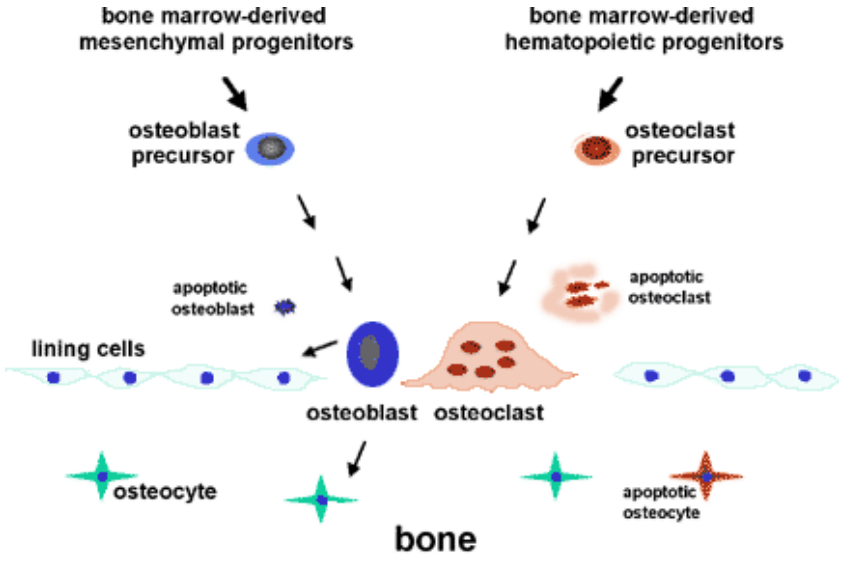


Figure 3- Bone cells origin.

The osteoblast lineage cells are responsible for the bone tissue formation during embryonic development, growth, remodelling, fracture repair or any bone defect. The differentiation process starts with an osteoprogenitor and then there's a progressive differentiation – preosteoblast, osteoblast and osteocyte.

Osteoblasts can migrate from neighbouring tissues or through the vascular system into the target area and are responsible for the production of the extracellular bone matrix (osteoid) and its subsequent mineralization through hydroxyapatite (HA) deposition that occur when the content of these vesicles is released by exocytosis. These cells are responsible for the production of bone tissue specific proteins, cell adhesion proteins, bone growth factors, proteoglycans and for storing enzymes, calcium and phosphate ions in vesicles. Osteoblasts can remain active cells; become embedded by the extracellular matrix that is subsequently mineralized (turning to osteocytes); or become relatively inactive and, eventually, develop into bone lining cells [5].

Osteocytes are the most abundant cell type in bone and are considered the most mature osteoblastic cells, resulting from the process whereby the osteoblasts become entrapped by mineralized bone matrix and stop its production. As osteocytes mature, they become smaller by cytoplasmic loss and by becoming entrapped deeper within the tissue. The *lacunae* where these cells are located have collagen fibrils which support cytoplasmic process that are responsible for intercellular crosstalk, via the established canaliculi. Besides allowing cell-mediated exchanges of minerals, this network is also believed to sense mechanical deformation within bone that leads to the activation of bone formation or resorption. Osteocytes are non-mitotic cells and their turnover is only achieved during the remodelling process (a combining process of bone resorption and formation). There is a direct relationship between the amount of osteocytes and the bone formation process where the higher bone formation, the greater the amount of trapped osteoblasts, so the number of formed osteocytes will increase.

Bone lining cells are inactive osteoblasts with dense nuclei and a more flattened shape, which cover most of areas in the bone tissue surface not being remodelled. These cells compactly associated with each other and with osteocytes by thigh junctions or cytoplasmic extensions and have less cytoplasmic organelles than osteoblasts, as they are metabolically less active. Its function is not clear but they seem to be responsible for the removal of the osteoid thin layer that coats the surface bone, thus exposing the bone to bone resorption performed by osteoclasts. Further, they also seem to take part in typical osteoblasts functions, under appropriate activation.

Osteoclasts are giant multinucleated bone-resorbing cells resulting from the fusion of several mononuclear haematopoietic precursor cells. Generally these cells are located on the surface of bone undergoing resorption and present a high mobility which allows them to move along the bone surface, from one site to another [6].

Osteoclasts are responsible for the bone resorption process, which occurs through the release of acidic substances that form an acid microenvironment and dissolve the bone tissue [7]. The products originating from bone resorption are endocyted by osteoclasts and then transported and released by the cell antiresorptive surface.

The **bone matrix** is composed by an organic component and an inorganic component.

The organic part makes 40% of the dry weight of bone and is composed by [8]:

- collagenous proteins – which are responsible for tensile strength and are synthesized, secreted and assembled by osteoblasts, while in the extracellular environment. Collagen type I, the most abundant, forms a triple helix of polypeptide chains that are organized into highly ordered fibrils establishing several crosslinks that contribute to the formation of a porous structure from which the bone derives its yield strength.
- proteoglycans - composed of glycosaminoglycan-protein complexes are responsible for compressive strength and inhibiting mineralization.
- matrix proteins (e.g. osteocalcin, osteonectin and osteopontin) - include non-collagenous proteins that are responsible for promoting mineralization and bone formation.
- cytokines and growth factors - present in small amounts are responsible for helping the bone cells differentiation, activation, growth, and turnover.

The inorganic part makes 60% of the dry weight of bone and is composed, essentially of calcium hydroxyapatite (HA). Calcium (Ca^{2+}) and phosphate (PO_4^{3-}) ions form crystals called HA crystals - $Ca_{10}(PO_4)_6(OH)_2$, arranged along the collagen fibrils and surrounded by proteoglycans and glycoproteins. Magnesium, sodium, potassium, bicarbonate and citrate ions also exist in the bone matrix but in lowest amount. The combination of HA with collagen fibrils gives the hardness and strength to the bone tissue that characterize it [9].

1.2.3- Bone metabolism

Bone tissue is being continuously remodeled, a lifelong process that maintains skeleton integrity and where old bone is removed from the skeleton by osteoclasts – bone resorption – and new bone is formed by osteoblasts to replace it – ossification [5].

Cortical bone remodels by osteoclastic tunneling through the bone, forming a cutting cone. The osteoclastic resorption is followed by layering capillaries, osteoblasts, *lamellae* and the cement line. The osteoblasts lay down the osteoid to fill the cutting cone until they become entrapped and differentiate into osteocytes, which will lead to the formation of a new Haversian system. Thus, cortical bone continues to change over time and its area decreases as age increases, while the medullary canal volume increases with age.

Cancellous bone has a lack of mature osteons and a large surface are of trabeculae, so it remodels by recruitment of osteoclasts to the bone surface, followed by their activation. Following, osteoblasts start to lay down extracellular matrix that is subsequently mineralized.

The bone metabolism is the complex sequence of bone turnover (osteoclastogenesis) and bone formation (osteoblastogenesis). The metabolic functions of bone largely involve the homeostasis of calcium and phosphate. Release or absorption of calcium by bone is largely regulated by calcitonin, parathyroid hormone, estrogen and androgens, vitamin D, and other cytokines, growth factors, prostaglandins and other molecules (REFs).

1.2.4- Osteogenesis and angiogenesis

Skeletal development and damage repair includes the coordination of multiple events such as migration, differentiation, and activation of multiple cell types and tissues [10]. The development of a microvasculature and microcirculation is critical for the homeostasis and regeneration of living bone, without which, the tissue would simply degenerate and die [11].

The vasculature transports oxygen, nutrients, soluble factors and numerous cell types to all tissues in the body. In mammalian embryonic development, the nascent vascular networks develop by aggregation of *de novo* angioblasts into a primitive vascular plexus – **vasculogenesis** – that undergoes a complex remodelling process in which sprouting, bridging and growth from existing vessels – **angiogenesis** – lead to the onset of a functional circulatory system [12].

The factors and events that lead to the normal development of the vasculature are recapitulated during situations of **neoangiogenesis** in the adult. The main pro-angiogenic agents are vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), various members of the transforming growth factor beta (TGF-β) family and hypoxia (through hypoxia-inducible transcription factor, HIF). Other factors with angiogenic properties include the angiopoietins (Ang-1), hepatocyte growth factor (HGF), platelet-derived growth factor (PDGF-BB), insulin-like growth factor family (IGF-1, IGF-2) and the neurotrophins (NGF) [13].

In the mature established vasculature, the endothelium plays a pivotal role in the maintenance of homeostasis of the surrounding tissue providing the communicative network to neighbouring tissues to respond to requirements as needed. Furthermore, the vasculature provides the necessary factors such as growth factors, hormones, cytokines, chemokines and metabolites needed by the surrounding tissue and acts, when needed, as a barrier to limit the movement of molecules and cells. Signals and attractant factors expressed on the bone endothelium help recruit circulating cells, particularly haematopoietic and mesenchymal stem cells [14]. Thus, any alteration in the vascular supply to the bone tissue can lead to skeletal pathologies such as osteonecrosis, osteomylitis and osteoporosis.

1.2.5- Interaction of endothelium and bone

In the cartilaginous region during embryogenesis, endothelial cells develop into immature vascular networks that enter the bone structure via cartilage canals already established in the expanding cortical bone. During postnatal bone growth, canal and vessel formation for blood vessels develops simultaneously at the growth plate region [15].

The bone vasculature comprises an arteriole, which divides into a complex anastomosing network of capillaries and sinusoidal vessels characterized by a highly permeable thin wall consisting of a single layer of endothelial cells, which are supported in part by a vascular membrane, pericytes, and reticular cells. These capillaries then double back and connect to a single venule, which follows the arteriole route returning to the perichondral plexus [16]. These vascular networks supply nutrients required by the chondrocytes, and mesenchymal cells for chondrogenesis and osteogenesis in order to maintain a functional secondary ossification centre [17]. Therefore, the development of these vessel networks is integral to the homeostasis of bone.

The intercellular signalling between endothelium and bone cells plays a critical role in:

- the homeostasis of bone integrity. For instance, human umbilical vein endothelial cells (HUVECs) conditioned medium was found to enhance the proliferation of human bone marrow stromal cells (hBMSCs) and when hBMSCs and HUVECs are co-cultured *in vitro*, the expression and activity of the early osteoblastic marker alkaline phosphatase (ALP) is elevated, but only when cell populations are grown in direct contact [18]. HUVEC can communicate with hBMSC via a gap junctional channel and affect dexamethasone-induced hBMSC differentiation into osteoblasts, by initiating the recruitment of osteoprogenitor cells at sites of bone remodelling and maintaining them in a pre-osteoblastic stage, hence avoiding mineral deposition within the vessel. Once these osteoblast precursors have migrated from the vessels towards a remodelling/bone forming site, the onset of rapid differentiation should occur into mature functional osteoblasts intent on laying down new osteoid.
- the functions of the osteoclastic lineage where osteoclast precursors need to adhere and migrate through the endothelium to areas of bone resorption. Alterations in the micro-vascular supply network will ultimately affect the tightly regulated resorption sequence resulting in decreases in bone formation, regeneration and repair, as well as altered osteointegration of orthopaedic and dental implants [19].

Bone endothelial cells display their own distinctive characteristics, with a capacity to respond to bone regulators such as cytokines, estrogen, PTH and others.

Within this context, VEGF plays a fundamental role. VEGF has multifarious activities in bone development, regulating the survival and activity of endothelial, chondrogenic and osteogenic cells, and promoting vascularisation during endochondral bone formation [20]. Elevated levels of VEGF and HIF-α overexpressing osteoblasts have been demonstrated to be critical in coupling angiogenesis and osteogenesis during long bone formation [21]. Although VEGFs' main effects are on endothelial cells, they also bind to VEGF receptors expressed on monocytes, neurons, chondrocytes and osteoblasts [22]. VEGF binds to the extracellular matrix and is made available by matrix metalloproteinase-9 (MMP-9) which is critical for early bone development [23]. It has been hypothesized that MMP-9 makes VEGF bioavailable from hypertrophic cartilage, functionalizing as a chemoattractant to promote vascular invasion.

Hypoxia and VEGF lead to an upregulation of bone morphogenic protein 2 (BMP-2) in microvascular endothelial cells demonstrating the intricate signalling pathways affecting the interactive relationship of endothelial cells and cells of the osteoblastic lineage [24]. These stress/strain and oxygen sensitive pathways may play a role in the response of endothelial cells at a site of healing and/or remodelling and hence be at the forefront of the repairing mechanistic cascade producing numerous factors such as BMPs that can act reciprocally on the osteoblast lineage and affect the rate of bone tissue repair.

1.2.6- Bone healing and angiogenesis

Bone has the unique capacity to regenerate without the development of a fibrous scar, which assists on the maintenance of the mechanical properties of the skeleton.

Following damage and/or trauma with damage to the musculoskeletal system, the following events take place in an ordered fashion (Figure 4):

- disruption of the vasculature leads to acute necrosis and hypoxia of the surrounding tissue, which leads to the activation of thrombotic factors in the coagulation cascade, resulting in haematoma formation [19];
- the inflammatory response and tissue breakdown activate factors that recruit osteoprogenitor and mesenchymal cells to the damaged site;
- the stimulation of the endosteal circulation in the damaged bone allows mesenchymal cells associated with growing capillaries to invade the wound region from the endosteum and bone marrow [25];
- the transiently formed granulation tissue at the edge is replaced by fibrocartilage;
- the periosteum directly stimulated intramembranous bone formation, leading the establishment of an external callus [26]; while internally, the tissue is being mineralised to form woven bone;
- the cell mediated remodelling cascade, involving BMP-2 and 4, VEGF, bFGF, TGF-β and PDGF is activated. Osteoclastic removal of necrotic bone tissue is accompanied by angiogenesis, which in turn is followed by the replacement of the large fracture callus by lamellar bone;
- the callus size is reduced and the normal vascular supply is restored.

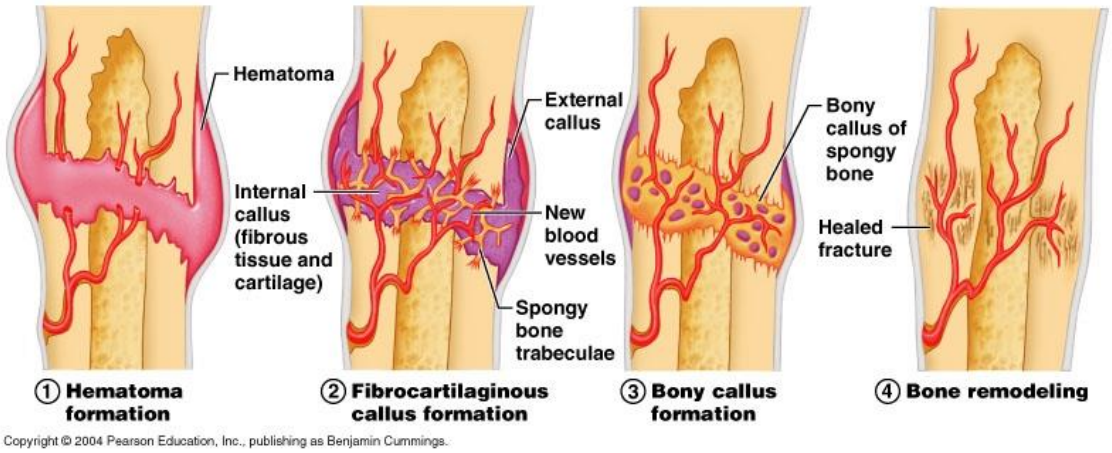


Figure 4- Bone healing steps.

VEGF expression is detected on chondroblasts, chondrocytes, osteoprogenitor cells and osteoblasts in the callus, where it is highly expressed in angioblasts, osteoprogenitor and osteoblast cells during the first seven days of healing but decreases after eleven days [25]. Additionally, osteoclasts release heparinase that induces the release of the active form of VEGF from heparin, activating not only angiogenesis, but also osteoclast recruitment, differentiation and activity leading to the remodelling of the callus during endochondral ossification [27].

The critical sequential timing of osteoclast differentiation and activation, angiogenesis, recruitment of osteoprogenitor cells and the release of growth factors in osteogenesis and damage repair maybe enhanced by the synchronized endogenous production of angiogenic and osteogenic mediators [19].

Disruption of the normal bone vasculature can result in skeletal problems such as osteopetrosis, metastatic bone disease and inflammatory bone loss in rheumatoid arthritis and apical periodontitis. Thus, we are now beginning to understand the intimate relationship

between the vasculature and bone, and the important role played in maintaining bone homeostasis, offering the possibility of novel new approaches to solving the problems of fracture healing, particularly in delayed and non-union pathologies.

1.3- Endodontics

The word endodontic is derived from the Greek language *endo* "inside" and *odons* "tooth" meaning it concerns processes that take place primarily within the pulpal chamber. Endodontics comprises not only theoretical thinking but also the skills and the practical thinking needed for clinical and moral judgment [28].

Historically, the main task of treatment has been to cure toothache as the consequence of inflammatory reactions in the pulp and periapical tissue. A commonly used method to remedy pain was to cauterize the pulp by a red-hot wire or by chemicals, such as arsenic, to devitalize the pulp. Removal of pulp procedures were introduced with the creation of hooked instruments and the advent of local anesthesia made treatment a painless procedure.

Pain relief is still a primary goal of endodontic treatment, but patients may also want to exclude the compromised tooth which means that intra- and extraradicular infections should be eradicated and that materials implanted in the root canal should not cause adverse tissue reactions. Using modern endodontic treatment procedures, these goals can be accomplished to a great extent [2].

1.3.1- Endodontic treatment procedures

The **vital pulp** is protected from injurious elements in the oral cavity by enamel, dentin and an intact periodontium. When these barriers are breached for any reason, microorganisms and the substances they produce may enter in direct contact with the pulp and adversely affect its stable condition [2].

One of the most common diseases of the world dental caries which result from bacterial infections caused by the *mutans streptococci*, most prominently *Streptococcus mutans* and *Streptococcus sobrinus*, and *lactobacilli*, that cause demineralization and destruction of the hard tissue encasement of the tooth, usually by production of acid by bacterial fermentation of the food debris accumulated on the tooth surface. If demineralization exceeds the buffering effect of saliva and other remineralization factors, these hard tissues progressively break down, producing cavities and holes in the teeth. Like any connective tissue, the pulp responds with inflammation (pulpitis) to neutralize and eliminate the agents and organize subsequent repair of the damaged tissue. The pulp may react in a manner that allows it to sustain the irritation and remain in a functional state. However, when caries has extended to the vicinity of the pulp, the response may take a destructive course and result in severe pain and death of the tissue (pulp necrosis that can be partial or total) [2].

If the pulp is in a state where an inflammatory breakdown is deemed imminent, there are measures that need to be taken in order to prevent an infectious development in the root canal system. **Pulpectomy** is the recommended procedure and involves the surgically removal and replacement of the pulp with a root filling material to prevent microbial growth and multiplication, within the pulpal chamber and pulp canals. It is done under local anesthesia and with the use of specially designed root canal instruments that remove the diseased pulp and

prepare the canal system so that it can be filled properly [29]. It may also be carried out when a pulp is directly exposed to the oral environment which may occur after:

- infectious pulpitis due to caries or exposure of the dentin or pulp;
- traumatic pulpitis due to traumatic injuries of the teeth;
- iatrogenic pulpitis due to improper and sometimes proper dental treatment.

If the exposure is recent and the pulp judged not to be seriously inflamed it may not have to be removed. If the open wound is treated with a proper dressing and protected from the oral environment by pulp capping, healing and repair of the wound are possible [29].

The **necrotic pulp** is defenceless against microbial invasion and due to an open direct exposure (either by uncovered dentinal tubules, cracks in the enamel and dentin or lateral canals exposed as a result of progressive marginal periodontitis), microorganisms in the oral cavity will reach the pulp chamber. The specific environment in the root canal, characterized by the degrading pulp tissue and lack of oxygen, will favour a microbiota dominated by proteolytic, anaerobic bacteria that organize in clusters and in communities, attached to the root canal walls and inside the dentinal tubules of the root. This environment confers protection from host defence mechanisms and favours a fast bacteria proliferation. Microorganisms attempt to invade the periodontal tissues and may do so before the host defence has been effectively organized via the apical foramen or any other exit from the root canal. Then, bacteria will usually be held back by the immune-inflammatory response, but not eliminated from the root canal space, which lead to the formation of a chronic inflammatory lesion. This evolution may or may not be associated with pain, tooth tenderness and various degrees of swelling, and remain for as long as no treatment is initiated [2].

To minimize the development of this clinical condition **root canal treatment** (RCT) (Figure 5) is performed to treat the necrotic pulp and is aimed to eliminate the intracanal infection, by cleaning the canal with files to remove the microbiota and its growth substrate. The instruments used cannot reach all parts of the canal system due to the complex anatomy of the root, therefore, antimicrobial substances are added to perform canal disinfection. The canal system is finally sealed with a root filling to avoid reinfection and to prevent surviving microorganisms from growing.

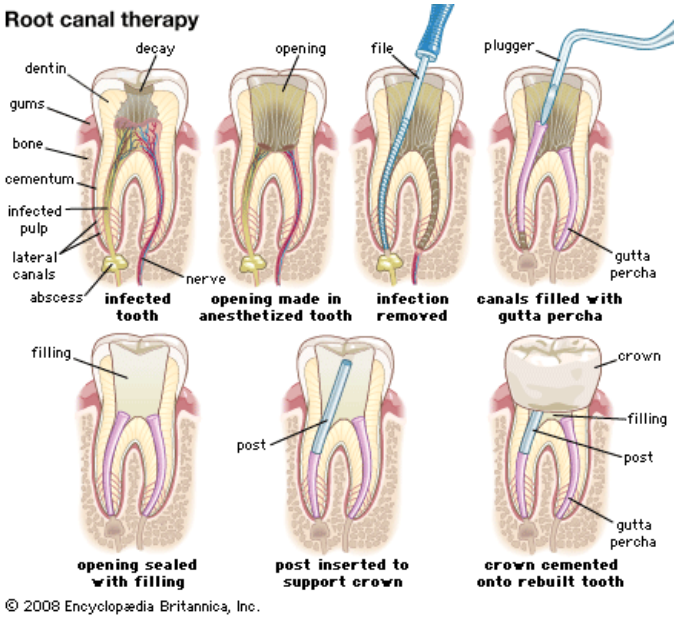


Figure 5- Root canal therapy stages.

Pulpectomy and RCT do not always lead to a successful clinical outcome. Treatment failures may occur, as a tooth may continue to be tender or periapical inflammation may persist which will allow original organisms to survive in the root canal or new bacteria to enter via leakage along the margins of the coronal restoration. The root canal may be following retreated [2]:

- non-surgically, where the root filling is removed, the canal is reinstrumented, antimicrobial substances are applied to eliminate the microbiota and the space is refilled. It may also be carried out to improve the technical quality of previous root fillings.
- surgically when the presence of crowns, bridges and posts may mean it is not feasible to reach the root canal in a conventional way. A mucoperiosteal flap is raised and access to the apical part of the root made through the bone. It often involves cutting of the root tip, instrumentation of the apical root portion and placement of a filling at the apical end.

1.3.2- Repair in the pulp

When reparative dentistry is possible, it is because pulpal inflammation heals when external irritants from a carious lesion are removed, and the resultant cavity is treated with a well-sealed restoration. The tissue reaction characterizing pulpal repair is usually similar to those in other connective tissues with the difference that the odontoblasts form secondary dentin in response to external irritants offering limited protection of the pulp. It is called reparative dentin, however, since it forms during the period of external irritation and tissue destruction, and not during the period of repair, having little bearing on the process [2].

When external irritants have been removed, pulpal repair may occur and could be complete if the inflammatory reaction is mild. However, if the inflammation is severe there may be extensive and irreversible tissue changes in the pulp where the inflamed tissue may be replaced by a fibrous tissue with only a few cells and the odontoblast layer may be completely destroyed in the area of reaction [2].

Abscesses are often present in the pulp of carious teeth and they are said to be a sign of irreversible pulpal inflammation, which may not be true. If the exogenous irritants are removed and the abscess is small (which often occurs within the pulp), it may become filled with a granulation tissue, and then by a fibrous tissue, or even become calcified.

A predictable reaction in immature teeth with ischemic necrosis is revascularization of the pulp with new vessels starting to grow within the necrotic tissue 4 days after, in the apical half after 10 days and in the coronal pulp and the pulp horns, after 30 days. The root canal and pulp chamber contain again vital, cell-rich connective tissue that it is not pulp tissue but periodontal tissue grown into the pulp space from the periodontal ligament. After a period of a few months to a year, bone- and cementum-like tissue will form. However, since the new pulp tissue does not contain odontoblasts, dentin formation will not occur. Nonetheless, if the root sheath has remained vital at the apex, induction of new odontoblasts may be verified , which in turn leads to dentin formation and to the organization of a complete root tip [2].

The hard tissue formation in revascularized teeth will ultimately lead to a clinically and radiographically complete obliteration of the pulp chamber and root canal space. However, microscopic inclusions of soft tissue will always be present in the hard tissue throughout the original pulp space.

1.4- Root canal filling materials

Bacteria play a significant role in the etiology and pathogenesis of apical periodontitis. The root canal has to be provided with a tight and long-lasting obturation to prevent bacteria and toxic products from spreading from or through the canal system, to the periapical area. Root canal obturation involves the three-dimensional filling of the entire root canal system with a root canal filling material and has two main purposes:

- elimination of all possible leakage pathways from the oral cavity or the periradicular tissues into the root canal system;
- sealing the root canal system of any irritants that remain after appropriate shaping and cleaning of the canals, thereby isolating these irritants.

Root canal filling materials may be divided into three main types [2]:

- gutta-percha cones that are prefabricated filling materials of a given shape and size;
- sealers that are pastes and cements mixed and hardened by a chemical setting reaction after a given amount of time which varies between preparations;
- a combination of cones and sealers that is currently recommended.

Less commonly used are thermoplastic materials (in combination with a sealer) heated or melted for better adaptation to the root canal wall and injected in a liquid state, and then hardened by cooling.

1.4.1- Root canal filling materials requirements

Root canal filing materials may be considered as implants; therefore, they should fulfil the requirements of such a device concerning technical, biological, handling and biocompatibility properties [2].

Technical properties are related with sealing aspects, taking into account that the success of a material depends on the prevention of infection or reinfection of the apical and lateral periodontal ligament and the adjacent bone. The material should be easily introduced into the canal and seal the canal laterally as well as apically; it should not shrink after being inserted and it should not be soluble in tissue fluids being impervious to moisture; it should not have pores and minimize water absorption, and should not stain tooth structure.

These materials should have a good sealing ability because apical and coronal leakage facilitates bacterial growth. *In vitro* test methods used to analyze leakage include dye penetration, in association with pressure, centrifugation or vacuum, however results are contradictory for the same material using different methods [30]. *In vivo* usage tests provide more relevant results but are more difficult to perform with the inclusion of more uncontrollable variables. This evaluation require a set of different test methods and this information should be regarded with caution as it is necessary other properties to characterize the clinical usefulness of the new material.

Biological properties are related with preventing local and systemic tissue irritation for both the patient and dental personnel and with stimulating regeneration of the apical region. The material should not cause or adverse reactions or allergies both for patients and dental personnel; it should not cause irritation of local tissues; it should be sterile or quickly and easily

sterilized before application, should possess antimicrobial properties; and it should stimulate the periapical healing process.

The material should have an acceptable level of **biocompatibility** and according to regulations, successfully pass a clinical risk assessment before being allowed to be marketed.

Different *in vitro* test methods have been designed to test biocompatibility [2]:

- cytotoxicity tests are a measure of the basic material properties regarding their ability to cause cell damage in terms of cell viability, proliferation and functional activity;
- genotoxicity/mutation assays analyses the possible influence of a material on the genetic information of cells;
- antimicrobial assays test the antimicrobial properties by assessing the efficacy of the materials on the inhibition of the proliferation of different bacterial strains.

The clinical relevance of these *in vitro* test methods is limited, because they do not take into account the complexity of a living organism, as well as the particular clinical situation of the apical region. So, this evaluation allows for the exclusion of potential toxic products, despite that further evaluation, within *in vivo* setting is mandatory to determine the biocompatibility of a material.

In vivo biocompatibility test methods are mainly performed on laboratory animals with implantation of a material into the subcutaneous/muscle tissues of rats, mice or rabbits to test the local toxic potential. Other tests include endodontic usage tests in dogs and monkeys where the material is applied as used later within the clinical settings, i.e., for filling root canals and where the apical repair and the formation of hard tissue after treatment of teeth with open apices is analysed. Although these tests are closer to the clinical situation than *in vitro* tests, they also have disadvantages because results depend strongly on the treatment method and there are indications that they do not provide a sensitive discrimination among endodontic materials with different chemical composition [2].

None of the test models described so far for assessing the biological properties of root canal filling materials can be said to be identical to the clinical situation under which the material is used. Clinical trials and x-rays with the human tooth are essential but unsatisfactory as they do not allow for histological evaluation. This demonstrates that the biocompatibility of a new material cannot be evaluated by one test alone, but a set of tests if necessary to cover the different aspects of biocompatibility, and that further the assessment of the material behaviour throughout routine clinical practice is fundamental [2].

Handling properties shall facilitate the actual use of the material and the control of the technique or treatment result. The material should have radiopacity needed for x-ray control; setting conditions must be adjusted to the special situation (regular fillings require slow setting allowing for condensation and eventual correction after x-ray control and retrograde fillings require fast setting for better moisture control during the operation); it should be easy to apply and remove if necessary using solvents, heat or mechanical instrumentation.

1.4.2- Gutta-percha cones

In the past, silver cones were broadly used but their application was limited by the bad sealing qualities and high corrosion leading to tooth discoloration and local reactions. Titanium cones have good biocompatibility however show low radiopacity and poor adaptation to the

root canal wall. Gutta-percha cones are the material of choice for filling the major part of the canal volume but do not fit the intricacies of the shape of the canal and therefore must be compacted and used together with sealers to improve the adaptation and sealing capabilities (Figure 6A) [2].

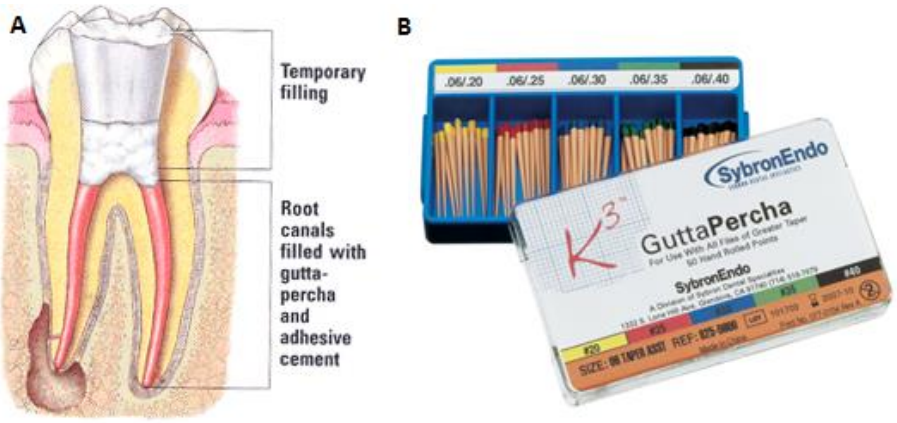


Figure 6- (A) Root canal filling with gutta-percha; (B) Commercial gutta-percha.

Gutta-percha is a natural product consisting of purified coagulated exudate of mazer wood trees (*Isonandra percha*) from the Malay archipelago or from South America. It is a high-molecular-weight polymer based on the isoprene monomer (Figure 6B). There are two forms relevant for dental products: β-form used in most cones and the α-form used for injectable products because of its improved flow characteristics. Their composition varies considerably between manufacturers [31]: formerly, cadmium-based dyes were added to provide a yellow color, which should facilitate removal; now, preparations use other colorants free of cadmium compounds. Some contain calcium hydroxide or chlorhexidine to enhance their antibacterial activity and stimulate apical healing. They are supplied in different sizes (length, diameter, taper) and may be used cold in combination with a sealer, in a heated state, allowing closer adaptation to the canal walls, or liquefied at temperatures of 70°C or 160/200°C and injected directly into the root canal [2].

Gutta-percha cones are elastic/flexible at room temperature, and becomes plastic at about 60°C, which insures the constancy of its volume under the oral cavity. Heating leads to expansion, a fact that reduces the sealing quality of warm or liquid gutta-percha application (when used without a sealer). They do not display systemic toxicity and allergic reactions are extremely rare. In cell culture studies, they proved to be non-cytotoxic or only a little cytotoxic depending on the product [32]. Generally, it is well tolerated by animal tissues, inducing the formation of a collagenous capsule with almost no inflammation, and revealed some antimicrobial properties [2].

These cones are usually supplied in a non-sterile way and their radiopacity is considered to be sufficient. Storage in disinfectants may have a negative influence on the mechanical properties of the cones and should be avoided. They should be stored cool and dark in order to prevent enhanced hardening and brittleness due to further crystallization and/or oxidation. The cones can be removed mechanically owing to its comparatively soft consistency [2].

1.4.3- Sealers

Root canal sealers (RCS) are used to fill voids and minor discrepancies of fit between the gutta-percha cones and the root canal wall. The use of sealers without gutta-percha cones leads to increased leakage due to the shrinking of the sealers during setting, which develops into pore formation and enhances the solubility of the sealer. Therefore, the use of sealers without any cone, as recommended in the past, is today obsolete [2].

Many endodontic sealers are now used in clinical practice, however, none of them meets all the appropriate requirements. To overcome the shortcomings of these materials, several materials have been proposed for clinical use in endodontic practice [33]. Sealers commonly used in clinical practice nowadays include zinc-oxide eugenol (ZnOE), polyketone, epoxy resin, glass ionomer cements (GIC), calcium hydroxide (CH) and calcium silicate (CH) sealers.

1.4.3.1- Zinc-oxide eugenol (ZnOE) sealers

ZnOE sealers have been used for many years with an ample clinical experience. However, sealing ability and biological properties are, in general, inferior compared with other RCS having a tendency for disintegration. Formaldehyde-releasing ZnOE RCS should not be used because of their inherent toxicity potential [28].

ZnOE sealers comprise a large group of different preparations with, sometimes, addition of thymol or thymol iodide to increase the antimicrobial effects, hydroxyapatite or calcium hydroxide to improve apical healing and colophony to give body structure, to impart adhesiveness to the sealer and to reduce the solubility/disintegration. Modified ZnOE preparations are tipically composed of: around 60% zinc oxide, 35% alumina and 5% natural resin (powder); 62.5% ortho-erhoxy benzoic acid and 37.5% eugenol (liquid). These preparations harden in a humid environment by forming a ZnOE chelate compound: the mix sets within 24h and the setting reaction is reversible, releasing eugenol and zinc ions under hydrolytic conditions [2].

Several studies showed apical leakage that increased with storage time in thick layers more than in thin layers [34]. Sealing properties were inferior in comparison to other sealers as coronal leakage was greater for a ZnOE sealer than for a CH sealer, probably due to the relatively high solubility of the ZnOE sealer [35]. Modified ZnOE cements appear to have better sealing abilities.

As for the biological properties, systemic toxicity was evaluated to be low despite known allergens released include eugenol and formaldehyde [36]. Eugenol is cytotoxic which has been shown frequently for ZnOE with different cell culture systems, especially after mixing but also following setting. Formaldehyde-containing ZnOE sealers were classified as highly/extremely cytotoxic even after several elutions of the hardened species and caused permanent damage of the nerve *in vivo*. In addition to allergic reactions, they may influence the immune system evoking and accentuating an inflammatory reaction in the apical region [37]. A ZnOE sealer has shown an initial inflammatory effect on the periapical tissues after teeth obturation, in ferret root canals, whereas only three out of ten showed inflammation after root filling with a calcium hydroxide formulation [38]. Antimicrobial properties can be demonstrated even 7 days after mixing on a variety of microorganisms, including *Enterococcus faecalis* suspensions and anaerobic bacteria [39].

ZnOE sealers are easy to handle and can be mixed to a smooth paste, which allows enough time for obturation and control (x-ray) before setting. Removal can be performed with organic solvents and the radiopacity of different ZnOE sealers can be regarded as sufficient.

Some examples of commercial ZnOE sealers, observed in Figure 7, include Tubli-Seal (Kerr, SybronEndo, Orange, CA, USA) and Pulp Canal Sealer (Kerr, SybronEndo, Orange, CA, USA).

Figure 7- Examples of ZnOE sealers: (A) Tubli-Seal; (B) Pulp Canal Sealer.

1.4.3.2- Polyketone sealers

A polyketone-based RCS commonly used is composed of a powder with 97% zinc oxide and 3% bismuth phosphate and a liquid of around 76% propionylaceophenone, 23.3% copolymers of vinyls, 0.5% dichlorophen and 0.2% triethlanolamine [2]. Polyketone sealers were shown to have acceptable technical properties (sufficient strength, low shrinkage and good adhesion to dentine) with lower microleakage scores than ZnOE sealers.

There are no reports indicating any systemic toxicity effect or allergic reactions, and cell culture experiments consistently showed cytotoxic reactions less pronounced than those reported for formaldehyde-containing ZnOE sealers and that decreased after setting. When mixed to sealer consistency, and following implantation in rat molars, it was shown to cause chronic inflammation that resolved totally or partially with increasing postoperative observation periods [40]. The sealer showed marked antibacterial properties and antimicrobial activity was dependent upon the used bacterial strain and was generally less distinct than with epoxy sealers [41].

As for handling properties, the sealer hardens rather rapidly (relatively short period of setting), which may create a problem with complex lateral condensation methods or may be an advantage when more than one canal needs to be filled. Radiopacity is considered to be sufficient but removal of the sealer is difficult, thus, it must be used with gutta-percha cones.

1.4.3.3- Epoxy resin sealers

The original preparation of epoxy resins was AH26 that is still marketed in some countries. Nonetheless, it has been broadly replaced because of the silver content that lead to tooth discoloration due to the formation of black silver sulfides. Its composition include 60% bismuth (III) oxide, 25% hexamethylene tetraamine, 10% silver and 5% titanium dioxide in the powder and bisphenol-A-diglycidylether (BADGE) in the liquid [2]. Preparations are now available

without silver and bismuth oxide is added for radiopacity. New follow-up products were developed, such as AH Plus, which is also based on BADGE but contains a different catalyst.

The setting reaction of AH26 is a polymerization process that lasts about 1-2 days (at body temperature) during which formaldehyde is released but in concentrations inferior to that on formaldehyde-releasing ZnOE formulations. The AH Plus is set after 8 hours and there are indications that it does not release formaldehyde [2].

These sealers showed good mechanical properties as well as excellent adhesion/adaptation to dentine. After *in vitro* and *in vivo* studies, these materials showed improved sealing properties than with any other sealers tested. Nonetheless, an increasing storage time (up to 2 years) was found to decrease the sealing quality [34].

Epoxy resins are biologically active molecules but no reports are available on systemic toxicity reactions with them, no effects on general health are expected but local and allergic reactions [42]. Cytotoxicity of AH26 was related to the initial release of formaldehyde during the setting reaction: freshly mixed the material is cytotoxic but after setting it is not toxic or only slightly toxic [43].

- AH26 was mutagenic [44], especially in a freshly mixed state [45], perhaps due to the formaldehyde or the epoxy monomer (BADGE) released.
- AHPlus was also mutagenic but only immediately after mixing [46]. After exposure to an enzyme mix containing esterases, BADGE is further hydrolyzed to a compound that is no longer mutagenic and because the set material in most studies was non-imunogenic, it can be used but care should be taken for who may come into frequent contact with the unpolymerized material. After implantation into laboratory animals, it proved to be toxic (again, due to the setting reaction) initially but the reaction resolved partially or even totally with prolonged post-operative periods [47].

Antimicrobial properties were found to be good, especially in a freshly mixed state and compared with ZnOE, CH and GIC sealers on the model of infected root dentine, AH26 showed the strongest antimicrobial effect [48], probably due to the initial release of formaldehyde.

Their handling properties are usually considered to be good and the radiopacity sufficient. The material set to a hard mass is virtually non-soluble even within organic solvents, therefore, it must be used with gutta-percha cones.

Some examples of commercial GIC sealers, observed in Figure 8, include EZ Fill (Essential Dental Systems, South Hackensack, NJ, USA), AH26 (Dentsply, Tulsa, OK, USA) and AH Plus (Dentsply, Tulsa, OK, USA).

Figure 8- Examples of epoxy resin sealers: (A) EZ Fill; (B) AH26; (C) AH Plus.

1.4.3.4- Glass ionomer cement (GIC) sealers

GICs broadly contain in their composition ground silicates (calcium-sodium-fluor-phosphor-silicate) in the powder and polyacrylic acid, malenic acid or tartaric acid in the liquid. The setting reaction, which is sensitive to both moisture and desiccation, starts as dissolution of the particles' surfaces followed by an acid-base reaction by which the metal ions from the powder replace the protons of the carboxylate groups and form a non-soluble matrix into which the remnants of the particles are embedded [2].

These sealers have good adaptation and chemical adhesion to the dentine and have shown good mechanical properties in other applications. However, the presence of pores seems to reduce the sealing quality, considerably. *In vitro* leakage studies revealed greater dye penetration than other sealers such as ZnOE and AH26, which may be due to problems of the test method or to the sensitivity of the setting process of GICs, with respect to moisture. It was observed that leakage increased with increasing storage time (2-year observation period) [34].

As for the biological properties, there are no reports available about systemic toxicity or allergic reactions. Cell culture experiments with GICs consistently show some cytotoxicity of the freshly mixed material but only minimal cytotoxicity after setting. For optimal setting, the correct water balance is necessary: either too much or too little moisture leads to insufficient setting combined with enhanced solubility and cytotoxicity which may be a problem in a root canal with an open apex and with the sealer in direct contact with the living tissue. In a study, a GIC RCS proved to be non-mutagenic [44]. After implantation, a mild inflammation appeared which further diminished with time, compared with a ZnOE sealer that produced a stronger inflammation, even after a longer implantation time [49]. Antimicrobial properties on anaerobic bacteria may be due to the initially low pH and the fluoride release. Comparing with other sealers, the antibacterial properties were somewhere between the ZnOE sealers (strong activity) and the CH preparations (weak activity) [50].

GIC sealers seem easy to handle, however the limited working time in the oral cavity may cause problems if used with lateral condensation technique on teeth with several root canals. Therefore the material should not be stored much longer than 7 minutes outside the capsule (where it can be kept for 40min), because the water evaporates and the paste tends to thicken. The radiopacity is considered to be sufficient. This material cannot be removed from the canal once it has set, therefore it must be used with gutta-percha cones.

Some examples of commercial GIC sealers, observed in Figure 9, include GICS (Shofu Dental Corporation, San Marcos, CA, USA), Ketac Cem (3M ESPE) and Ketac Fil Plus (3M ESPE).

Figure 9- Examples of GIC: (A) Glass Ionomer Base Cement; (B) Ketac Cem; (C) Ketac Fil Plus

1.4.3.5- Calcium hydroxide (CH) sealers

CH sealers were introduced in an attempt to stimulate periapical healing with bone repair through the release of CH. Their base paste is broadly composed by 32% calcium hydroxide, 32% colophony, 8% silicon dioxide, 6% calcium oxide, 6% zinc oxide and 16% of other components and their catalyst past is broadly composed of 36% disalicylates, 18% bismuth carbonate, 15% silicone dioxide, 5% colophony, 5% tricalcium phosphate and 21% of other components [2]. The setting reaction is based on the activation of salicylate compounds and after contact with fluids calcium oxide is partially transformed into CH with an increased potential of intratubular penetration and removal of unmineralized extracellular matrix. CH sealers release OH^- and Ca^{2+} ions, mainly in suspensions, and evoke an increase of pH when in distilled water (48h after setting). When CH sealers are used together with lateral condensation of gutta-percha, the outer dentine surface does not become alkaline, contrarily to CH suspensions, due to the fact that no OH^- ions are available for diffusion through dentine [51].

Mechanical properties are inferior compared with the other sealers, perhaps due to the desired release of OH^- ions that causes degradation of the sealer, enhancing leakage. After long-term studies, they showed significant volumetric expansion, disintegration and high solubility. Some sealers dissolve at a relatively high rate, especially when used in a thick layer [52], and the bond to dentine is weak [53]. Some *in vitro* studies showing less leakage for a CH sealer than for an epoxy and a ZnOE sealer could not be confirmed *in vivo*. Coronal leakage for bacteria after up to 90 days of exposure proved to be diminished for a CH sealer than for a ZnOE sealer, when used with the lateral condensation technique [54]. After 2 years of storage, leakage increased [34].

As for the biological properties, there are no reports available about systemic-toxic or allergic effects. When tested in different cell culture systems, their cytotoxicity was generally low compared with other sealers [55] and when in a more complex cell culture system able to demonstrate both cytotoxicity and the influence on immunocompetent cells it was nearly innocuous [37]. A salicylate-based CH sealer was non-mutagenic in an *in vitro* bacterial test system [44], however induced a fast and complete inhibition of nerve conductance when in direct contact with the tissue, which after 30min was irreversibly blocked by the material [56]. After implantation in laboratory animals, CH sealers initially caused a severe reaction that diminished after several months and was finally lower than a ZnOE sealer [38]. A CH sealer evoked the most extensive apical hard-tissue formation, whereas a pure CH preparation induced less hard tissue and gutta-percha reported minimal effect [57]. Antimicrobial properties were shown *in vitro* and were found to possibly increase with time, due to partial disintegration of the sealer. The mechanism was related to the high pH, and the buffer capacity of body fluid will reduce the effect in time. Comparing with ZnOE sealers, CH sealers exhibited weaker antimicrobial effect regardless of the microorganisms tested [50], as they did not disinfect the dentinal tubules infected with *Enterococcus faecalis*, which is in line with the frequently isolation of Enterococci from persistent infections.

CH sealers have the property of 'root-end closure' which is the induction of calcified tissue formation to obturate the dental apical foramen, a process that is not yet elucidated. It is apparently related to the high pH and the release of calcium ions from the material, which promotes a state of alkalinity of the adjacent tissues, arresting root resorption and favouring repair, with the inhibition of osteoclastic activity. Ca^{2+} acts on the process of cell differentiation

and on macrophage activation. Acidic products produced by osteoclasts are neutralized and calcium phosphate complexes are formed. It was suggested that activation of ATP, which accelerates bone and dentine mineralization, and the induction of TGF-ϐ (transforming growth factor ϐ) signalling pathway, play a central role in the attained biomineralization [58].

Handling properties of CH sealers are adequate, radiopacity is considered sufficient and the material can be easily removed with common rotary instruments from the root canal.

Some examples of commercial CH-based sealers, observed in Figure 10, include CRCS (Coltène Whaledent), Apexit (Ivoclar Vivadent, Schaan, Liechtenstein), Sealapex (Kerr, SybronEndo, Orange, CA, USA), Dycal (Dentsply, Milford, DE, USA), Calcicur (Voco GmbH, Cuxhaven, Germany) and Calcimol LC (Voco GmbH, Cuxhaven, Germany).

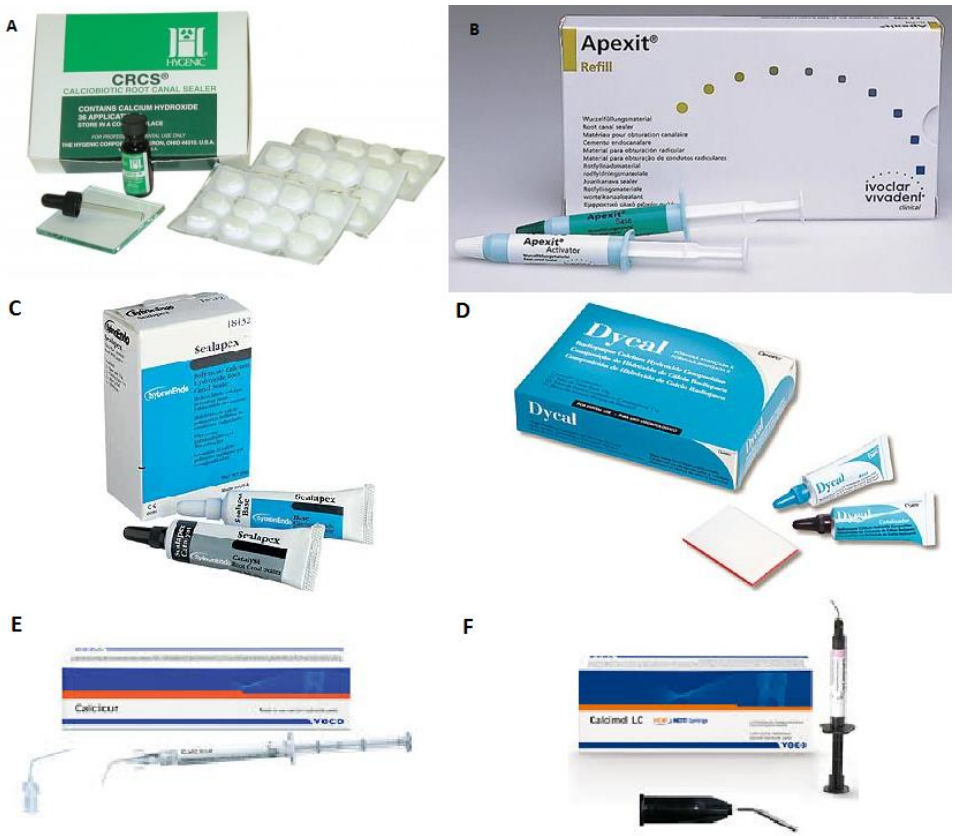


Figure 10- Examples of CH-based sealers: (A) CRCS; (B) Apexit; (C) Sealapex; (D) Dycal; (E) Calcicur; (F) Calcimol LC.

1.4.3.6- Calcium silicate (CS)-based sealers

Calcium silicate-based filling materials have been the most recent developed sealers. The original formulation, mineral trioxide aggregate (MTA), was developed in 1993 by Dr. Mahmoud Torabinejad and approved by the U.S. Food and Drug Administration for clinical use in 1998 [59]. These were designed to obtain new endodontic sealers and root-end filling materials with adequate workability and consistency, being used in many other clinical applications which include pulp capping, furcal repair, root resorption and apexification (Figure 11) [60].

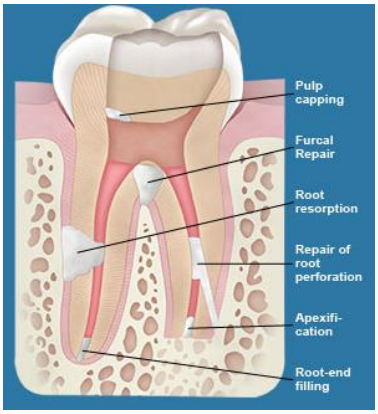


Figure 11- Clinical applications of MTA

1.4.3.6.1- Chemical properties

MTA was formulated from commercial Portland cement (PC) combined with bismuth oxide powder for radiopacity and consists of a powder formed by fine hydrophilic particles that sets in the presence of moisture (several liquids have been used to hydrate MTA powder). The MTA patent [61] shows that it contains calcium oxide (CaO) and silicon (SiO), and several studies reported that its main components are calcium, silica, as well as bismuth oxide [62].

MTA is currently marketed in two forms: it was introduced as Grey-MTA (GMTA), but because of the discoloration potential, White-MTA (WMTA) was developed, where lower amounts of iron, aluminium and magnesium are present [63] (detailed composition can be seen in Table 1). The actual composition of GMTA is 75% PC, 5% calcium and 20% bismuth oxide and AMTA is composed of 80% PC and 20% bismuth oxide [64].

Table 1- Electron probe microanalysis results of GMTA and WMTA [63]

Chemical	GMTA (%wt)	WMTA (%wt)
CaO	40.45	44.23
SiO_2	17.00	21.20
Bi_2O_3	15.90	16.13
Al_2O_3	4.26	1.92
MgO	3.10	1.35
SO_3	0.51	0.53
Cl	0.43	0.43
FeO	4.39	0.40
P_2O_5	0.18	0.21
TiO_2	0.06	0.11
H_2O+CO_2	13.72	14.49

The primary differences between MTA and PC are a lack of potassium and the presence of bismuth oxide [60]. An investigation evaluated the dry powder of GMTA and WMTA, as well as ordinary PC (OPC) and white PC (WPC), finding that all tested materials have similar major constituents: tricalcium silicate, tricalcium aluminate, calcium silicate, and tetracalcium aluminoferrite [65]. GMTA basically consists of dicalcium and tricalcium silicate and bismuth oxide, whereas WMTA is primarily composed of tricalcium silicate and bismuth oxide [62].

When MTA powder is mixed with water, calcium hydroxide and calcium silicate hydrate are initially formed and eventually transform into a poorly crystallized and porous solid gel; calcium precipitate is formed and is affected by bismuth after MTA hydration [66]. Bismuth oxide in MTA provides its radiopacity and is present in both hydrated and nonhydrated MTA and is also a part of calcium silicate hydrate [66].

1.4.3.6.2- Physical properties

Hydration of MTA powder results in a colloidal gel that solidifies into a hard structure. Characteristics of the mixture can be influenced by the powder/liquid ratio, method of mixing (amount of entrapped air), pressure used for condensation, humidity of the environment, type of MTA, type of storage media, pH value of the environment, type of vehicle, length of time between mixing and evaluation, thickness of the material, and temperature [66]. Some of these factors cannot be controlled easily; therefore, different results might be obtained during a study on physical properties of MTA [67].

MTA is prepared by mixing its powder with sterile water in a 3:1 powder-to-liquid ratio and its mean setting time is 165 ± 5 minutes, which is longer than other materials [61]. MTA's long setting time is one of the major drawbacks of the material.

Most investigations reported low or no solubility for MTA [68] and different results are caused by different types of PC or MTA [69], powder-to-liquid ratios as higher liquid-to-powder ratios increased porosity and solubility [67] and addition of bismuth oxide as a cause for MTA insolubility [66].

Compressive strength [61], flexural strength and push-out strength [70] of MTA are significantly reduced, in comparison to other materials after 24 hours, however, after 3 weeks, there is no significant difference. Available data suggests that MTA gains optimal physical properties when it receives enough moisture after being placed in an operation site.

The pH value of MTA is 10.2 after mixing, which rises to 12.5 at 3 hours [61]. In a long-term study, MTA maintained its high pH value, perhaps due to the constant release of calcium and the formation of CH (which results from mixing MTA with water). The mean radiopacity of MTA has been reported at 7.17 mm of an equivalent thickness of aluminium [61].

The physical properties of cement might be influenced by crystal size, as smaller particles increase surface contact with the mixing liquid and lead to greater early strength as well as ease of handling. The amount of porosity in mixed cement is related to the amount of water added to make a cement paste, entrapment of air bubbles during the mixing procedure, or the environmental pH value [71]. Microhardness can be adversely affected by less humidity, low pH values [72], the presence of a chelating agent [60] and more condensation pressure [73].

The handling of MTA has been viewed as one of its shortcomings [74]. Various carriers have been used to enhance the ease of handling, including Teflon sleeves and pluggers specially designed for placement of MTA, specially designed carriers for dispersing MTA, and

scooping out MTA from grooves [75]. Current data show that the method of MTA insertion has great impact on its physical properties and using more condensation pressure does not necessarily improve these properties [73].

1.4.3.6.3- Antibacterial and antifungal properties

The persistence of microorganisms in dentinal tubules, lateral canals and apical ramifications after root canal treatment has been reported [76][77]. If the filling provides a good seal, it will only impair the exit of bacteria entrapped in the root canal system. However, to eradicate the remaining microorganisms, the antimicrobial activity could play an important role [78].

The agar diffusion test (ADT) is used extensively to assess the antimicrobial effect of endodontic sealers, despite its well-known limitations. Its results are influenced by the solubility and diffusibility of the material in the culture medium and the fact that this test cannot distinguish between bactericide and bacteriostatic properties of the material [79]. On the other hand, the direct contact test (DCT) does not have these disadvantages and it can be used to assess the antimicrobial effect of water-insoluble materials, providing quantitative and reproducible results [80].

Several investigations reported that MTA has limited antimicrobial effect against some microorganisms [81]. An investigation on facultative and strict anaerobic bacteria showed that MTA has an antibacterial effect on some facultative bacteria and no effect on any species of strict anaerobes [82]. However, Super EBA and ZOE pastes exhibited some antibacterial effect on both types of tested bacteria [82]. In an antimicrobial study on MTA and PC against *Staphylococcus aureus*, *Enterococcus faecalis*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Candida albicans*, and a mixture of these bacterial and fungal species, both materials exhibited diffusion in agar without inhibition of microbial growth [81]. Some investigations showed that GMTA [83] and WMTA [84] have an antifungal effect and, in contrast, others showed that GMTA has limited or no antifungal effect [81].

Conflicting results from investigations on MTA might be attributed to the various tested species of the microorganisms, the source of the preparing material, as well as the concentration and the type of MTA used in these studies [85]. Furthermore, the alteration of the powder-to-liquid ratio might significantly affect the antibacterial and antifungal properties of MTA [86][87].

1.4.3.6.4- Portland cement

PC is an inexpensive material and because of its chemical similarity to MTA [65] some investigations suggested PC as a substitute material for MTA [62]. As previously said, a number of investigations showed a similar composition between PC and MTA, except for the bismuth oxide [64][88] and it was demonstrated that both are composed of tricalcium and dicalcium silicate, which on hydration, produce calcium silicate hydrate gel and CH [89].

However, there are many differences between the materials in terms of setting expansion, chemical composition, surface chemical composition, porosity, compressive strength, radiopacity, cation releases, and particle sizes [90]. A recent study investigated the effect of adding bismuth oxide on PC physical properties [91], reporting an influence on

porosity and compressive strength, an increase in porosity, solubility, and degradation of the material with increasing amounts of bismuth oxide, and an increase in the amount of cracks in the set material because of the presence of more flaws in the composition. An investigation confirmed the similarity of PC and MTA, except for the presence of potassium and lack of bismuth oxide [60].

The biocompatibility of some types of PCs is evaluated in several studies: three separate studies using endothelial, L929 fibroblast, and human osteosarcoma cell cultures compared PC and MTA [92][93][94] and revealed no significant differences between the tested materials. MTA, unmodified PC, and modified PC samples with added 10% and 15% CC for acceleration of setting time show the same effect on SAOS2 osteosarcoma cell line [95].

However, a study comparing MTA and PC containing varying ratios of bismuth oxide on immortalized human periodontal ligament cells showed that PC without the addition of bismuth oxide, shown a similar cell viability as MTA at 12 and 24 hours. The addition of bismuth oxide to PC powder at all ratios significantly lowered cell viability during early evaluation time [96]. Other studies observed lower particle size in WMTA and a large area of CH in WPC, and did not observed in WMTA, as assessed by SEM observation [88]. Further, lower amounts of the aluminate phase in WMTA [66], and an efficacy of PC for pulp capping comparable to that of MTA were reported [97]. A recent review concluded that both MTA and PC exhibit no genotoxicity [98].

The reasons that PC cannot substitute MTA in clinical applications are [99]:

- PC is manufactured widely all around the world, and it is impossible to control the quality, composition, and biocompatibility of all its components.
- PC is composed of an amount of lead and arsenic which are known to be toxic [70] and WMTA contains fewer heavy metals such as copper, manganese, and strontium. A recent study showed that OPC contains more than 6 times the amount of arsenic compared with GMTA [100]. It is impractical to evaluate the amount of these elements in all types of PC but because of the release of toxic elements into the surrounding tissues [69], its long-term safety is questioned.
- The higher solubility of some types of PC is concerning, as it is possible that PC might degrade after clinical application and jeopardize the seal of the material [69].
- The compressive strength of OPC and WPC to be stable against occlusal pressure for repairing perforations and pulp capping is significantly lower than WMTA and GMTA 28 days after hydration [90].
- Excessive expansion that might result in a cracked root is an undesirable property when a material is used as a root-end filling substance [90]. The setting expansion of PC is a matter of controversy in the literature which might be attributed to the differences of chemical composition among various types of PC [101].
- Carbonation, important for perforation repair or pulp capping, occurs when the amount of carbon dioxide increases in inflamed tissues, forming calcium hydrogen carbonate [101] and resulting in a drop in tensile strength and resiliency of PC which might cause the material to crack and buckle under high stress, instead of deforming.

- The amount of calcium release in WMTA is reported to be much more than that released by WPC, and the mechanism of hydration is different in these materials [102]. It is important to note that complete healing occurs only when the proper amounts of elements and signal molecules are present in tissues after injury and a lack of an element or an imbalance between elements might prevent complete regeneration.
- MTA is manufactured in laboratories as a medical material under close supervision in terms of its composition and prevention of contamination [101] and is approved by the U.S. Food and Drug Administration for use in human beings [60].

A recent review article highlighted the need for more investigations on PC as a medical material. The authors explained their concern regarding the exact type of PC, which is not mentioned in some investigations. They believe that if PC is intended for use in clinical applications, the material needs to be sterile, the amount of toxic heavy metal ions of the material should be detected, and the material particle size should be similar and homogenous. Despite some similarities between PC and MTA, it is not safe to use PC, which has not been formulated for human use, in place of a bioactive medical material such as MTA [101].

1.5- Types of calcium silicate-based sealers

Because calcium silicate cements set in the presence of moisture, such as blood and other fluids with a great clinical advantage, it appeared interesting to develop endodontic sealers based on calcium silicate hydraulic cements.

Different MTA-based root canal sealers are:

- **ProRoot MTA** (Dentsply; Tulsa Dental Products, Tulsa, OK, USA);
- **MTA Plus** (compounded by Prevest Denpro, Jammu, India for Avalon Biomed Inc. Bradenton, FL, USA): mixture of finer particle size powder composed of tricalcium silicate, dicalcium silicate and bismuth oxide with an anti-washout gel (replacing the mixing water) that reduced the setting time of the cement and enhanced the compressive strength.
- **MTA Fillapex** (Angelus, Angelus Odontológica, Londrina, PR, Brazil);
- **MTA Angelus** (Angelus, Angelus Odontológica, Londrina, PR, Brazil);
- **MTA Obtura** (Angelus, Angelus Odontológica, Londrina, PR, Brazil): mixture of white MTA with a proprietary liquid resin.
- **CPM Sealer** (EFEO SRL, MTM Argentina SA, Buenos Aires, Argentina): developed in 2004. The powder is composed of tricalcium silicate, tricalcium oxide, tricalcium aluminate and other oxides and the liquid consists of saline solution and calcium chloride. Presented as a modified WPC-based material, its most significant difference is the presence of large amount of calcium carbonate (reduces pH from 12.5 to 10 after setting), which intends to increase the release of calcium ions and offer good sealing properties, adhesion to dentinal walls adequate flow rate, and biocompatibility.
- **Theracal** (Bisco Inc., Schaumburg, IL, USA): new light-cured, resin-modified, calcium silicate-filled base/liner material designed with direct and indirect pulp-capping. It contains polymerizable methacrylate monomers, Portland cement type III, polyethylene glycol dimethacrylate, and barium zirconate.
- **MTAS experimental sealer MTAS** (an association between 80% of WPC and 20% bismuth oxide) with an addition of water soluble polymers).

Other types of bioceramic calcium silicate-based sealers not MTA related include:

- **Quick-Set** (Primus Consulting, Bradenton, FL): is a calcium aluminosilicate cement originated from Capasio powder after refinal and removal of the cationic surfactant. Capasio is composed primarily of calcium aluminosilicate powder, dental glass, and bismuth oxide as a radiopacifier and is supplied with a water-based gel.
- **BioAggregate** (Innovative BioCeramix, Vancouver, BC, Canada): marketed after Food and Drug Administration approval in 2006, it promotes a complicated set of reactions upon mixing with BioA Liquid (deionized water), which leads to the formation of a nano-composite network of gel-like calcium silicate hydrate intimately mixed with hydroxyapatite bioceramic, and forms a hermetic seal when applied inside the root canal. It has excellent handling characteristics after mixing with water, which aids in the repair process of the affected tooth. It has good radiopacity properties, convenient setting and hardening time, easy workability and good handling properties.
- **Biodentine** (Septodont, Saint Maur de Fossés, France): marketed after FDA approval in 2009, it is an alternative to BioAggregate, being aluminium-free (non-tricalcium

aluminate) and composed by zirconia (ZrO2) instead of bismuth trioxide for radiopacity. It is pure tricalcium silicate and dicalcium silicate with calcium carbonate by 20% and high concentrated calcium chloride solution as hydration accelerant.

- **iRoot SP** (Innovative BioCeramix, Vancouver, BC, Canada): it is also available as EndoSequence BC Sealer (Brasseler USA, Savannah, Georgia, USA) since 2009. It is a convenient premixed ready-to-use injectable white hydraulic cement paste developed for permanent root canal filling and sealing applications. It is user-friendly, highly biocompatible, non-toxic, aluminium-free, radiopaque (by tantalum pentoxide and zirconia or any other radiopacifying agents except bismuth trioxide), anti-bacterial, hydrophilic and provides outstanding sealing properties. Unlike conventional sealers on the market, it uses the moisture naturally present in dentinal tubules to initiate its setting reaction. This highly radiopaque and hydrophilic sealer forms hydroxyapatite upon setting, and readily forms chemical bonds to both dentin and bioceramic filling material points.

Since the following formulations will be examined in this experimental work the composition and properties of these materials are detailed below

1.5.1- ProRoot MTA

Figure 12- ProRoot® MTA (Dentsply; Tulsa Dental Products, Tulsa, OK, USA).

ProRoot MTA (Figure 12) was initially commercialized in 1998, in cooperation with and based on prior work by Dr. Mahmoud Torabinejad. Unlike commercial PC, ProRoot MTA is manufactured under FDA guidelines for medical devices outlined in the ISO 9001 specifications and the strict European Medical Device Regulation to ensure the quality, purity and efficacy for use as a dental material. The composition is certified for purity and the absence of heavy metal contamination. It is composed mainly of 53,1% tricalcium silicate, 22,5% dicalcium silicate, 21,6% bismuth oxide, and small proportions of tricalcium aluminate and calcium sulfate [102]. The bismuth oxide is added to enhance the radiopacity of the material [103].

In response to customer requests, White ProRoot MTA (WMTA), introduced in 2002 and patented in 2008, was also developed for reducing discoloration to the tooth structure (eliminating tetracalcium aluminoferrite as iron's main discoloration induced element). It contains significantly lesser amount of aluminium oxide (Al_2O_3), magnesium oxide (MgO) and ferric oxide (Fe_2O_3) than Grey ProRoot MTA (GMTA).

1.5.2- MTA Plus

Figure 13- MTA Plus™ (Prevest Denpro, Jammu, India).

MTA Plus (Figure 13) is a calcium silicate cement with a finer particle size based on tricalcium silicate, dicalcium silicate and bismuth oxide, derived from advanced material research in inorganic hydraulic powder technology. MTA Plus is provided with either water or an anti-washout gel that enhances handling and application as well as improved resistance [104]. An ideal anti-washout agent should (i) inhibit the decay of cement paste in liquid; (ii) not interfere with the hydration reaction or significantly reduce the bioactivity of the cement; (iii) not decrease the mechanical strength of the cement once set; (iv) not worsen the handling properties of the cement paste; (v) not significantly extend the setting time; and (vi) not reduce the radiopacity [105].

Because of its variable stoichiometric properties [106], the powder/gel ratio of MTA Plus may be adjusted to enable more diverse applications, ranging from perforation repair, root-end filling, direct pulp capping (thicker consistency) to sealing of the cleaned-and-shaped root canal space (thinner consistency).

1.5.3- MTA Fillapex

Figure 14- MTA Fillapex® (Angelus, Londrina, PR, Brazil).

MTA Fillapex (Figure 14) is composed of resins (salicylate, diluting, natural), radiopaque bismuth oxide, silica nanoparticles, mineral trioxide aggregate (MTA) and pigments. According to the manufacturers, it requires moisture that originates from dentinal tubules or periapical

tissues in order to set and harden. It has the following physical properties: working time, 35 min, flow capacity, 27.66 mm, setting time, 130 min, optical density, 77%, and solubility, 0.1%.

MTA Fillapex has easy handling and manipulation and it was created in an attempt to combine the physicochemical properties of a root canal sealer with the biological properties of MTA. Recent studies showed that this sealer has suitable physicochemical properties to be used in endodontic therapy, such as excellent radiopacity, flow, and alkaline pH [107] [108].

1.5.4- Biodentine

Figure 15- Biodentine™ (Septodont, Saint Maur de Fossés, France).

Biodentine (Figure 15) consists of a powder and liquid. The powder mainly contains tricalcium and dicalcium silicate (3CaO SiO_2 and 2CaO SiO_2), the principal component of Portland cement, as well as calcium carbonate (CaCO3). Zirconium dioxide (ZrO_2) serves as contrast medium. The liquid consists of calcium chloride ($CaCl_2 \cdot 2H_2O$), which is used as a setting accelerator and water-reducing agent in aqueous solution with an admixture of polycarboxylate (a superplasticizing agent).

The consistency of Biodentine is similar to that of phosphate cement. The material can be applied directly in the restorative cavity with a spatula as a bulk dentin substitute without any conditioning treatment [109].

Biodentine is a new calcium silicate–based restorative cement with dentin-like mechanical properties, which can be used as a dentin substitute on crowns and roots similar to how MTA is used [110]. Some main benefits for Biodentine over other products are the reduced setting time (several minutes compared with several hours for MTA), better mechanical properties and sealing ability confirmed *in vitro* with a very low silver nitrate penetration. This agent is characterized by the release of calcium hydroxide in solution, which when in contact with tissue fluid forms hydroxyapatite [102]. It offers the advantages for direct pulp-capping and, in properly selected cases, may contribute to the long-term maintenance of tooth vitality [111].

II: RESEARCH HYPOTHESIS AND OBJECTIVES

Root canal sealers are intended to be contained within the root canal space, but they may extrude through the apical constriction, or eluents from the sealers may come into contact with periradicular tissues. This might cause irritation and delay in wound healing. Moreover, the contact of root canal filling materials with periapical tissues can also affect the normal bone metabolism and regeneration. Regarding this, it is known that angiogenesis is critical in the bone environment for the normal bone remodeling and regenerative events. Accordingly, biological evaluation of endodontic sealers benefit from the biocompatibility profile concerning bone formation and angiogenesis.

In this context, the aim of this study was to compare four calcium silicate-based sealers, namely ProRoot MTA, MTA Plus, MTA Fillapex and Biodentine, regarding the effects of the sealers' extracts on:

- osteoblastic cell behaviour, by using (i) human bone-marrow derived mesenchymal stem cell cultures and (ii) an *ex vivo* osteogenic assay (regeneration of critical size defects created on the parietal bone of neonatal mice), and
- endothelial cell behaviour, by using (i) human endothelial cell cultures and (ii) an in vivo angiogenesis assay (CAM assay).

With this approach, it is expected to have information on the biocompatibility of these calcium silicate-based sealers regarding bone formation and angiogenesis, two key events in maintaining normal bone remodelling and regenerative ability.

III: MATERIALS AND METHODS

1- Materials

All cell culture chemicals and supplies were acquired from Merck and Sigma Aldrich (St. Louis, MO) unless otherwise noted. All tissue culture flasks and plates were obtained from Corning (Corning, NY). Calcium silicate-based root canal sealers were acquired from their manufacturers (Table 2).

Table 2- Manufacturers and composition of calcium silicate-based root canal sealers.

Group	Manufacturers	Composition
ProRoot® MTA	Dentsply Tulsa Dental, Tulsa, OK, USA	**Powder:** tricalcium and dicalcium silicate, tricalcium aluminate, tetracalcium aluminoferrite, free calcium oxide, bismuth oxide; **Liquid:** distilled water
MTA Plus™	Prevest Denpro Limited, Jammu city, India	**Powder:** tricalcium and dicalcium silicate, bismuth oxide, calcium sulfate, and silica; **Liquid:** distilled water *instead it can be mixed with an anti-washout gel
MTA Fillapex®	Angelus, Londrina, PR, Brazil	salicylate resin, natural resin, diluting resin, bismuth oxide, nanoparticulated silica, MTA and pigments.
Biodentine™	Septodont, Saint-Maur-des-Fosses, France	**Powder:** tricalcium and dicalcium silicate, calcium carbonate and zirconium oxide **Liquid:** water, calcium chloride (setting accelerator), modified polycarboxylate (superplasticising agent)

2- Preparation of the sealers' extracts

ProRoot MTA and MTA Plus, composed of white Portland cement and bismuth oxide, were prepared according to the manufacturers' instructions. MTA Fillapex, distributed in a base paste and a catalyst paste, was also mixed accordingly to the instructions.

Biodentine consists of a powder in a capsule and liquid in a pipette, which were mixed with an Amalgam carrier for 30 seconds. Once mixed, Biodentine sets in about 12 minutes, during which calcium hydroxide was formed.

Table 3- Sealers' preparation characteristics.

RCS	Components	Instruments	Working time	Setting time
ProRoot® MTA	Powder + Water	Spatula	5 min	120-240 min
MTA Plus™	Powder + Water/Gel	Spatula	12 min	60 min
MTA Fillapex®	Base paste + Catalyst paste	Spatula	30 min	120 min
Biodentine™	Powder + Water	Amalgam carrier	6 min	10-12 min

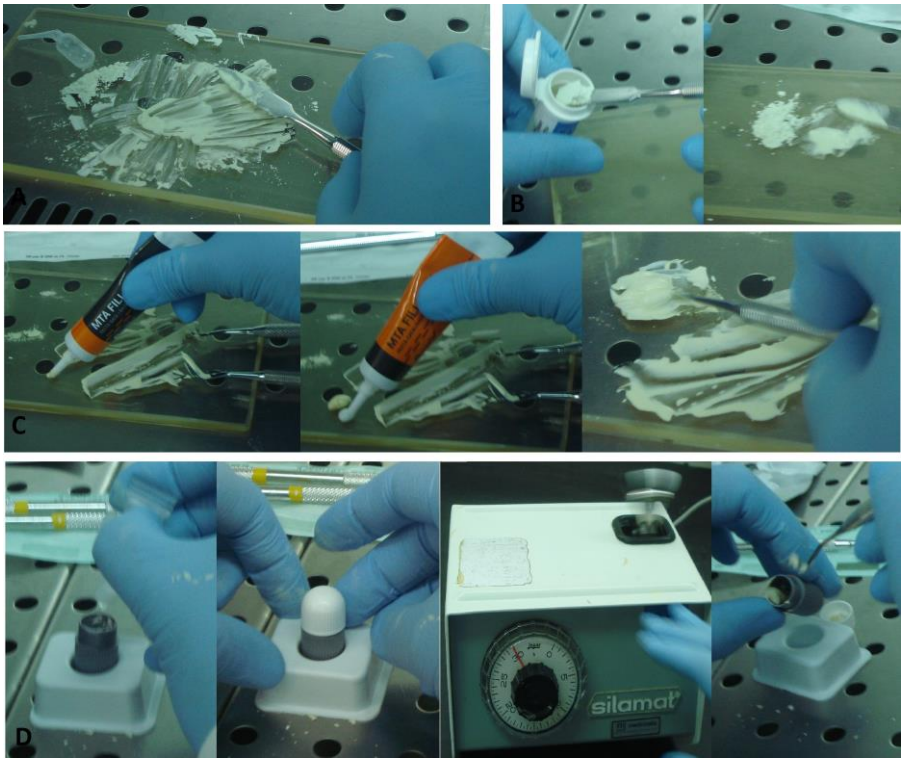


Figure 16- Sealers' preparation method: (A) ProRoot MTA; (B) MTA Plus; (C) MTA Fillapex; (D) Biodentine.

After mixed (Figure 16), the sealers were placed at the bottom of a 24-well plate at approximately 0.1g/well. 2 mL of Eagle's minimal essential medium alpha (α-MEM; Invitrogen) were added to each well (Figure 17) and the plate was incubated for 24h at 37 °C in an air atmosphere containing 5% CO_2 and 95% relative humidity to prepare the cement extract.

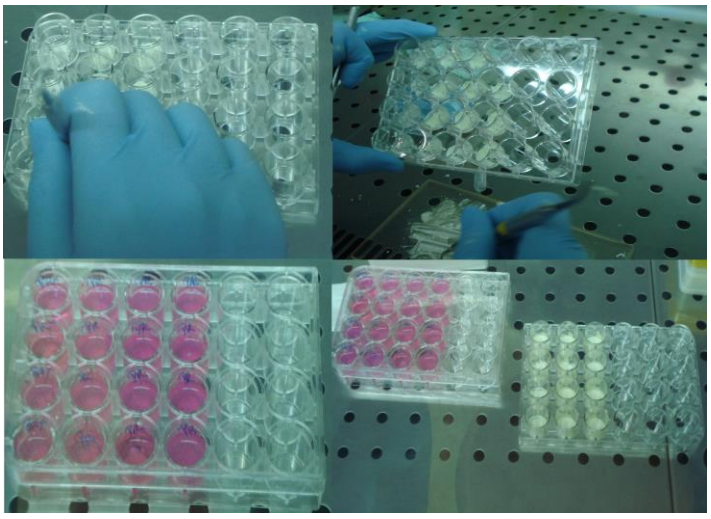


Figure 17- Placement of the sealers over the wells surface with the addition of α-MEM.

The α-MEM containing extract was collected for further use. After, the extracts were diluted in α-MEM: 1:2, 1:5, 1:10 and 1:20.

3- Cytotoxicity evaluation of the sealers' extracts

3.1- Cell culture

L929 mouse fibroblasts (L929; ATCC Cat. No. CCL-1) were cultured in 90mm culture plates and were maintained in standard culture conditions, i.e. in Eagle's minimal essential medium alpha (α-MEM; Invitrogen) supplemented with 10% fetal bovine serum (FBS; Gibco/BRL), 2.5µg/mL fungizone (Fungi), 100 IU/mL penicillin-2.5µg/mL streptomycin (Pest) and 50 µg/mL ascorbic acid (AA). Cultures were incubated in a humidified atmosphere of 5% CO_2 in air at 37°C, and culture medium was changed twice a week. Adherent cells at a logarithmic growth phase were detached by trypsinization (0.04% trypsin in 0.25% EDTA). Cellular viability was assessed by the exclusion method of blue trypan and cells were counted in a Neubauer Improved Chamber.

L929 mouse fibroblasts were seeded at 5 x 10^3 cell/cm^2 in 96-well plates, and were cultured in the experimental conditions described above for 2 days. Subsequently, cultures were exposed to the sealers' extracts (1:20 – 1:2 dilutions) during 7 days. Untreated cells were used as control and three wells were used for each extract and for the control.

Cell cultures' monitoring was conducted periodically using phase contrast optical microscopy. Cultures were maintained during 7 days and characterized for cell proliferation.

3.2- Cell proliferation assay (DNA content)

Cell proliferation was estimated by the DNA content at specific time points (days 1, 3 and 7). DNA content was analysed by the PicoGreen DNA quantification assay (Quant-iT™ PicoGreen® dsDNA Assay Kit, Molecular Probes Inc., Eugene), according to manufacturer's instructions. PicoGreen® is an ultrasensitive fluorescent nucleic acid stain that binds double-stranded DNA and forms a highly luminescent complex when compared to the free dye in solution [112].

Cultures were treated with Triton X-100 (0.1%) (Sigma) and fluorescence with a maximum excitation of 480 nm and an emission peak of 520 nm was measured and corrected for fluorescence of reagent blanks on an ELISA reader (Synergy HT, Biotek). The amount of DNA was calculated by extrapolating a standard curve obtained by running the assay with the given DNA standard. Results are expressed as ng/mL.

4- Effect of the sealers' extracts on Osteoblastic cell behaviour

4.1- Human mesenchymal stem cells

4.1.1- Cell culture

Human bone marrow-derived mesenchymal stem cells (hMSCs; Lonza, Wakersville, MD, USA) were cultured in 90mm culture plates and were maintained in standard culture conditions, i.e. in α-MEM supplemented with 10% FBS, 2.5µg/mL Fungi, 100 IU/mlLpenicillin-2.5µg/mL streptomycin and 50 µg/mL AA. Cultures were incubated in a humidified atmosphere of 5% CO_2 in air at 37°C, and culture medium was changed twice a week.

Adherent cells at a logarithmic growth phase were detached by trypsinization (0.04% trypsin in 0.25% EDTA). Cellular viability was assessed by the exclusion method of blue trypan and cells were counted in a Neubauer Improved Chamber.

hMSCs were seeded at 10^4 cell/cm^2 in 96-well plates, and were cultured in the experimental conditions described above for 2 days. Subsequently, cell cultures were exposed to the sealers extracts (1:20 – 1:2 dilutions). Cultures were maintained for 21 days, and the medium with extract was renewed twice a week. Untreated cells were used as control and three wells were used for each extract and for the negative control.

Cell culture monitoring was conducted periodically using phase contrast optical microscopy. Cultures were maintained during 7 days characterized for cell proliferation at days 1, 7, 14 and 21 after the addition of the sealer's extracts, for cell proliferation, F-actin cytoskeleton, total protein content, alkaline phosphatase activity and staining and collagen staining and quantification.

4.1.2- Characterization of the cell response

4.1.2.1- Cell proliferation assay (DNA content)

Cell proliferation was estimated by the DNA content at specific time points (days 1, 7, 14 and 21). Cultures were treated with Triton X-100 (0.1%) and fluorescence with a maximum excitation of 480 nm and an emission peak of 520 nm was measured and corrected for fluorescence of reagent blanks on an ELISA reader (Synergy HT, Biotek). The amount of DNA was calculated by extrapolating a standard curve obtained by running the assay with the given DNA standard. Results are expressed as ng/mL.

4.1.2.2- F-Actin cytoskeleton and cell morphology

Cell cultures were observed by confocal laser scanning microscopy (CLSM) following staining of the F-actin cytoskeleton and nucleus counterstaining. For CLSM assessment, cell cultures were fixed in 3.7% paraformaldehyde during 15 minutes and permeabilized with Triton X-100 (0.1%). Then, cells were incubated with albumin (10 mg/ml) to reduce non-specific staining. Cell cytoskeleton filamentous actin (F-actin) was visualized treating the cells with Alexa Fluor 488®-conjugated phalloidin (1:20 dilution in PBS for 20 minutes) and counterstained with propidium iodide (1 µg/mL for 10 min) for cell nuclei labelling. Labelled cultures were mounted in Vectashield® and examined with a Leica SP2 AOBS (Leica Microsystems) microscopy.

4.1.2.3- Total Protein Content

The total protein content was determined using the Lowry method [113][114]. This is based on the reaction of Folin-Ciocalteau reagent with aromatic amino acids, resulting in the formation of a colored product, which is measured by spectrophotometry. After removing the culture medium, cells were washed twice with PBS buffer ("Dulbeco's Phosphate Buffered Saline," Sigma D-1480), pH 7.4. Triton X-100 (0.1%) was added to each well and plates were placed in an incubator at 37°C for one hour, to lyse the cell layer. After, samples were incubated with an alkaline copper solution (10 min) prior to the addition of the phenol reagent, and the mixture was placed in the dark for one hour. Once this time, absorbance was read in a spectrophotometer (ELISA reader, Synergy HT, Biotek) at 750nm. Absorbance was compared with values obtained for a series of standards of bovine serum albumin, made from a solution of 0.5 mg/mL in NaOH 0.1 M.

4.1.2.4- Alkaline phosphatase activity

Alkaline phosphatase (ALP) is a metalloenzyme present in various tissues, including bone where it is produced by osteoblasts and serves as an indicator of their activity, being considered a marker of earlier osteogenic differentiation *in vitro* and a useful parameter for monitoring changes in bone formation [115]. The activity of this enzyme is determined by using a specific substrate, the p-nitrophenylphosphate (pNPP). In a buffered solution, and at alkaline pH, the hydrolysis of pNPP occurs mediated by ALP, resulting in p-Nitrophenol (yellowish compound) and a phosphate group [116]. The amount of p-Nitrophenol formed is determined by the absorbance at a wavelength of 400nm and is proportional to the catalytic concentration of ALP present in sample, according to Beer's law [116].

The culture medium was removed and cells were washed twice with PBS buffer, pH 7.4. Triton X-100 (0.1%) was added to each well to induce cell lysis. Thereafter, the buffer substrate (p-NPP 25 mM) was added to the samples and the plate was incubated for one hour at 37°C with 5% CO2 in the air. NaOH 5M was added to stop the reaction and the absorbance was measured in an ELISA plate reader (Synergy HT BioTek) at 400nm. Results were expressed as nanomoles of p-nitrophenol produced per minute per microgram of protein (nmol/min.µg protein) as compared with the respective calibration curve.

4.1.2.5- Alkaline phosphatase staining

The culture medium was removed and cell cultures were washed twice with PBS. Cells were fixed with glutaraldehyde at 1.5% during ten minutes. Thereafter, sodium cacodylate buffer 0.14 M was added to preserve the cells at 4°C, until being stained.

Cell cultures were stained to identify the presence of ALP to assess the osteogenic differentiation of the hMSCs. The method was based on the hydrolysis of sodium naphthyl phosphate by ALP, and precipitation of phosphate liberated by reaction with a diazonium salt (Fast Blue RR). Hence, this reaction originates a colored product that, depending on amount of enzyme, may be yellow, brown or black. Fixed cultures were incubated in the dark for one hour with 2 mg/mL of sodium naphthyl phosphate and 2 mg/mL of Fast Blue RR in a Tris buffer solution 0.1 M, pH 10. After the incubation, samples were washed with distilled water and allowed to dry under ambient conditions. Stained cultures were photographed in a Nikon TMS Inverted Phase Contrast microscope with magnifications of 40x and 100x.

4.1.2.6- Collagen staining and quantification

The culture medium was removed and cell cultures were washed twice with PBS. Cells were fixed with glutaraldehyde at 1.5% during ten minutes. Thereafter, sodium cacodylate buffer 0.14 M was added to preserve the cells at 4°C, until being stained.

Collagen assay is based on the binding of the dye Sirius red F3BA (BDH, UK) – with the triple helical collagen fibril. This dye stains collagen through a reaction of its sulphonic acid groups with basic groups present in the collagen molecule [117].

After removing the buffer, fixed cells were incubated with 0.1% Sirius red F3BA in saturated picric acid at room temperature, during one hour, under mild shaking. Afterward, the dye solution was removed and the stained cell layers were intensively washed with 0.01 N hydrochloric acid to remove all non-bound dye. Stained cultures were photographed in a Nikon TMS Inverted Phase Contrast microscope with magnifications of 40x and 100x.

Collagen quantification was performed on the stained cultures, after the addition of 0.1 N sodium hydroxide to dissolve the Sirius red staining. Absorbance was read in a spectrophotometer (ELISA reader, Synergy HT, Biotek) at a wavelength of 550nm. Results are expressed as absorbance values.

4.2- *Ex vivo* Osteogenic assay

The effect of the sealer's extracts was evaluated in an *ex vivo* osteogenic assay by assessing the regeneration of a critical size bone defect performed in the parietal bone of neonatal mice.

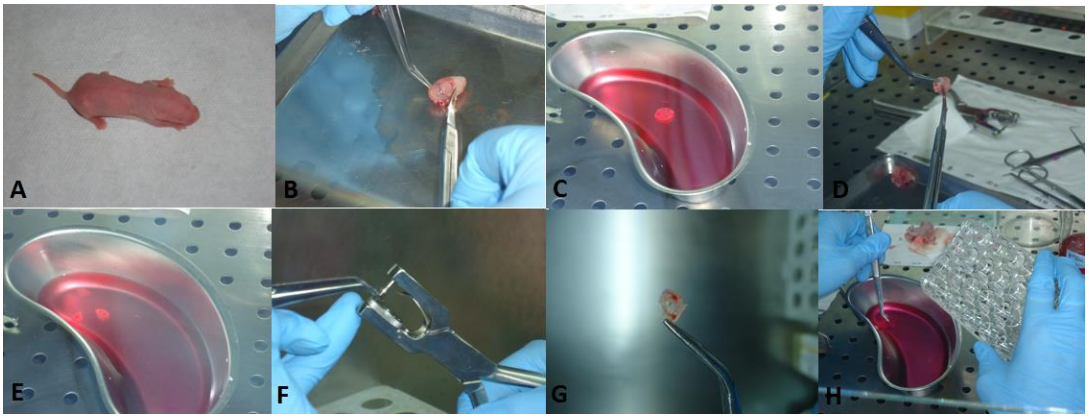


Figure 18- Procedure to obtain the parietal bone of neonatal mice.

The neonatal mice (Figure 18A) were washed and euthanized by decapitation. The heads were washed in ethanol solution and the skin surrounding the parietal bone was carefully removed (Figure 18B). The parietal bone was carefully cut out so that no lesions were created, cut in the middle and washed in α-MEM (Figure 18C-E).

In each half, a full-thickness, 0.8mm in diameter, critical bone defect was created through the parietal bone using a hollow steel tube (Figure 18F). The two halves were washed again and were cultured concave side down in 24-well plates (Figure 18G-H), in α-MEM supplemented with 10% fetal bovine serum (FBS; Gibco/BRL), 2.5µg/mL fungizone (Fungi), 100 IU/mL penicillin-2.5µg/mL streptomycin (Pest) and 50 µg/mL ascorbic acid (AA) and incubated in a humidified atmosphere of 5% CO_2 in air at 37°C for 14 days. Medium was changed on day 2, being replaced with a medium containing two extract dilutions (1:5 and 1:20) of each sealer. The bones were cultured for 14 days, and the medium (containing the sealer extracts) was changed on days 4, 6, 10 and 12.

Cell growth observed within the bone defects and also on the culture plate underneath the bone specimens was monitored by phase contrast microscopy, and alkaline phosphatase and collagen staining.

4.2.1- Alkaline phosphatase staining

On days 7 and 14, the parietal bone specimens were transferred to another 24-well plate, and were fixed and stained for alkaline phosphatase. The cell layer that grew underneath the bone specimens was also stained for alkaline phosphatase at the same time points. Stained samples were photographed in a Nikon TMS Inverted Phase Contrast microscope with magnifications of 40x and 100x.

4.2.2- Collagen staining

On days 7 and 14, the parietal bone specimens were transferred to another 24-well plate, and were fixed and stained for collagen. The cell layer that grew underneath the bone specimens was also stained for collagen at the same time points. Stained samples were photographed in a Nikon TMS Inverted Phase Contrast microscope with magnifications of 40x and 100x.

5- Effect of the sealers' extracts on Endothelial cells and Angiogenesis

5.1- Human endothelial cells

5.1.1- Cell culture

Human umbilical vein endothelial cells (HUVECs; tebu-bio) were cultivated in 90mm culture plates, previously coated with a 0.2% gelatin solution, and were maintained in standard culture conditions, i.e. medium 199 (M199) (Sigma) supplemented with 20% FBS, 2.5µg/mL Fungi, 100 IU/mL penicillin-2.5µg/mL streptomycin and 25 µg/mL of ECGS-25 µg/mL of heparin and incubated in a humidified atmosphere of 5% CO_2 in air at 37°C.

Culture medium was changed twice a week. Adherent cells at a logarithmic growth phase were detached by trypsinization (0.04% trypsin in 0.25% EDTA). Cellular viability was assessed by the exclusion method of blue trypan and cells were counted in a Neubauer Improved Chamber.

HUVECs were seeded at 10^4 cell/cm^2 in 96-well plates and were cultured in the experimental conditions described above. At day 2, cell cultures were submitted to the addition of the sealers' extracts. Untreated cells were used as the negative control and three wells were used for each extract and for the negative control.

5.1.2- Characterization of the cell response

5.1.2.1- Cell proliferation assay (DNA content)

HUVECs were cultured for 2 days before the addition of the extract. Cultures were maintained for an additional 7 days. Cell proliferation was estimated by the DNA content at specific time points - days 1, 4 and 7 - after the addition of the extract. Cultures were treated with Triton X-100 (0.1%) and fluorescence with a maximum excitation of 480 nm and an emission peak of 520 nm was measured and corrected for fluorescence of reagent blanks on an ELISA reader (Synergy HT, Biotek). The amount of DNA was calculated by extrapolating a standard curve obtained by running the assay with the given DNA standard. Results are expressed as ng/mL.

5.1.2.2- Matrigel coating assay

HUVECs were seeded at 10^4 cell/cm^2 in 96-well plates and were cultured in the experimental conditions described above for two days before the addition of the extract. Cells were exposed to two extract concentrations of each sealer (1:20 and 1:5). After 24 h, the culture medium was removed and 50µl/cm^2 of BD Matrigel Matrix was added to each well, and the plate was incubated at 37°C for 30 minutes. After the incubation period, the culture medium was added to each corresponding well, and the plate was incubated again at 37°C. After 18 hours of incubation, cultures were photographed in a Nikon TMS Inverted Phase Contrast microscope with magnifications of 40x and 100x.

Untreated cells were used as the negative control.

5.2- *In vivo* Angiogenesis Assay

The *in vivo* CAM assay was used to address the effect of the sealer´s extracts on angiogenesis. This procedure is described in detail below.

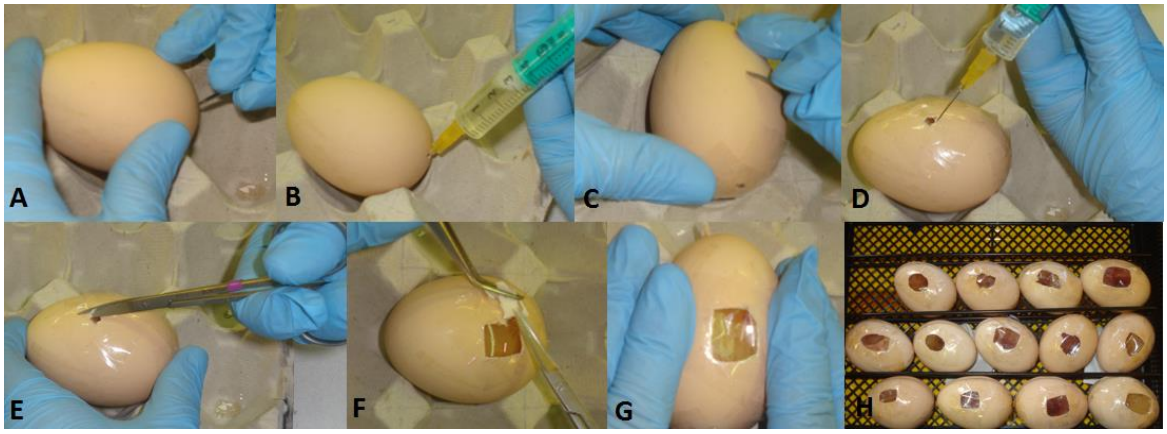


Figure 19- CAM assay procedure on day 4.

- White fertilized chicken eggs (Pintobar, Portugal) were kept under constant humidity of 60% at 37 °C, as soon as embryogenesis starts.
- At day 4, the surface of the eggs is disinfected with a 70% ethanol solution and a small hole was created in the pointed end of the egg (Figure 19A) to remove 2-3mL of albumen using a 5-cc syringe and 18-gauge needle o allow dissociation of the CAM from the egg shell membrane (Figure 19B). In addition, a small hole is created in the top of the egg (Figure 19C), to allow injection of 1mL of isotonic solution to ensure the drop of the membrane (Figure 19D) and then a square window is opened into the shell with the aid of small dissecting scissors to observe the development of the vessels along the incubation time (Figure 19E-F). The opening is then sealed with transparent tape to prevent dehydration (Figure 19G) and the eggs were returned to the incubator until the day of the experiment (Figure 19H).

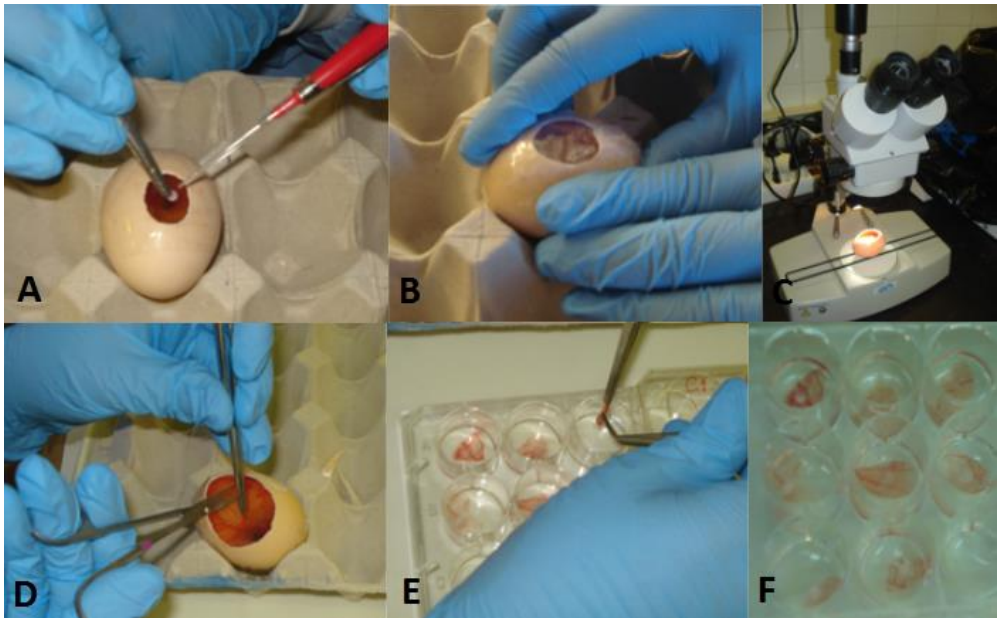


Figure 20- CAM assay procedure on days 8 (A and B) and 11 (C through F).

- At day 8 of incubation, the window was opened and sterile 0.5cm diameter circle filter papers were implanted in the CAM with 5µl of the test substance (Figure 20A). Four sealers were tested at pure concentration with the control group. The egg was closed again with tape and placed back on the incubator (Figure 20B).
- At day 11, the window was open and images of the CAM implants were acquired *in ovo* at 0.7x and 1x (Figure 20C). As complementary, the embryos and their membranes were fixed *in ovo* by adding a small volume of formaldehyde 3.7% solution to be frozen in a -20 °C refrigerator during 10 minutes so that blood vessels stay unaffected. CAM surrounding the filter paper was cut from the egg by means of using forceps and a small dissecting scissor (Figure 20D). Then, CAM portions were transferred to 6-well plates containing formaldehyde solution to obtain *ex ovo* images of each CAM implant directly from the plate (Figures 20E-F).
- The *in ovo* and *ex ovo* images obtained at day 11 of embryonic development with Nikon TMS Inverted Phase microscope (magnifications of 0.7x and 1x) were processed using the WCIF ImageJ software program (Wright Cell Imaging Facility, US National Institutes of Health) for facilitating the evaluation of the angiogenic response by analysing the total number of blood vessels converging toward the implanted discs.

6- Statistical analysis

The data presented within this work is the result of two separate experiments. Multivariate analysis of variance (MANOVA) was conducted and no significant differences were verified between experimental settings. Accordingly, the shown results, are the ones from a representative experiment. Further, within quantitative data (ALP activity, DNA) each point represents the mean values ± standard error. For quantitative assays, four replicates were conducted except where otherwise noted. Qualitative analysis and microscopical evaluation were conducted in triplicates. Statistical analysis was done by t-student test in the software Excel. P values ≤0.05 were considered significant.

IV: RESULTS

1- Cytotoxicity evaluation of the sealers' extracts

In the first part of this study the principal aim was to evaluate the cytotoxicity of the extracts of four different calcium silicate-based sealers - ProRoot MTA, MTA Plus, MTA Fillapex and Biodentine - on L929 mouse fibroblasts.

Cells exposed to a concentration range of the extracts were evaluated for cell proliferation throughout 7 days of culture.

1.1- Cell proliferation

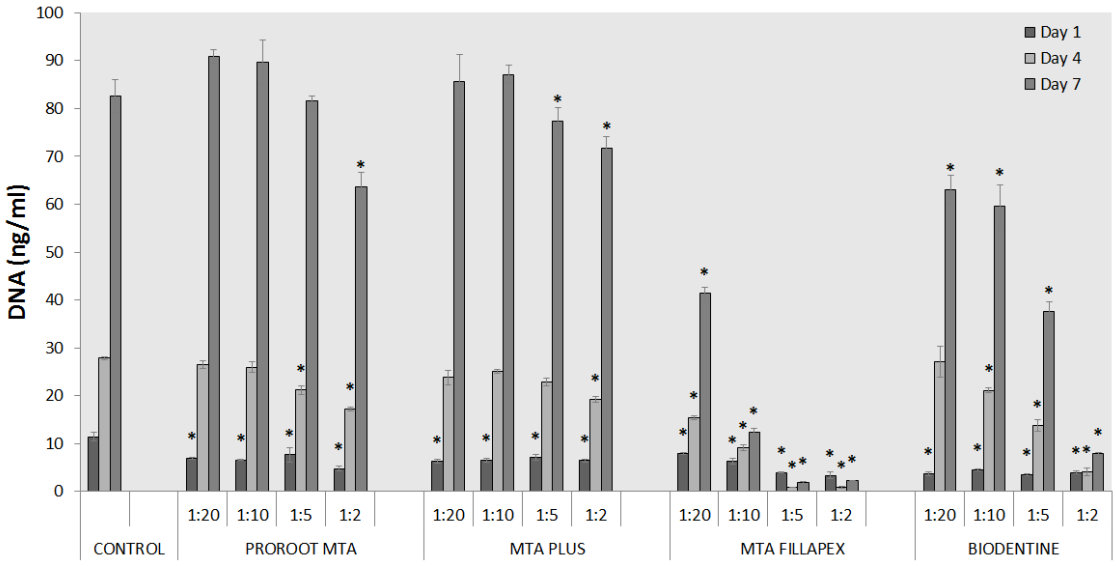
Cell proliferation was assessed by the DNA content of the cultures on days 1, 4 and 7, after the addition of the extract. Results are represented in Graphic 1.

Control cultures showed DNA values that increased with the culture time. Comparing to control, all extracts from all sealers presented a lower cell proliferation on day 1. This inhibition in cell proliferation was higher in the extracts with higher concentration of sealer, such as extracts with the dilutions 1:5 and 1:2.

On the ProRoot MTA and MTA Plus groups, a small inhibition on the cell proliferation was observed with the extracts on day 1, but values were similar to control on day 4 and day 7. Extract 1:2, on both sealers, caused a small inhibition.

On the MTA Fillapex group, only the cultures exposed to the extracts 1:20 and 1:10 showed some cell proliferation, while the cultures exposed to the extracts 1:5 and 1:2 showed very low DNA values.

On the Biodentine group, there was an inhibition with the extracts on day 1, but cells tend to recover along the culture time. Still, the extracts caused a dose-dependent inhibition. This inhibition was low for the dilutions 1:20 and 1:10, higher for the dilution 1:5 and very high for the dilution 1:2 (in this case, cell proliferation was very low).



Graphic 1- Proliferation of L929 fibroblasts cultured for 7 days, in the absence (control) and in the presence of the extracts from the endodontic sealers. *- significantly different from control (p≤0.05)

2- Effect of the sealer's extracts on osteoblastic cell behaviour

In the second part of this study, the principal aim was to assess the effect of the sealers' extracts on the osteoblastic behaviour, by performing two studies, i.e. (i) evaluation of the behaviour of human bone marrow derived mesenchymal stem cells in the presence of the extracts, and (ii) evaluation of the effect of the extracts in the regeneration of critical size bone defects in an *ex vivo* model.

2.1- Human mesenchymal stem cells

Human mesenchymal stem cells were exposed to the sealers' extracts (1:20 to 1:2 dilutions) during 21 days, and were characterized throughout the culture time for cell proliferation, F-actin cytoskeleton and cell morphology, alkaline phosphatase activity and staining and collagen staining and quantification.

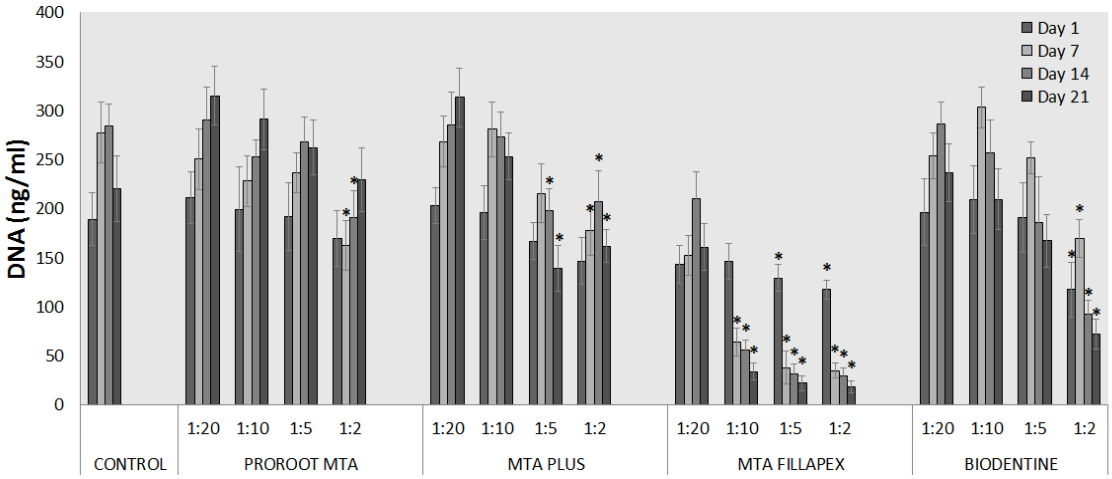
2.1.1- Cell proliferation

The DNA content was the method used to assess the cell proliferation of the cultures exposed to the extracts on days 1, 7, 14 and 21 (Graphic 2).

Control cell cultures showed an increasing proliferation until day 14, and a slight decrease after that.

On the ProRoot MTA and MTA Plus groups, mostly, cell proliferation increased throughout the culture time, and DNA values were similar to control for the dilutions 1:20, 1:10 and 1:5 (ProRoot MTA) and 1:20 and 1:10 (MTA Plus). Higher extract concentrations caused a small inhibitory effect, i.e. dilution 1:2 (ProRoot MTA) and dilutions 1:5 and 1:2 (MTA Plus). On the MTA Fillapex group, cell proliferation was lower than in the control at all extracts concentrations. The inhibition was dose-dependent and also time-dependent.

Biodentine group showed a behaviour similar to control at the extract dilutions 1:20, 1:10 and 1:5. The more concentrated extract (dilution 1:2) caused an inhibitory effect.



Graphic 2- Proliferation of human mesenchymal stem cells cultured for 21 days, in the absence (control) and in the presence of the extracts from the endodontic sealers. *- significantly different from control (p≤0.05)

2.1.2- F-actin cytoskeleton and cell morphology

Assessment of cell morphology of hMSCs cultures on day 7 was performed by CLSM, Figure 21. Control cells showed expanded cytoplasm, an adequate F-actin cytoskeleton and nuclear organization and several cell-to-cell contacts. The cells proliferated adequately and, at this time point, a large area of the culture surface was already covered by cells.

On the ProRoot MTA and MTA Plus groups, cultures with extracts of lower concentration (1:20 and 1:10 dilutions) exhibited more expanded cytoplasm than cultures with the extracts 1:5 and 1:2. On the MTA Fillapex group, extract 1:20 showed cytoplasm expansion similar to some of the other sealers, however extracts with higher concentration (1:10, 1:5 and 1:2) exhibited little or almost none cytoplasm. On the Biodentine group, extracts with lower concentration (1:20, 1:10 and 1:5) showed a good amount of cytoplasm expansion, while extract 1:2 exhibited little expansion of cytoplasm.

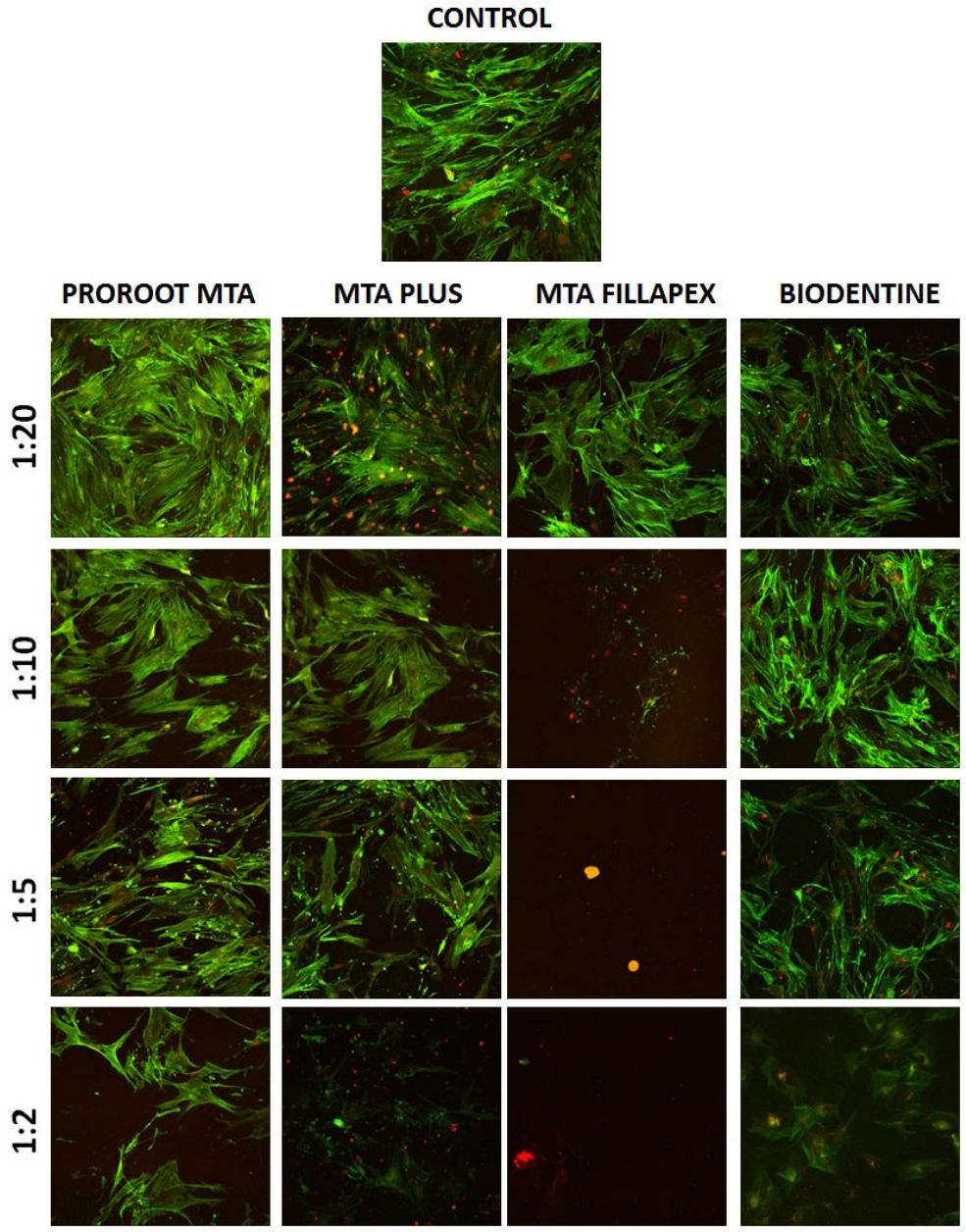


Figure 21- CLSM imaging of hMSCs cultures at day 7 in the absence (control) and presence of extracts of the endodontic sealers (extract dilutions 1:20 to 1:2).

2.1.3- Alkaline phosphatase activity

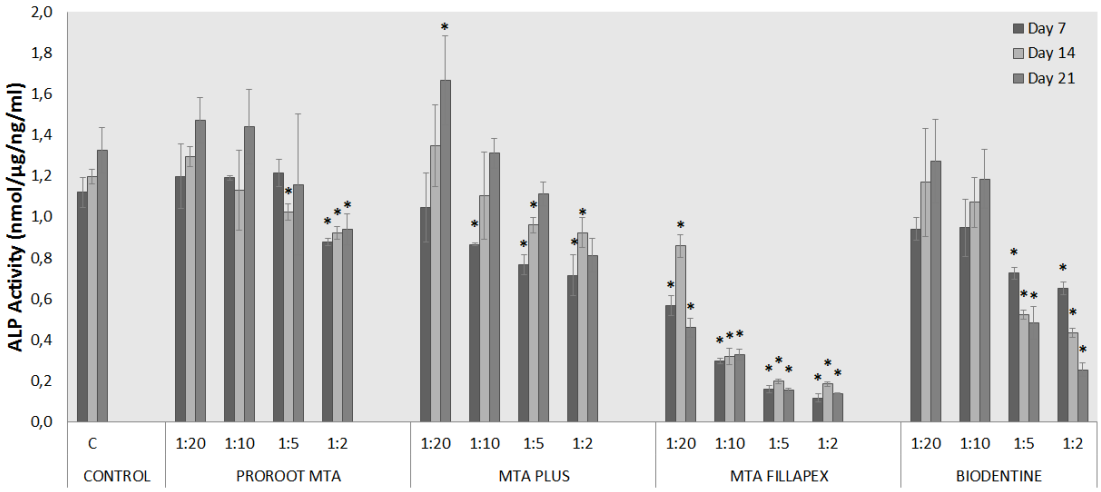
Results of ALP activity (normalized to total protein content) on days 7, 14 and 21 are represented in Graphic 3.

Control cultures showed a little increase in ALP activity throughout the culture time.

On the ProRoot MTA and MTA Plus groups, a similar pattern was observed. However, a slight inhibition was seen for ProRoot MTA at 1:2 dilution. With MTA Plus, ALP activity was lower in the presence of the extracts 1:10 to 1:2 at day 1, but values were similar to control on the days 14 and 21. In addition, exposure to the lower extract concentration (1:2) caused an increase in ALP activity at day 21.

On the MTA Fillapex group, ALP activity was lower than in control. Inhibition was dose-dependent, and ALP activity was very low in the presence of the extracts 1:5 and 1:2.

One the Biodentine group, ALP activity was lower than in control in the presence of the extracts 1:5 and 1:2.



Graphic 3- ALP activity of human mesenchymal stem cells cultured for 21 days, in the absence (control) and in the presence of the extracts from the endodontic sealers. *- significantly different from control (p≤0.05)

2.1.4- Alkaline phosphatase staining

Figures 22-25 are representative of alkaline phosphatase staining of hMSC cultures cultured for 7, 14 and 21 days in the absence (control) and in the presence of the extracts of the endodontic sealers. Control cultures presented nodular aggregates that stained intensively for ALP.

Figure 22 shows hMSC cultures in the absence (control) and presence of the extracts of ProRoot MTA sealers, dilutions 1:20 to 1:2, stained for ALP.

At day 7, ALP staining was lower than in the control, for all the extract concentrations. However, on day 14 and day 21 the intensity of the staining increased with all the extracts, particularly those with lower concentrations (1:20, 1:10 and 1:5), and was similar to that on control.

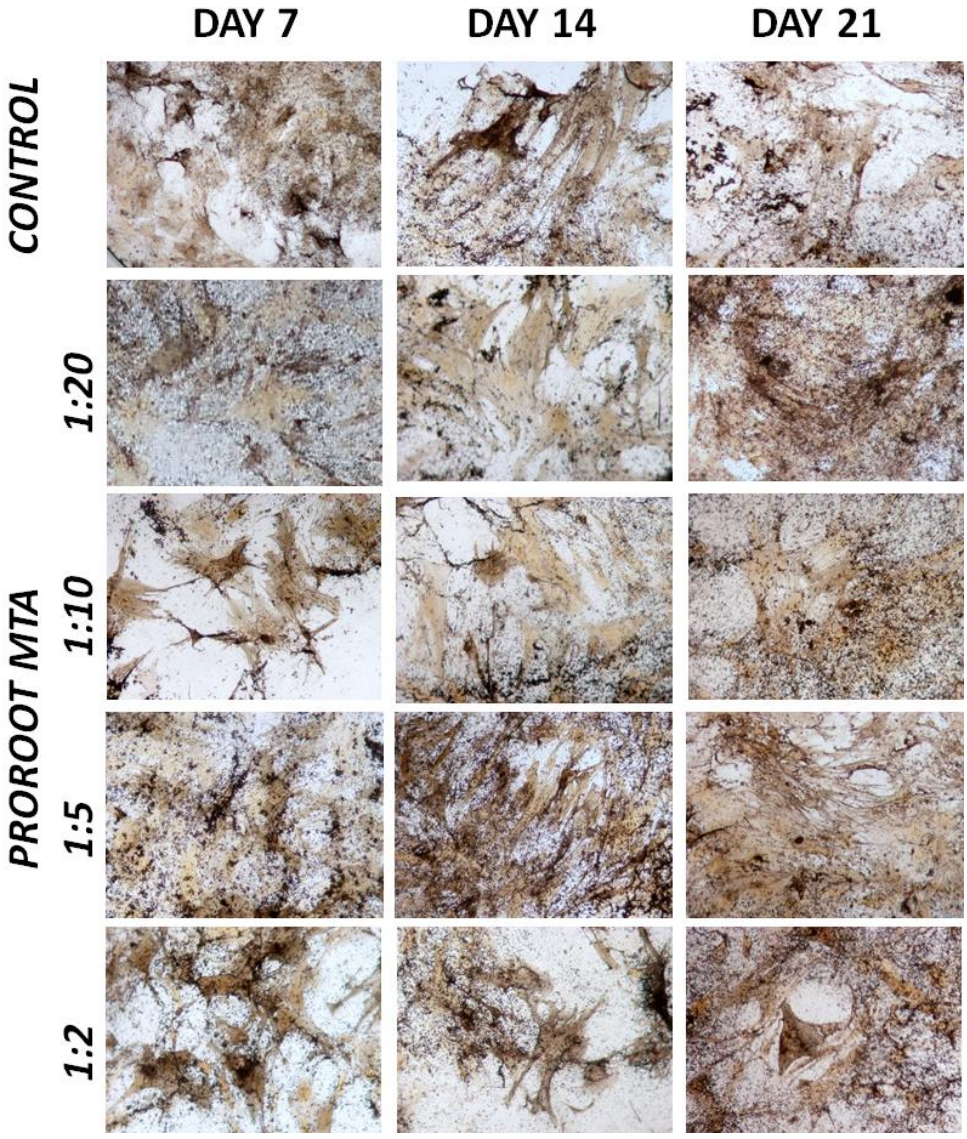


Figure 22- ALP staining of human mesenchymal stem cell cultures exposed to the extract of ProRoot MTA (1:20 to 1:2 dilutions) for 7, 14 and 21 days. Magnification: 100x.

Figure 23 shows hMSC cultures in the absence (control) and presence of the extracts of MTA Plus sealer, dilutions 1:20 to 1:2, stained for ALP.

ALP staining was similar for all the extracts on day 7 and lower than the control. By day 14, the staining increased mostly in the wells with lower concentration of extract (1:20 and 1:10) but also on extract 1:5, however less noticeable. By day 21, it is clearly visible that extract 1:20 produced the higher staining for ALP, and the cell layer covered all the surface of the well. By contrast, cultures exposed to the extracts 1:5 and 1:2 presented lower staining for ALP and a less abundant cell layer, from day 7 to day 21.

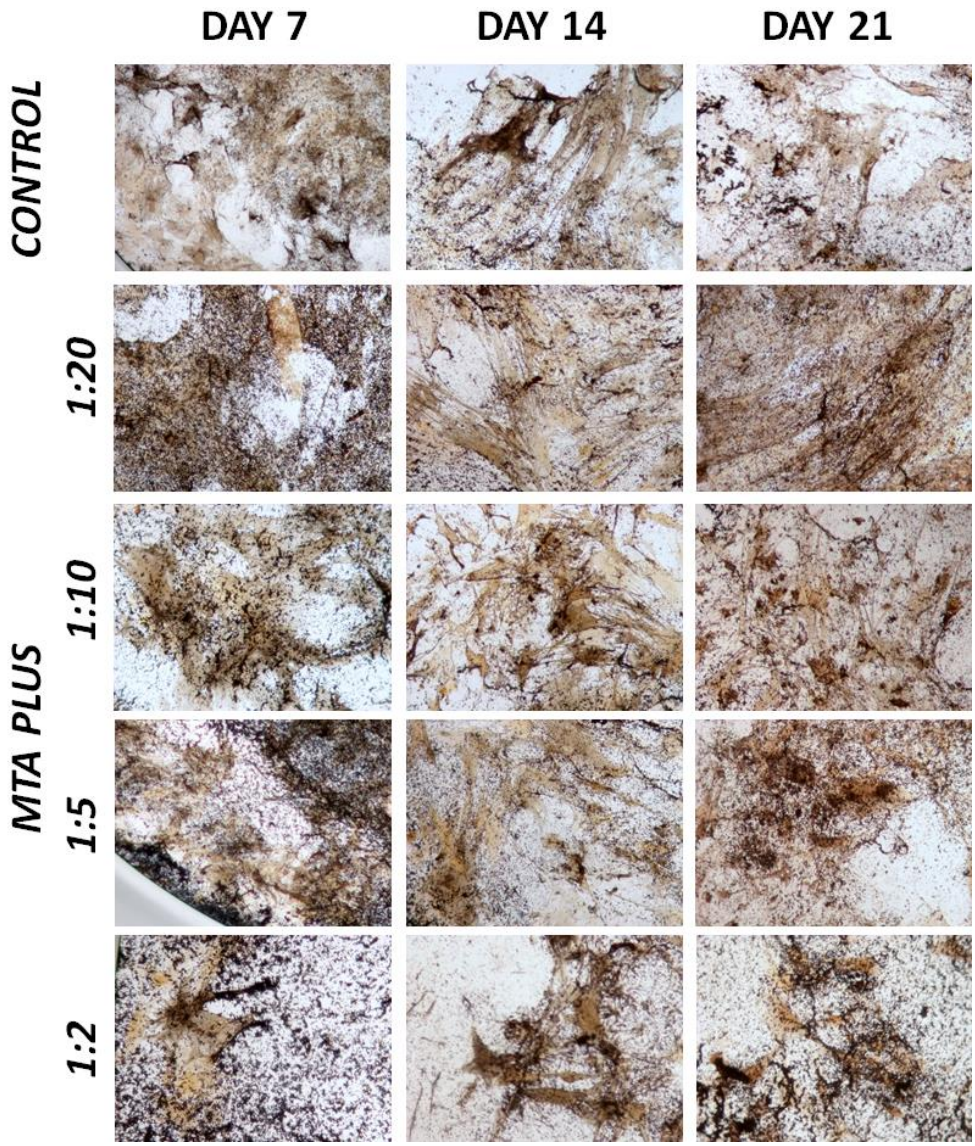


Figure 23- ALP staining of human mesenchymal stem cell cultures exposed to the extract of MTA Plus (1:20 to 1:2 dilutions) for 7, 14 and 21 days. Magnification: 100x.

Figure 24 presents hMSC cultures in the absence (control) and presence of the extracts of MTA Fillapex sealer, stained for ALP.

Production of ALP was significantly lower than in control for all the extracts on day 7, where a low amount of stained cell clusters were found on the wells. On day 14, an increase in ALP was observed particularly on extracts 1:20 and 1:10 which also increased on day 21. However, these increases in staining on days 14 and 21 were lower than the increase in control. Extracts with higher concentration (1:5 and 1:2) demonstrated little or none ALP production as cells were practically non-existent.

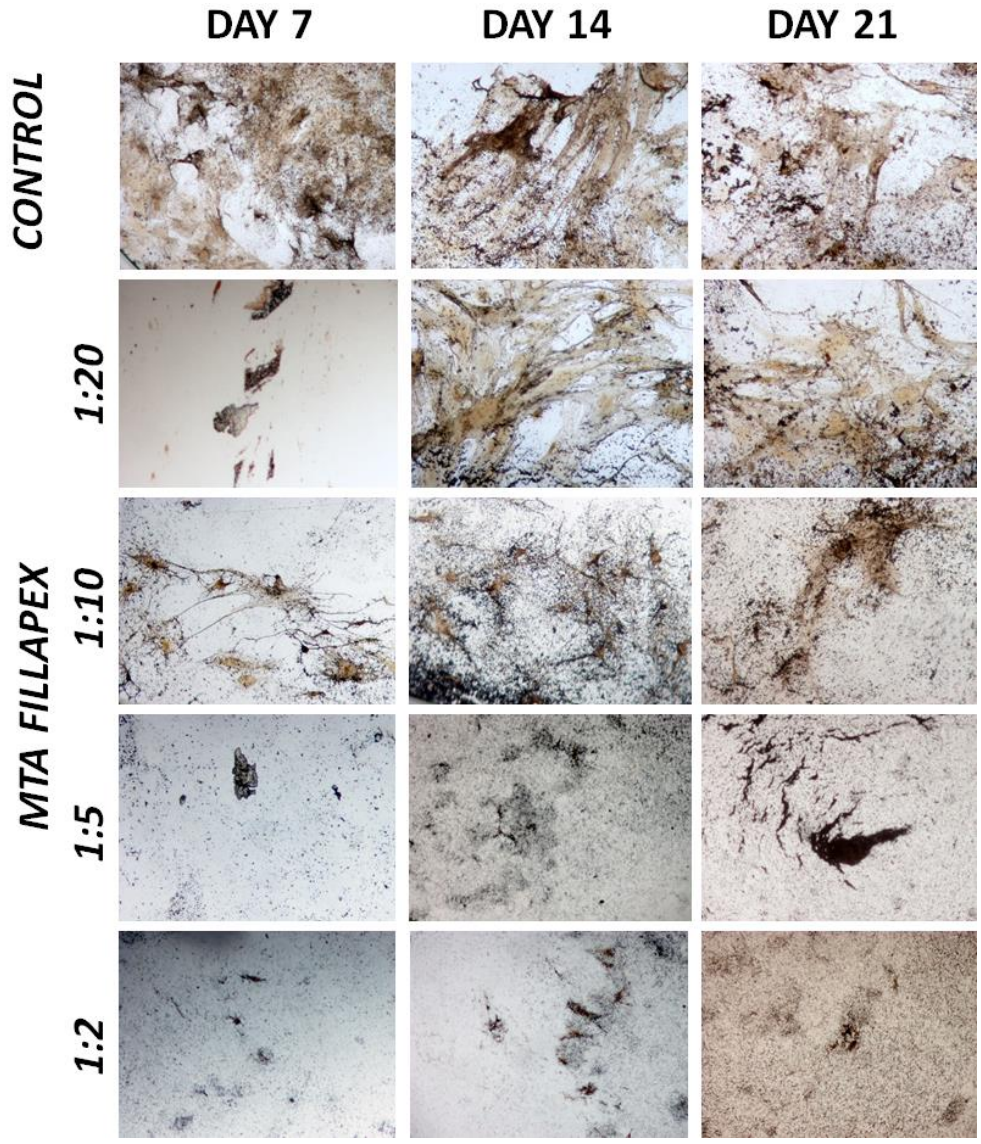


Figure 24- ALP staining of human mesenchymal stem cell cultures exposed to the extract of MTA Fillapex (1:20 to 1:2 dilutions) for 7, 14 and 21 days. Magnification: 100x.

Figure 25 presents hMSC cultures in the absence (control) and presence of the extracts of Biodentine sealer, stained for ALP.

ALP activity was similar for all the extracts on day 7 and also at the same level than that on the control, except on extract 1:2 which was lower. By day 14, the staining increased to a darker brown mostly in the wells with lower concentration of extract (1:20 and 1:10) but also on extract 1:5. By day 21, ALP activity on these three extracts was present as the staining was darker but with a lower amount and distribution. On extract 1:2, the staining clearly decreased from day 7 to day 21.

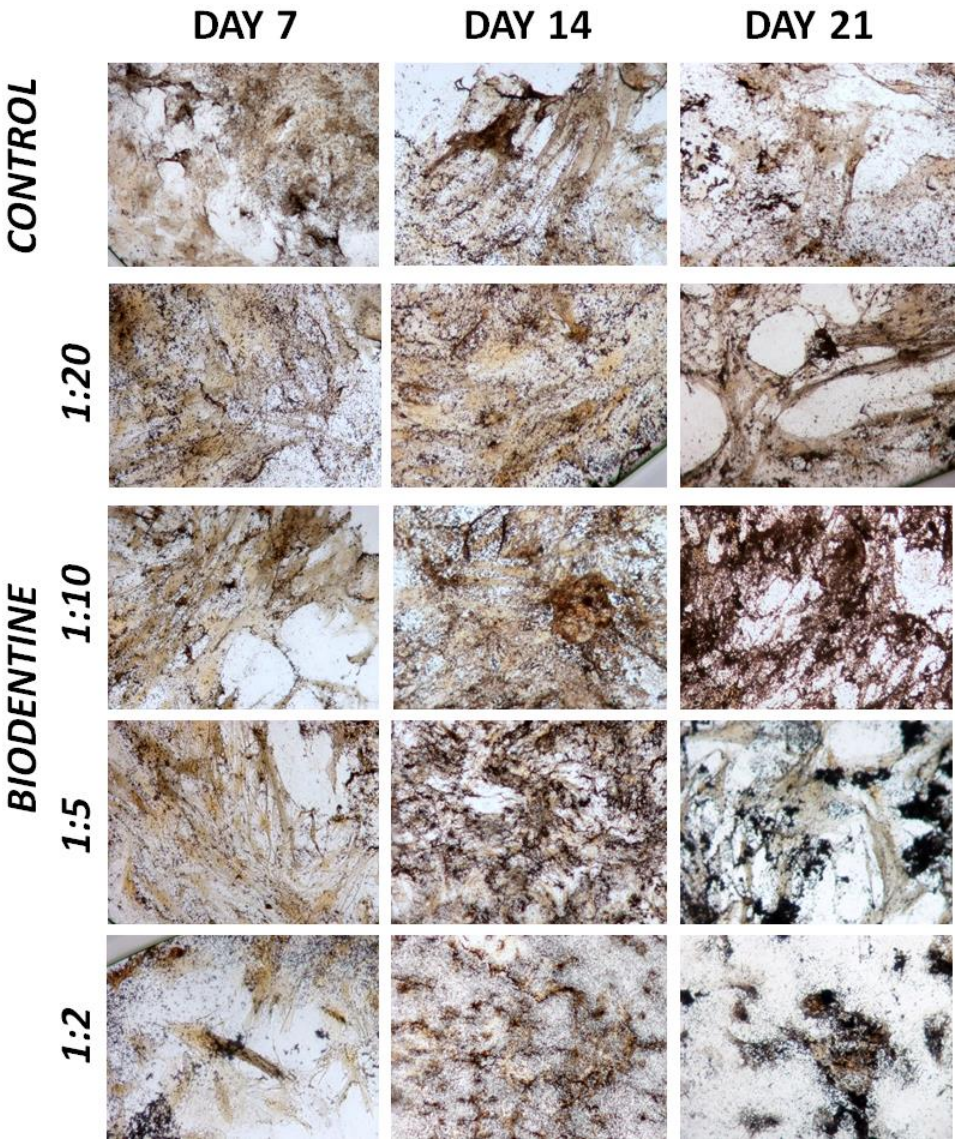


Figure 25- ALP staining of human mesenchymal stem cell cultures exposed to the extract of Biodentine (1:20 to 1:2 dilutions) for 7, 14 and 21 days. Magnification: 100x.

2.1.5- Collagen staining and quantification

Figures 26-29 are representative of collagen staining of hMSC cultures, established for days 7, 14 and 21 in the absence (control) and presence of the extracts of the endodontic sealers. In control cultures, the nodular aggregates were already visible at day 7 and increased over time, increasing also in the colour staining, covering the vast majority of the culture on day 14. By day 21, collagen staining was less intense as cells stopped proliferating.

Figure 26 shows hMSC cultures in the absence (control) and presence of the extracts of ProRoot MTA sealer, stained for collagen.

On day 7, it is visible that wells with lower extract concentrations (1:20 and 1:10) present collagen distribution with a lighter coloration than that on control. On day 14, an increase in staining was observable in extracts 1:20, 1:10 and 1:5 but not in the extract 1:2. By day 21, a darker staining was observed with distribution of collagen fibres covering all the surface of the wells in extracts 1:20 and 1:10, with a staining intensity higher than control; cultures exposed to extracts 1:5 and 1:2 presented lower collagen staining compared to control.

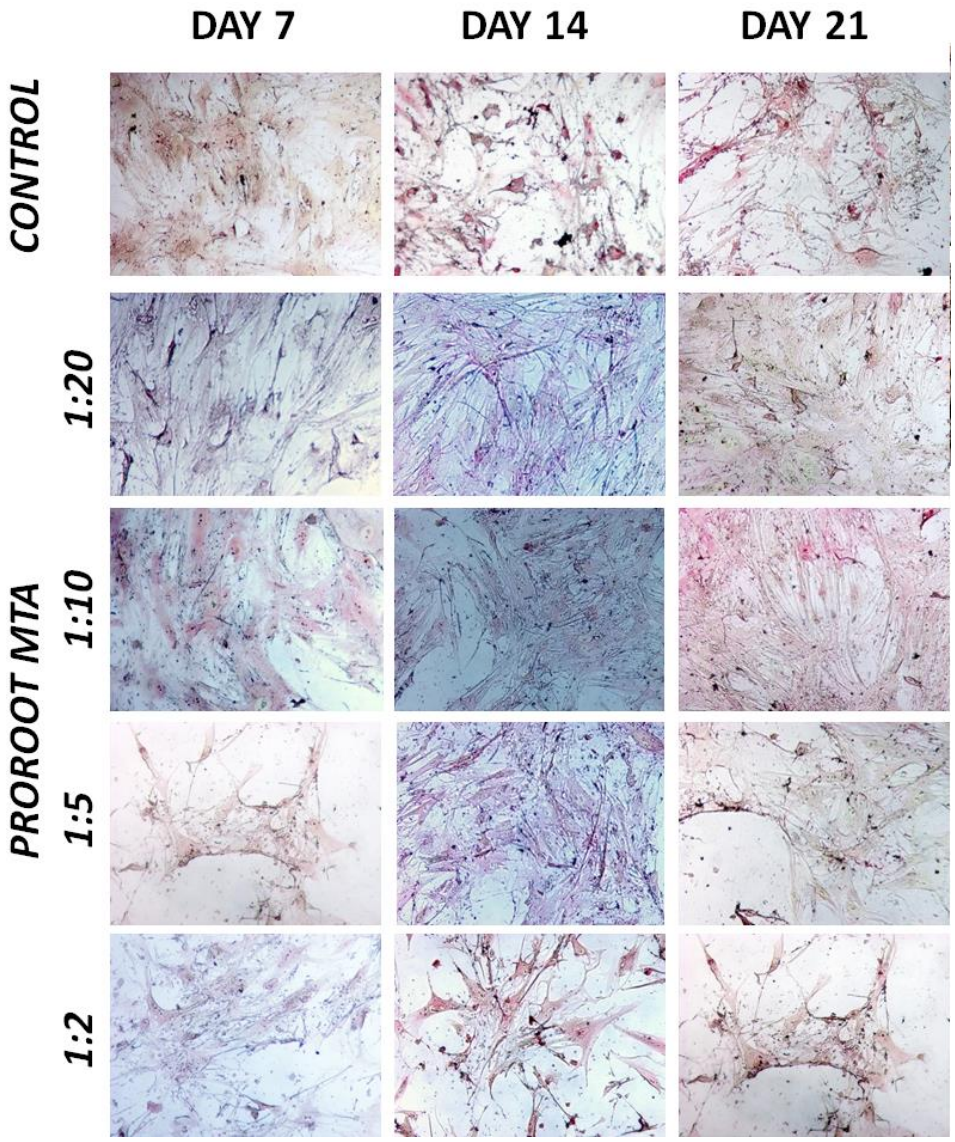


Figure 26- Collagen staining of human mesenchymal stem cell cultures exposed to the extract of ProRoot MTA (1:20 to 1:2 dilutions) for 7, 14 and 21 days. Magnification: 100x.

Figure 27 shows hMSC cultures in the absence (control) and presence of the extracts of MTA Plus sealer, stained for collagen.

Collagen staining in the cultures exposed to the extracts seems very similar to control on day 7. On day 14, a slight increase was observed with the extracts with lower concentrations (1:20, 1:10 and 1:5), but not with the extract 1:2 where the staining decreased. By day 21, cultures treated with extracts 1:20 and 1:10 increased in staining, even more than control, with the cell layer covering the vast majority of the wells; cultures exposed to the extracts 1:5 and 1:2 stayed with the same, or even lower, amount of staining. Overall, it can be observed that cultures treated with the extracts with lower concentration (1:20 and 1:10) presented increased staining over time and cultures performed with the extracts with higher concentration (1:5 and 1:2) showed decreased staining.

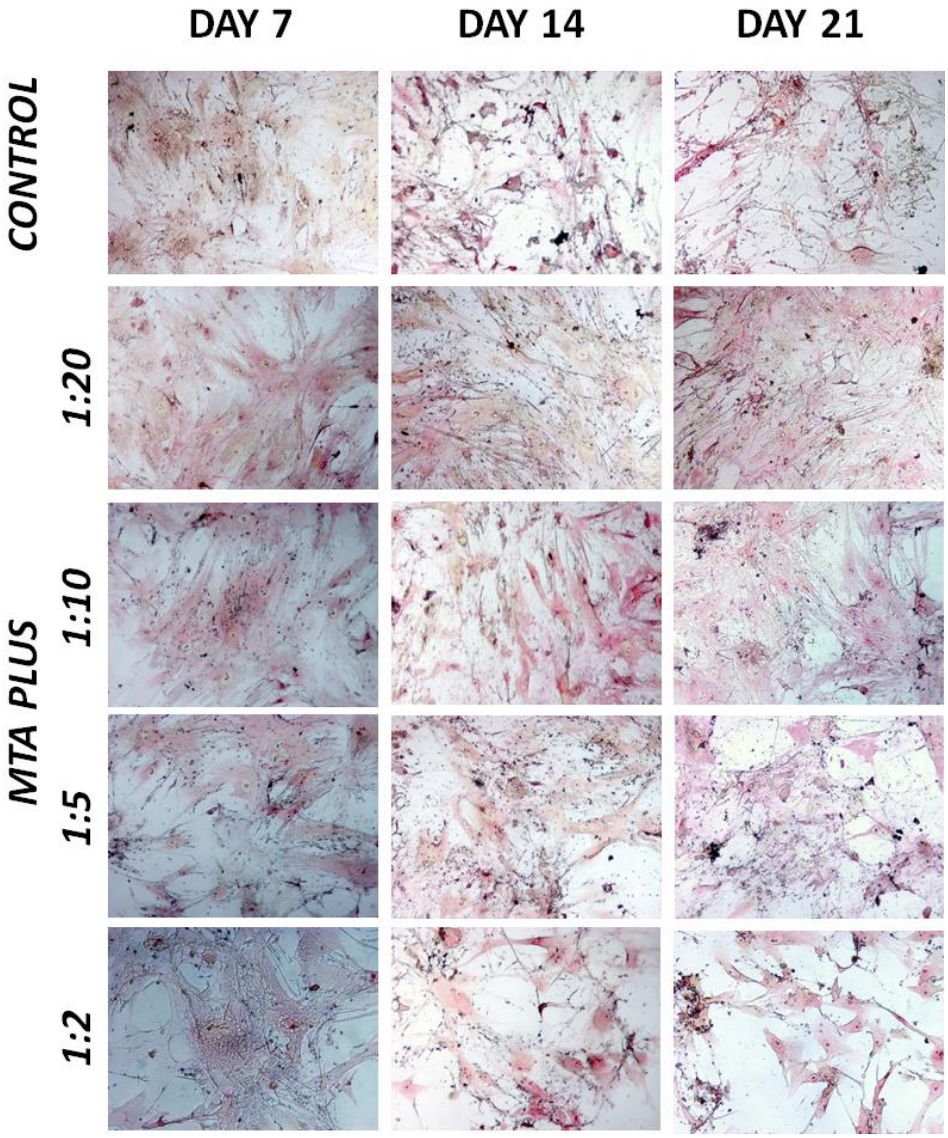


Figure 27- Collagen staining of human mesenchymal stem cell cultures exposed to the extract of MTA Plus (1:20 to 1:2 dilutions) for 7, 14 and 21 days. Magnification: 100x.

Figure 28 shows hMSC cultures in the absence (control) and presence of the extracts of MTA Fillapex sealer, stained for collagen.

On day 7, it is clearly visible that cultures exposed to all extracts presented lower collagen staining than control, with only extracts 1:20 and 1:10 presenting a relative good amount of collagen across the well. By day 14, extract 1:20 presented an increase in staining, while the other three extracts presented the same amount of collagen or even a decrease in the staining. By day 21, the staining was equal to that on day 14, where extract 1:20 displayed a distribution of collagen along the well with some blank spaces between, while the other three extracts presented a few fibres of collagen on the surface of the well.

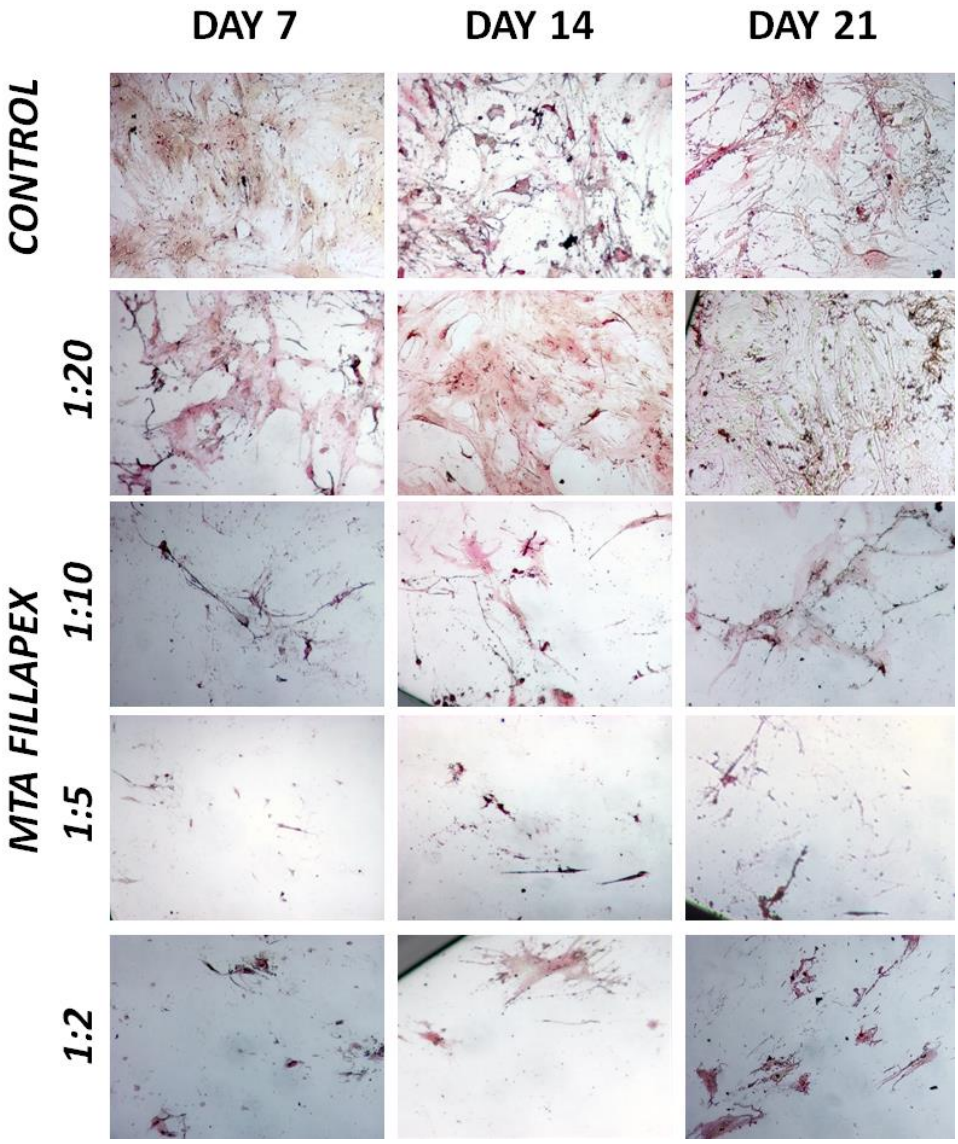


Figure 28- Collagen staining of human mesenchymal stem cell cultures exposed to the extract of MTA Fillapex (1:20 to 1:2 dilutions) for 7, 14 and 21 days. Magnification: 100x.

Figure 29 presents hMSC cultures in the absence (control) and presence of the extracts of Biodentine sealer, stained for collagen.

On day 7, it is visible that all extracts presented a good level of collagen staining, even though a little lower than that on the control. On day 14, an increase in collagen staining was observable for extracts with lower concentration (1:20 and 1:10) but not on extracts with higher concentration (1:5 and 1:2) where the staining stayed the same or even decreased. On day 21, extracts 1:20, 1:10 and 1:5 increased staining even more than the control, whereas extract 1:2 decreased collagen staining, remaining with a few fibres along the well.

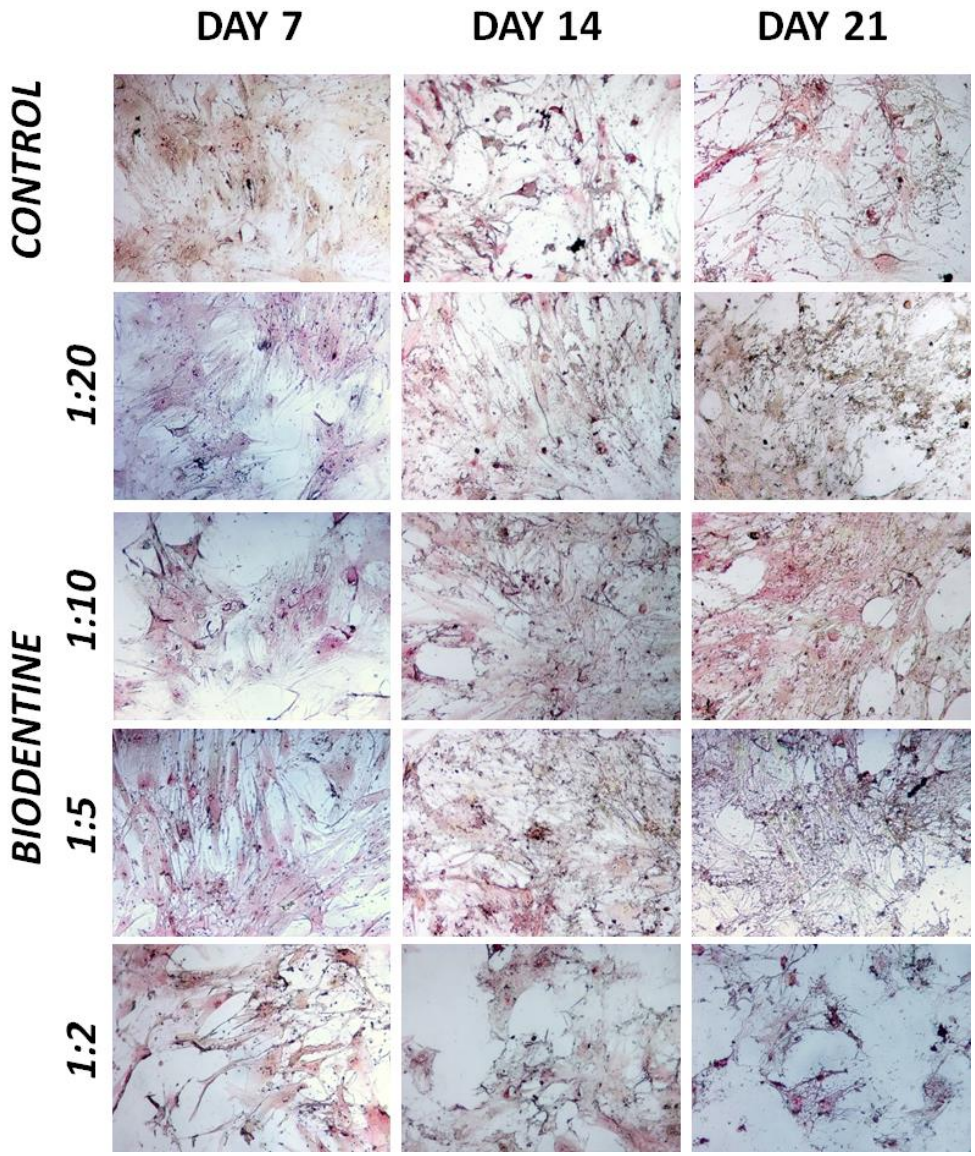


Figure 29- Collagen staining of human mesenchymal stem cell cultures exposed to the extract of Biodentine (1:20 to 1:2 dilutions) for 7, 14 and 21 days. Magnification: 100x.

Collagen quantification values were organized and are represented in Graphic 4.

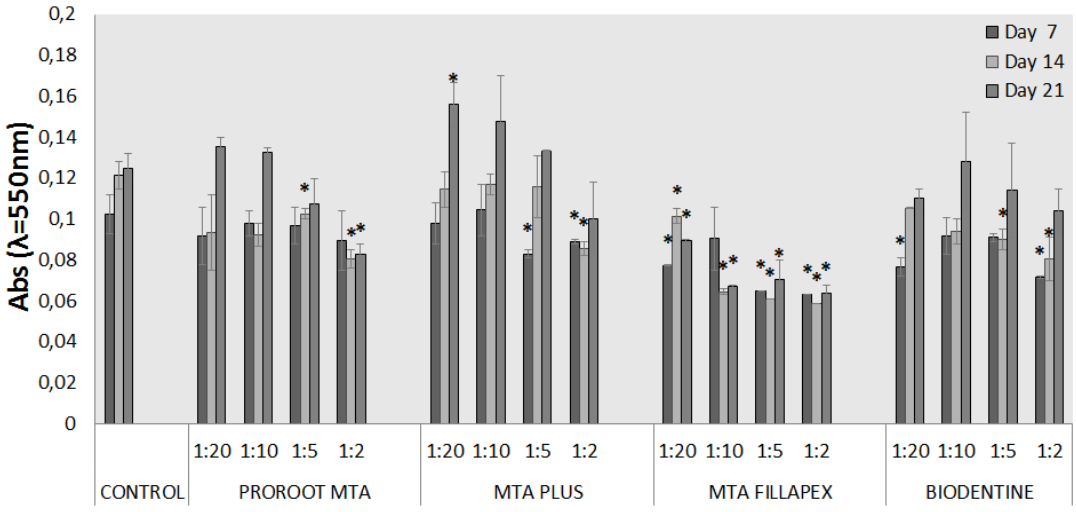
Control cultures exhibited an increase in the amount of collagen from day 7 to day 21, with days 14 and 21 being very similar.

On the ProRoot MTA group, an increase in collagen is clearly observable in extracts 1:20 and 1:10, mainly on day 21. On extract 1:5, collagen quantity was almost the same on the three experimental days and on extract 1:2 there is a little decrease in collagen quantity.

MTA Plus group displayed a similar behaviour as ProRoot MTA, with an increase in collagen in extracts 1:20, 1:10 and 1:5 from day 7 to day 21. On extract 1:2, a little increase was observed but not very significant. Overall, at the end of the culture time, MTA Plus displayed the most amount of collagen.

On the MTA Fillapex group, a little increase was only observable on extract 1:20, while extract 1:10 displayed a decrease in collagen and extracts 1:5 and 1:2 stayed the same from day 7. Overall, collagen quantity in all of the extracts of MTA Fillapex was lower than that of the other three sealers.

On the Biodentine group, an increase in collagen quantity was clearly observable on all extracts during the culture time. The higher the concentration of the extract, the lower the amount of collagen except on extract 1:20 on day 21 that stayed similar to that of day 14.



Graphic 4- Quantification of the collagen staining in the human mesenchymal stem cell cultures in the absence (control) and in the presence of the sealers' extracts for 7, 14 and 21 days. *- significantly different from control (p≤0.05)

2.2- *Ex vivo* osteogenic assay

Parietal bone specimens with the created circular defects were cultured in the absence (control) and in the presence of the extracts (1:20 and 1:5 dilutions) from the endodontic sealers, for a period of 14 days. The images of the regeneration of the defects at days 4, 6, 10 and 14 are shown in Figure 30. At the baseline (the first days), there was no difference among all the groups. After 14 days of culture, some differences on bone formation were noticed.

In the control group, on day 6, a well-defined regeneration was already observed. On days 10 and 14, the regeneration level evolved quickly, being almost fully filled by the mineralized tissue.

In the ProRoot MTA group, extract 1:5 showed mineralized tissue covering almost the entire defect by day 14 while on extract 1:20, the mineralized tissue did not covered the entire defect yet.

In the MTA Plus group, both extracts started developing regeneration of parietal bone with a higher effect on extract 1:20. However, by day 14, regeneration on extract 1:5 was better than on extract 1:20 as the mineralized tissue covered all the defect with a stronger layer than that of extract 1:20.

In the MTA Fillapex and Biodentine groups, only extracts 1:20 developed a well-defined regeneration of parietal bone while on extracts 1:5, a low level of regeneration can be observed on the edges but it did not even closed half of the defect by the last day of culture.

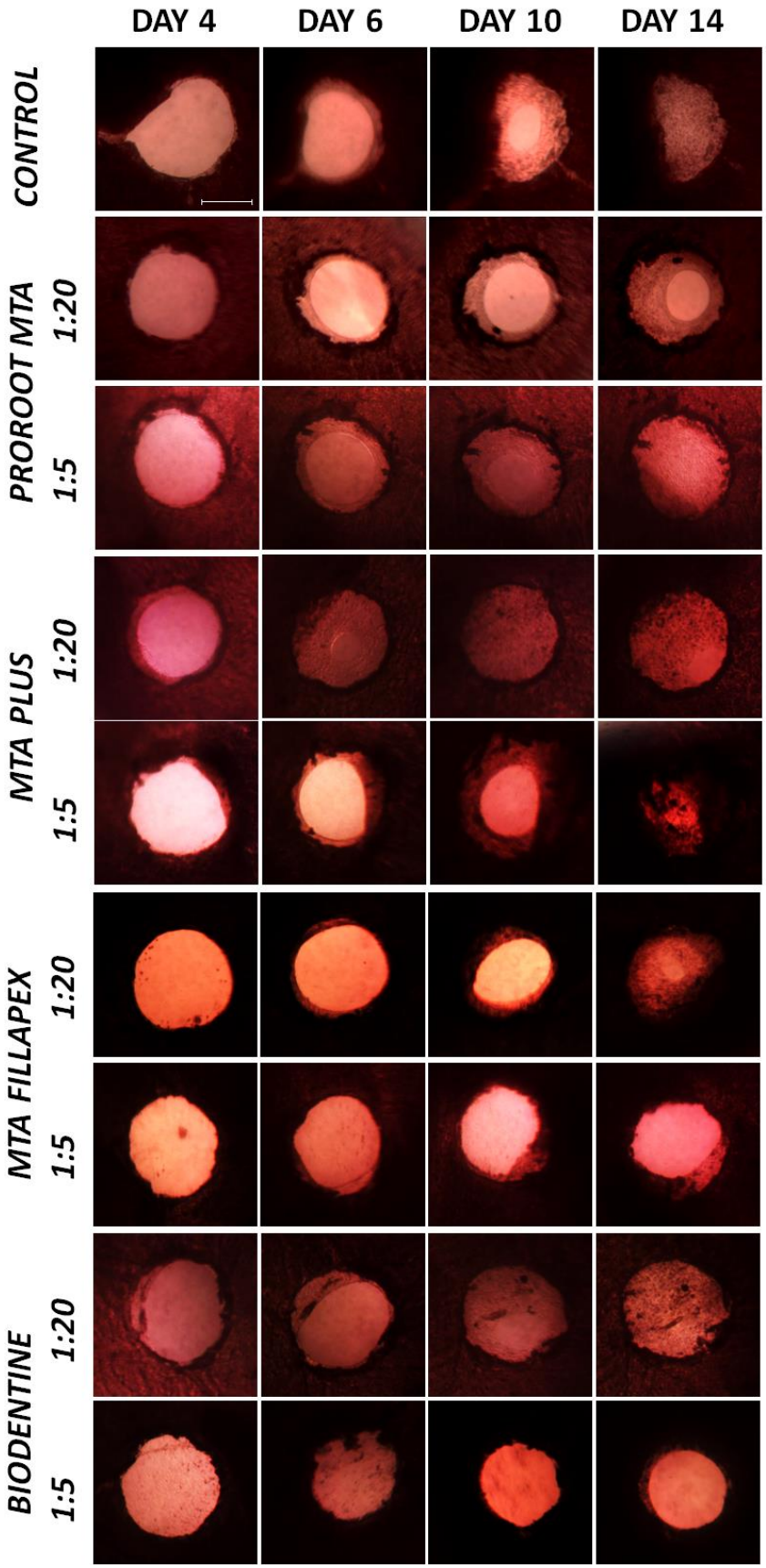


Figure 30- *Ex vivo* regeneration of neonatal rat parietal bone defects in the presence of the sealers' extracts (1:20 and 1:5 dilutions) over a period of 14 days. Magnification: 1x. Scale bar: 0.5mm.

2.2.1- Alkaline phosphatase staining

Parietal bone specimens were cultured in the absence (control) and in the presence of the extracts (1:20 and 1:5 dilutions) from the endodontic sealers, for a period of 14 days. Cells migrated from the parietal bone specimens to the culture plate surface and formed a well-organized cell layer. The resulting cell layer was stained to identify the presence of ALP on days 7 and 14 (Figure 31). On control, a dense monolayer organization can be observed from cells grown up to day 14.

On the ProRoot MTA and MTA Plus groups, staining increased from day 7 to day 14 and extracts 1:20 presented more staining than extracts 1:5 on both days. On day 7, with extracts 1:20, ALP staining covered more space in the well and on day 14 presented more clusters with darker staining than in the presence of extracts 1:5.

On the MTA Fillapex group, extract 1:20 presented a high amount of ALP staining on day 7, whereas extract 1:5 presented almost none. On day 14, staining diminished on extract 1:20 and on extract 1:5 it stayed the same.

On the Biodentine group, staining increased from day 7 to day 14 and extract 1:20 presented more staining than extract 1:5 on both days. On day 7, ALP staining covered more space in the well and on day 14 presented more clusters with darker staining.

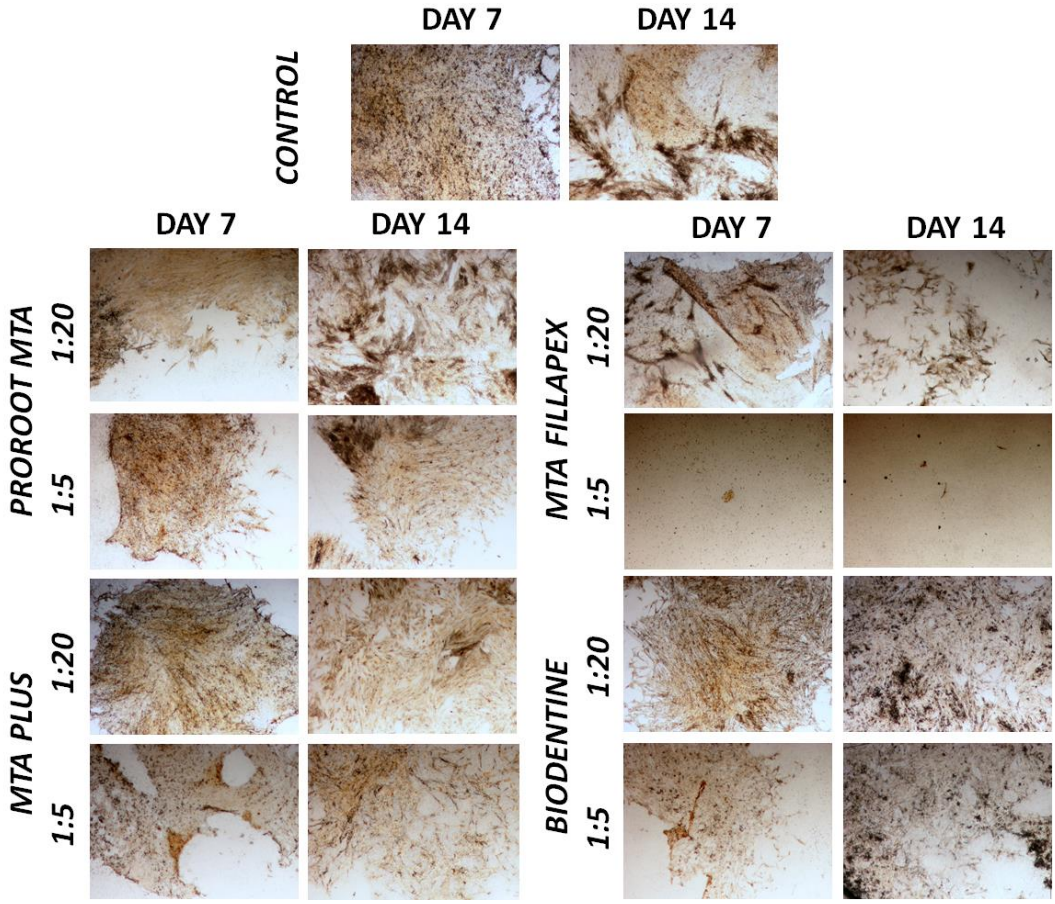


Figure 31- ALP staining of the osteoblastic cell cultures outgrown from the rat parietal bone cultured in the presence of the sealers' extracts (1:20 and 1:5 dilutions), at days 7 and 14. Magnification: 40x.

2.2.2- Collagen staining

Parietal bone specimens were cultured in the absence (control) and in the presence of the extracts (1:20 and 1:5 dilutions) from the endodontic sealers, for a period of 14 days. Cells migrated from the parietal bone specimens to the culture plate surface and formed a well-organized cell layer. The resulting cell layer was stained to identify the presence of collagen on days 7 and 14 (Figure 32). In control, a homogenous cell layer is observed at day 11, staining positive for collagen.

In the ProRoot MTA and MTA Plus groups, staining increased from day 7 to day 14 and extracts 1:20 presented more staining than extracts 1:5 on both days of the staining. On days 7 and 14, collagen staining on extract 1:20 covered more space in the well than on extract 1:5. Extract 1:20 displayed ALP staining similar to control.

In the MTA Fillapex group, extract 1:20 presented some collagen staining on day 7, whereas extract 1:5 presented almost none. On day 14, staining seemed to stay the same on extract 1:20 and on extract 1:5 it seemed to decrease even more.

In the Biodentine group, staining seemed to decrease from day 7 to day 14, extract 1:20 presented more staining than extract 1:5 on day 7. On day 14, staining was similar with both extracts.

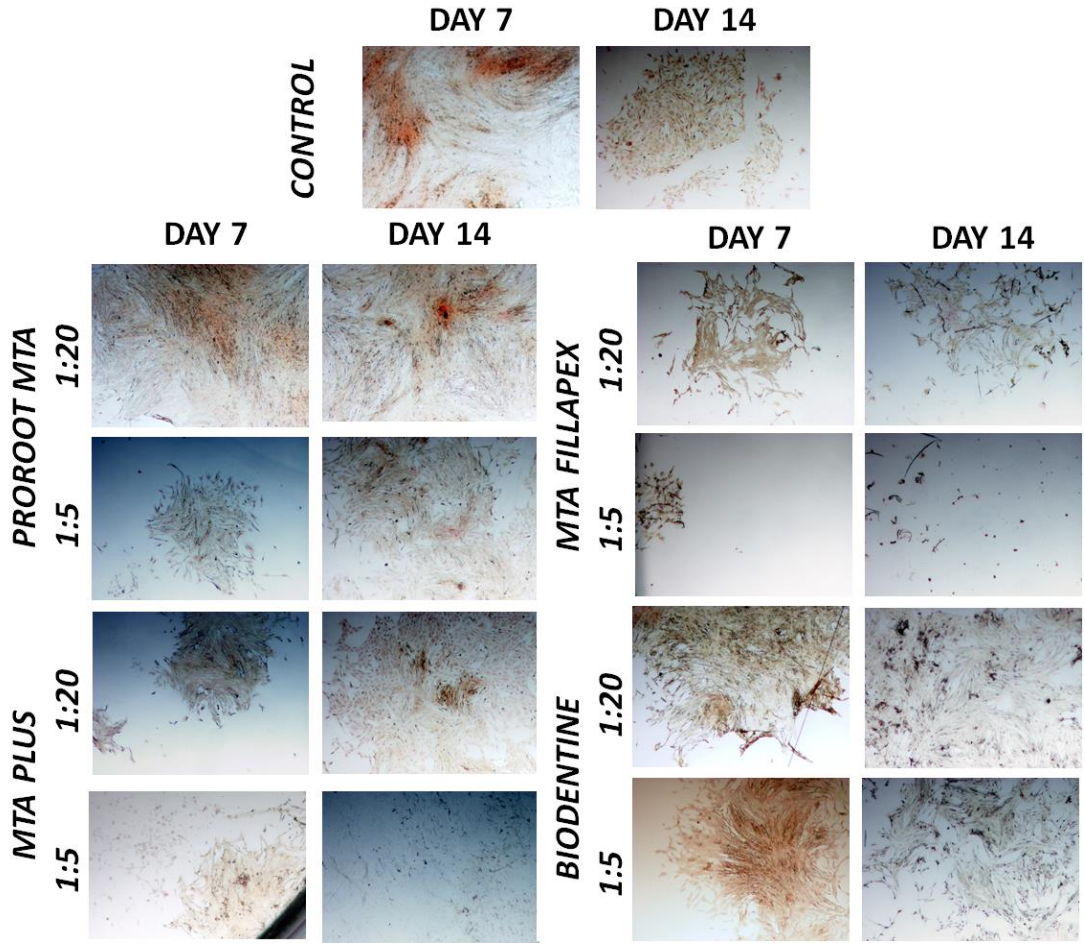


Figure 32- Collagen staining of the osteoblastic cell cultures outgrown from the rat parietal bone cultured in the presence of the sealers' extracts (1:20 and 1:5 dilutions), at days 7 and 14. Magnification: 40x.

3- Effect of the sealer's extracts on endothelial cells and angiogenesis

In the third part of this study the principal aim was to assess the effect of the sealers' extracts on human endothelial cells and angiogenesis. Two studies were performed. In the first one, human umbilical vein endothelial cells were cultured in the presence of the extracts for 7 days, and cell behaviour was assessed for cell proliferation, F-actin cytoskeleton and cell morphology and the ability of the cell layer in organizing in cord-like structures upon the addition of Matrigel. The second study consisted in an *in vivo* angiogenesis evaluation in the presence of the sealers' extracts, using the CAM assay.

3.1- Human endothelial cells

3.1.1- Cell proliferation

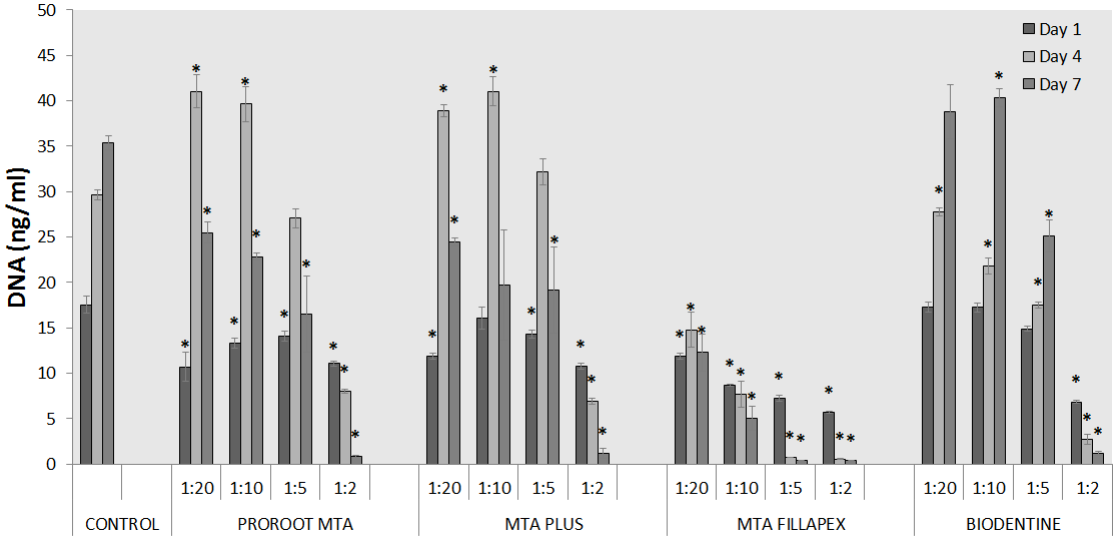
HUVECs proliferation was assessed by the DNA content and the results are in Graphic 5.

Control cell culture showed an increasing proliferation throughout the culture time.

On the ProRoot MTA and MTA Plus groups, an initial inhibitory effect was observed but cells recovered in day 4, where cell proliferation was at their highest in extracts 1:20, 1:10 and 1:5. The higher the concentration of the sealer, the lower the cell proliferation. On extract 1:2, cell proliferation decreased from day 1 to day 7.

On the MTA Fillapex group, results showed little cell proliferation in extracts 1:20, a decrease of proliferation in extract 1:10 and were severely cytotoxic at highest concentrations (1:2 and 1:5), where cell proliferation decreased on days 4 and 7.

On the Biodentine group, extracts were very similar to control on day 1, except for extract 1:2 where proliferation was lower and decreased on the next days. On the other extracts, cell proliferation increased from day 1 to day 7.



Graphic 5- Proliferation of human umbilical vein endothelial cells cultured for 7 days, in the absence (control) and in the presence of the extracts of the endodontic sealers. *- significantly different from control (p≤0.05)

3.1.2- Matrigel coating assay

Images shown in Figure 33 are representative of the cell layer organization upon the addition of a Matrigel coating over the HUVEC cultures performed in the absence and presence of two extract dilutions (1:20 and 1:5) of the endodontic sealers. In the control cultures, the cell layer organized through an extensive network of interconnecting cords following the addition of Matrigel.

On ProRoot MTA and MTA Plus groups, both extract dilutions of both sealers seemed to show the same level of cell layer organization.

On the MTA Fillapex group, extract 1:20 showed also an appearance similar to that observed on control, while extract 1:5 showed only few attached cells.

On the Biodentine group, both extracts presented some degree of cell attachment; the cell layer showed a tendency to be organized in cord-like structures but with a poorer appearance compared to that on control, especially in the presence of the extract 1:5.

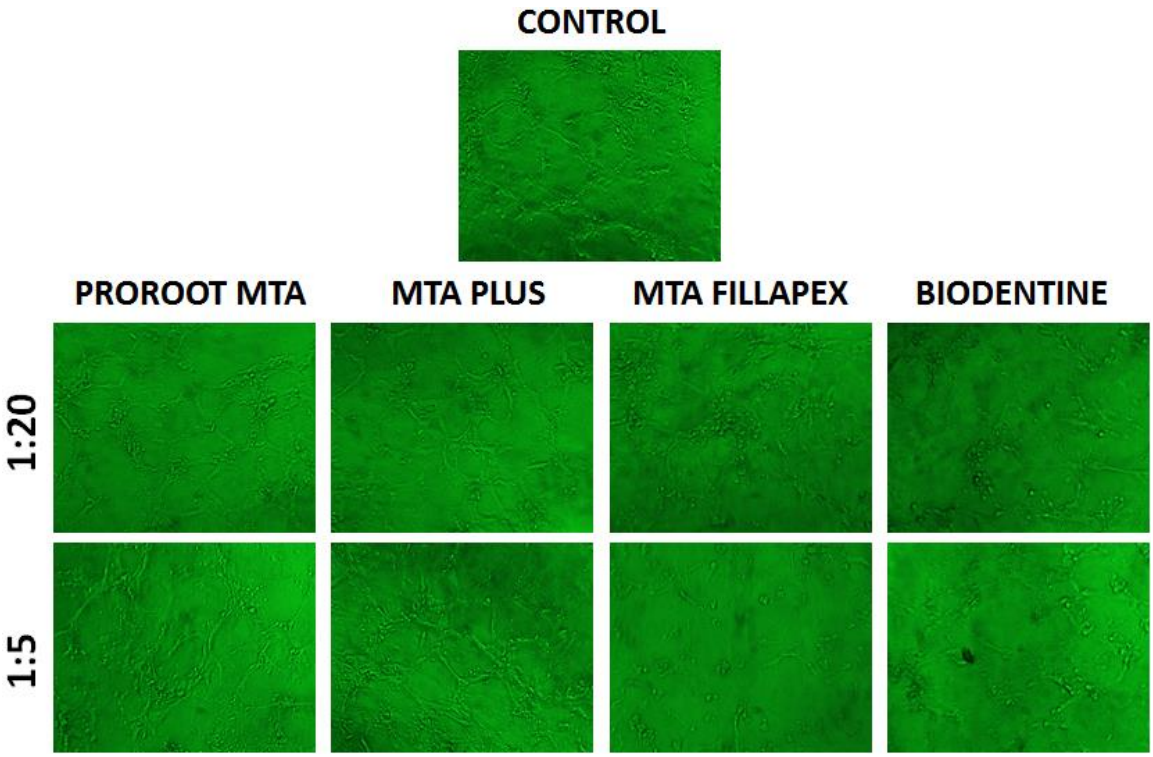


Figure 33- Matrigel assay performed on human umbilical vein endothelial cell cultures exposed to the sealers' extracts (1:20 and 1:5 dilutions). Magnification: 100x.

3.2- *In vivo* angiogenesis assay

The undiluted extracts from the four endodontic sealers were tested for the angiogenic response using the CAM assay. The extract was placed in circular paper filter samples, and the elicited angiogenic response was evaluated. Representative images are shown in Figure 34.

From a manual quantification of blood vessels view, no significant differences were observed between the control and the extracts.

From a qualitative view, it can be observed that both filters with extracts from ProRoot MTA and MTA Plus, along with the control, are surrounded by a thin layer of blood vessels, whereas MTA Fillapex and Biodentine do not present that feature.

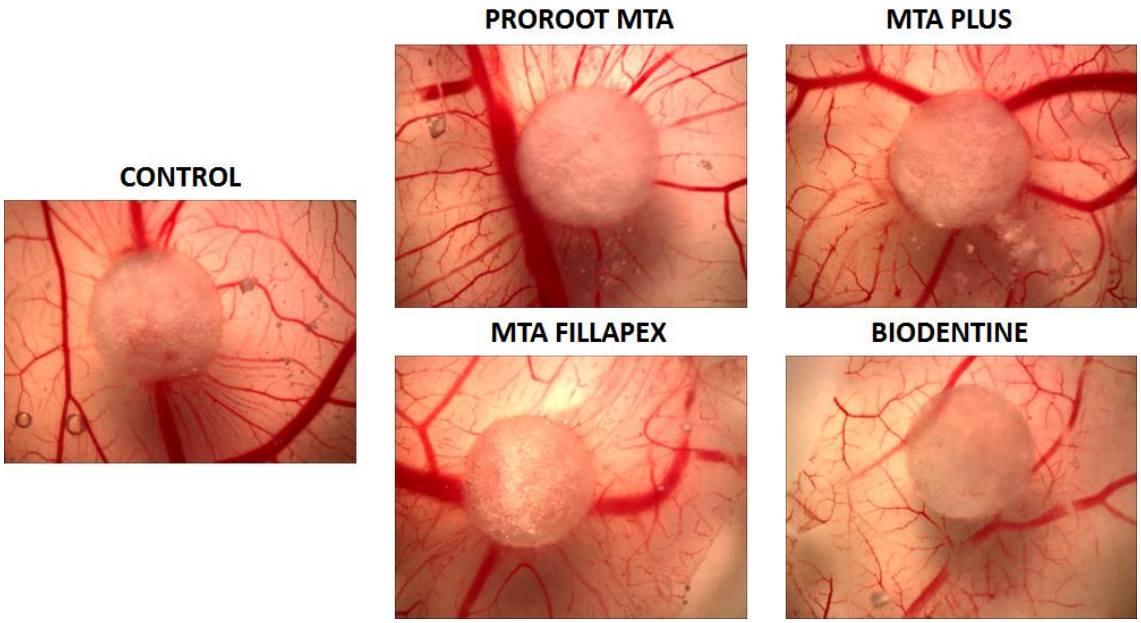


Figure 34- Representative images of CAMs containing the undiluted extract of the four endodontic sealers. Magnification: 1x.

V: DISCUSSION

Endodontic surgeries are performed to resolve inflammatory processes that cannot be successfully treated by conventional techniques, which may be due to complex canal and/or apical anatomy and external inflammatory processes observed in teeth structure. Endodontic treatment usually involves elimination of residual pulp, tissue breakdown products and microorganisms present at the root canal system, followed by the placement of a material designed to seal the root canal contents from the periradicular tissues and repair root defects. Repair of vital and necrotic pulp by pulpectomy and root canal treatment is critical for the treatment and eradication of bacterial infections. Although they are to be contained within the root canal space, they may extrude through the apical constriction, or the eluents from the sealers may come into contact with pulpal or periapical tissues. Proper selection of these materials with different compositions, used worldwide, plays a very important role in the success of the surgery.

An ideal orthograde or retrograde filling material should seal the pathways of communication between the root canal system and its surrounding tissues and should also be nontoxic, noncarcinogenic, nongenotoxic, biocompatible with the host tissues, insoluble in tissue fluids, and dimensionally stable. Furthermore, the presence of moisture should not affect its sealing ability; it should be easy to use and be radiopaque for recognition on radiographs. Many endodontic sealers are now used in clinical practice, including zinc-oxide-eugenol sealers, polyketone sealers, epoxy resins, glass ionomer cements, calcium hydroxide-based sealers and calcium silicate-based sealers, but none meets all the appropriate requirements and to overcome the shortcomings of these materials, several materials have been recently proposed for clinical use in endodontic practice. The materials chosen for our study are the calcium silicate-based sealers, and include the original ProRoot MTA, two modifications of MTA (MTA Plus and MTA Fillapex) and a new class of dental material (Biodentine). There is a lack of research on these sealers in direct contact with cells that should be undertaken to improve our understanding of the biocompatibility and bioactivity of these cements.

There are several *in vitro* and *in vivo* tests to evaluate the biocompatibility of dental materials such as testing the general toxicity profile of potential materials in a cell culture, implantation tests, and usage tests in experimental animals according to accepted clinical protocols. *In vitro* cytotoxicity assays have the advantages of being simple, reproducible, cost-effective, and suitable for the evaluation of basic biological aspects relative to biocompatibility. However, other factors such as the material's physical structure and surface characteristics, known to influence the tissue response to the materials, should also be considered. Many researchers have shown that the quality and quantity of cell attachment to the retrofilling materials could be used as a criteria for the evaluation of the biocompatibility of the materials.

The choice for sealer/cements extracts of different concentrations was performed to observe a possible dose-response relationship and based on the fact that, when placed into the root canals or as root-end fillings, sealers and cements release soluble components that may be diluted by tissue fluids and then carried to the surrounding cells and tissues. The extracts can also be experimentally subjected to serial dilutions allowing the evaluation of a possible dose-response effect in a similar manner to the increased dilution occurring *in vivo* [118][119][120]. It may also be assumed that the extract dilution might lead to a more accurate comparison of cytotoxicity among different materials.

Other important issue to consider during the experimental design for *in vitro* biocompatibility studies is the cell type. Osteoblasts [121], mouse fibroblasts [122] and human fibroblasts [118] are some of the cell types usually employed in such investigations.

The choice for cultured L929 mouse fibroblasts for the cytotoxicity assay in the present study is justified by the fact that they are a continuous cell line routinely used for the testing of cytotoxic properties of dental materials, because of their reproducible growth rates and biological responses. Human fibroblasts would be preferred based on their relevance to clinical conditions. If a tested material is proven to be cytotoxic to cultured cells *in vitro*, one may assume a similar behaviour *in vivo*. Additionally, human cells can be conveniently cultured with a low number of passages, resulting in minimal cell changes due to cell culture manipulation and would reduce bias concerning species origin and non-tissue specific cell lines.

Human bone marrow-derived mesenchymal stem cells were used as a model to address the effect of the extracts on the osteoblastic behaviour. Human umbilical vein endothelial cells (HUVECs) were chosen to study endothelial activity. Osteoblastic and endothelial cells are essential in the regeneration of the bone periapical tissues.

Cell culture studies evaluate cytotoxicity through morphologic observation of the cultured cells in the vicinity of the test materials or their extracts, recording the number of detached cells, ALP activity, SEM observation, fluorescent measurements, and cell viability [92] [93] [94]. Studies on MTA showed that the cell response to the material depends on many factors such as the cell types and the choice of study duration [130], use of a fresh or cured material [89], frequency of changing the medium [92], the use of direct contact or extract of MTA [92], and the concentration of the material in the cell culture media.

Cytotoxicity is only one aspect of biocompatibility and therefore cytotoxicity tests alone cannot characterize a material as biocompatible or not. In the present study, in addition to cell proliferation, we also sought to investigate the effect of sealer's extracts on osteoblastic behaviour through an *ex vivo* osteogenic assay and the effect of sealer's extracts on endothelial cells through Matrigel assay and on angiogenesis through CAM Assay.

The *ex vivo* osteogenic assay was performed to assess the effect of the sealers' extracts on the regeneration of defects created on the parietal bone of neonatal mice.

On HUVECs, a Matrigel coating assay was performed where capillary-like structures will be formed *in vitro* when plated under or on top of a reconstituted basement membrane extracellular matrix (BME, Matrigel, EHS matrix, etc.). This morphological differentiation has been demonstrated with a variety of endothelial cells and involves several steps in blood vessel formation, including cell adhesion, migration, alignment, protease secretion, and tubule formation. This assay is the most widely used *in vitro* angiogenesis assay as it is easy to perform, rapid, quantifiable and with numerous applications, such as in the study of angiogenic and antiangiogenic factors, definition of mechanisms and pathways involved in angiogenesis, and definition of endothelial cell populations. It has also been successfully used to study processes ranging from wound repair and reproduction to development and tumour growth.

BD Matrigel Basement Membrane Matrix is a solubilized basement membrane preparation extracted from the Engelbreth-Holm-Swarm (EHS) mouse sarcoma, a tumor rich in extracellular matrix proteins to include laminin (a major component), collagen IV, heparan sulfate proteglycans, and entactin/nidogen. It also contains TGF-beta, epidermal growth factor, insulin-like growth factor, fibroblast growth factor, tissue plasminogen activator, and other

growth factor which occur naturally in the EHS tumor. It is effective for the attachment and differentiation of both normal and transformed anchorage dependent epitheloid and other cell types, such as neurons, Sertoli cells, chick lens, and vascular endothelial cells, and hepatocytes.

An *in vivo* angiogenesis assay was also performed to observe the effect of the sealers' extracts on angiogenesis. Currently, tissue responses to biomaterials are evaluated *in vivo* using mammalian models such as mice, rats and dogs, however they are time and labour intensive, expensive and do not allow continuous evaluation of the tissue reactions to the implant, rather requiring surgical implantation, animal sacrifice, tissue procurement, processing, and histopathological evaluation. However, there is an alternative *in vivo* approach named chicken embryo CAM assay that is a non-mammalian model for studying angiogenesis since 1970s by Judah Folkmann [123]. CAM is an extraembryonic membrane formed in day 4 of incubation of fertilized eggs (consists in the fusion of allantois derived from the mesoderm with the chorion epithelium derived from the ectoderm) where primitive vessels proliferate and differentiate into an arteriovenous system until day 8, originating a network of capillaries that migrate to occupy an area beneath the chorion and mediate gas exchanges with the outer environment. Rapid capillary proliferation goes until day 11 where their mitotic index declines just as rapidly, and the vascular system attains its final arrangement on day 18, just before hatching [124]. The CAM serves as a support for the extraembryonic respiratory capillaries, actively transports sodium and chloride from the allantoic sac and calcium from the eggshell into the embryonic vasculature, and forms part of the wall of the allantoic sac, which collects excretory products. Thus, CAM is an excellent structure commonly used *in vivo* to study both new vessel formation and its inhibition in response to tissues, cells or soluble factors.

While there have been many variations developed over the years, such as *in vivo* use of CAM vasculature for embryonic or tumoral and *in vitro* use of CAM vasculature utilizing a Petri dish, the basic assay is performed by implanting a membrane or coverslip containing the compound of interest on the chick embryo CAM through a hole cut in the egg shell. The subsequent incubation period ranges from 1-3 days, depending on the compound, after which time angiogenesis can be quantified via image analysis or colorimetric detection methods. Although rabbit cornea is used just as often as an *in vivo* assay, CAM offers the advantage of being quicker, technically simpler, and inexpensive allowing several assays to be carried out simultaneously in the same embryo, especially when the *in vitro* approach is used instead. On the other hand, there are only very few restrictions to using CAM, essentially due to: non-specific inflammatory reactions that may develop and pre-existing vessels present which makes it hard to distinguish the extent of angiogenesis and anti-angiogenesis. Also, other drawbacks include limited time of implantation (7-10 days) and the fact that it is labour intensive due to the large number of eggs that are required to obtain consistent results. The investigator should note that this is a non-mammalian system which should be taken into consideration when interpreting results.

A huge number of scientific reports describe the properties of MTA, highlighting especially the biocompatibility of this cement in comparison with any other developed endodontic sealer or cement.

In this study, ProRoot MTA extracts presented some cellular cytotoxicity on day 1 of the culture with L929 fibroblasts, however on day 4, the cultures recovered and the proliferation increased in all the extracts.

In hMSCs cultures, ProRoot MTA extracts were very similar to control on day 1, and had increased in proliferation on the next time points. By the last day of culture, extracts with lower concentration (1:20 and 1:10) had more cell proliferation than control. Cell morphology, ALP activities, ALP and collagen staining results were also in agreement with proliferation results, being also dose and time-dependent. Along with MTA Plus, it was the sealer with the best behaviour of cell proliferation, morphology, ALP activity, ALP and collagen staining. Besides these assays, an *ex vivo* osteogenic assay was performed where defects of mouse parietal bone were put in contact with medium with the extracts which resulted in a faster regeneration with extract 1:5 than 1:20.

In HUVECs cultures, ProRoot MTA extracts presented some cellular cytotoxicity on day 1 of the culture however, the culture recovered by day 4 of culture and the proliferation increased in all the extracts except on extract 1:2 where proliferation decreased. Besides this assay, a matrigel coating assay was performed which showed a network of cell attachments similar in both extracts, which could suggest a good influence on angiogenesis. An additional CAM assay was performed to assess angiogenesis when in contact with the sealer extracts, where a thin layer of blood vessels surrounded the filter, which is perhaps suggestive of being an angiogenesis stimulator.

As a general rule, on all the cell lines tested, the higher the concentration of the sealer, the lower the cell proliferation. Other studies also observed a dose-response effect of MTA extracts on cell toxicity *in vitro* at 3 days [120][125].

It is suggested that the greater cytotoxicity of recently manipulated MTA was due to the increased release of toxic components from the cement in aqueous solutions, which would have affected the cells morphology and their ability to adhere. Thus, the toxic effect of MTA cement repair before hardening can determine the minimum irritant material even when used in direct capping, because this cement is applied on the pulp immediately after manipulation with incomplete setting.

By using various cell culture systems, a number of investigations comparing the cytotoxicity and biocompatibility between different types of sealers and with distinct cell lines have been made.

- various studies have shown that freshly mixed and set MTA is less cytotoxic on cells [126], on human periodontal ligament (PDL) cell cultures after 24 hours [118] and more biocompatible in cells [127] than amalgam, Super EBA or IRM.
- cytotoxicity and cell attachment investigations with various cell cultures showed better results with MTA in comparison to amalgam, Super EBA, IRM and various types of glass ionomers [128][129][130]. In contrast to these studies, an experiment with mouse fibroblast and macrophage cells on freshly mixed and set MTA, IRM, amalgam and Retroplast found no significant difference between amalgam and MTA [122]. In another study on murine pre-osteoblastic clone cell cultures, MTA and Super Bond resin showed no significant difference in cell adhesion and proliferation [131]. In a study on rat bone

marrow cells, MTA did not inhibit cell growth but suppressed osteoblast- like cell proliferation [132].

- one study reported that that MTA induces an osteogenic phenotype, which reflects up-regulation of the expression of ALP, osteonidogen, osteonectin, and osteopontin [133]. It also showed that MTA and amalgam showed more PDL fibroblasts proliferation when compared with gingival fibroblasts and that 24-hour cured MTA shows a more favourable response than freshly mixed MTA [133]. On the other hand, WMTA induces proliferation and DNA synthesis of murine odontoblast-like and undifferentiated pulp cells, with no noticeable difference between fresh and premixed WMTA [134].
- other study compared the cytotoxicity of MTA, Dycal, and an adhesive resin cement using rat dental pulp cell cultures. Their data indicated that MTA has no cytotoxicity after 72 hours, increases mineralization by stimulating dental pulp cells and also increases the amount of bone morphogenetic protein-2 (BMP-2) production. In contrast, Dycal decreases BMP-2 production and increases cell death, whereas dentin adhesive cement has no effect [135]. It also shown production of type I collagen and osteocalcin expression in the presence of MTA [135] and confirmed cement conductivity, cement inductivity, and osteoconductivity of WMTA [136].
- a study evaluated the morphology of human fibroblasts in contact with ProRoot MTA before (fresh) and after hardening (24h) and observed that only a small number of viable cells remained adhered to the fresh material and that some cells exhibited morphological alterations. This fact may suggest a possible cytotoxicity of some components released from MTA in contact with cells [137].
- other study evaluated the cytotoxicity effects of three root-end filling materials on the human ECV 304 endothelial cell line after three experimental time intervals (24, 48 and 72h) and concluded that all three cements (ProRoot MTA, MTA Angelus and PC) showed an elevated cytotoxic effect that decreased gradually with time allowing the cell culture to repair and similar cell reaction patterns [94];
- other study studied the *in vitro* biological compatibility evaluating cell viability and number, cellular adhesion and morphology of osteoblast-like cells derived from human osteosarcoma (Saos-2 cell line) after contact with cement extracts for 72h and showed the typical osteoblast morphology, well spread, and quite close to confluence. It was also observed some dividing cells and some migrating cells into the porosities of the cement material and partially immersed in the cement bulk [138].
- other study compared the cytotoxicity of four root-end filling materials in contact with rat pulp cells (RPC-C2A) and human lung fibroblasts (MRC-5) after two observation periods (24 and 72h) using the XTT assay and observed different degrees of cytotoxicity effects in the following ascending order: ProRoot MTA – MTA Angelus ≤ Super EBA (modified zing oxide-eugenol) ≤ Vitrebond (resin modified glass ionomer cement) [139].
- other study evaluated the biocompatibility of three root canal filling materials in contact with L929 mouse fibroblasts using the MTT assay after 24h, where ProRoot MTA was significantly less cytotoxic than the intermediate cytotoxic iRoot SP and the severely cytotoxic AH Plus [140].
- other study evaluated the biocompatibility and cytotoxicity of five root canal sealers (ERRM Putty, ERRM Paste, IRM, Cavit G and grey ProRoot MTA) with human gingival fibroblasts during three experimental periods (1, 3 and 7 days) and showed significantly

lower cell viabilities for IRM and Cavit G in contrast with higher cell viabilities for ERRM Putty, ERRM Paste and GMTA [120].

- another study compared the cytotoxicity of four root-end filling materials in contact with human osteosarcoma MG-63 cells using the 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetazolium-5-carboxanilide (XTT) assay after three observation periods (1, 3 and 7 days), where ProRoot MTA and GIC showed good biocompatibility, compared with Ortho MTA [141].
- a study examined the cytotoxicity of Quick-Set comparing to WMTA with a murine odontoblast-like cell line (MDPC-23) using the methyl-thiazol-diphenyltetrazolium assay, flow cytometry and vital cell staining and concluded similar cytotoxic profiles [142]. Other study examined the osteogenic/dentinogenic potential by quantitative reverse transcription polymerase chain reaction, ALP enzymatic activities and Alizarin red S assay and concluded no differences between Quick-Set and WMTA with increased matrix mineralization and increased extracellular calcium deposition [143].

Because of differences in the chemical composition of WMTA and GMTA [63], several investigations have been performed on the cytotoxicity of both types of MTA and there is some disagreement among investigators [89][144][145]. One investigation on osteoblast cell cultures compared cured samples of WMTA and GMTA [89]: 1-day cured samples of both GMTA and WMTA showed more biocompatibility than the 28-day cured samples. In contrast to these results, another experiment found that 12-day cured GMTA significantly increases cell proliferation in comparison with 1-day cured GMTA [144]. Other study with human alveolar bone, fibroblast, and macrophage cell cultures showed no difference between GMTA and WMTA in terms of cytotoxicity and cell proliferation [145].

Another important factor of sealer *in vivo* application is that the release of some components to the surrounding tissues might result in benefits to the healing process. In this context, it has been shown that calcium oxide, one of MTA's major components, is converted into calcium hydroxide in the presence of water, elevating the surrounding pH to an alkaline pH which has a destructive effect on protein structures and may promote enzyme denaturation and also cell membrane damage [171]. Intriguingly, the occurrence of chemical irritation by endodontic materials, such as calcium hydroxide, may not be so detrimental after all as the presence of a non-extensive inflammatory process on the underlying tissue may lead to the stimulation of tissue repair. A thin necrotic zone initiates inflammation, which in turn results in cell migration, proliferation and in new collagen deposition [172]. As demonstrated in one study, necrotic areas were observed at early stages after MTA subcutaneous insertion however, longer experimental periods demonstrate presence of connective tissue and absence of inflammatory infiltrate [173].

In this study, MTA Plus extracts also presented some cellular cytotoxicity on day 1 of the culture with L929 fibroblasts, however on day 4, the cultures recovered and the proliferation increased in all the extracts.

In hMSCs cultures, MTA Plus extracts were similar to control on day 1, and had increased in proliferation on the next time points, except on extracts 1:5 and 1:2 where it decreased by the last day of culture, while extracts 1:20 and 1:10 had more cell proliferation than control. Cell morphology, ALP activities, ALP and collagen staining results were also in agreement with proliferation results, being also dose and time-dependent. Along with ProRoot MTA, it was the sealer with the best behaviour of cell proliferation, morphology, ALP activity, ALP and collagen staining. Besides these assays, an *ex vivo* osteogenic assay was performed where defects of mouse parietal bone were put in contact with medium with the extracts which resulted in a faster regeneration with extract 1:5, which was almost closed, than with extract 1:20.

In HUVECs cultures, some MTA Plus extracts presented cellular cytotoxicity on day 1 of the culture the culture recovered by day 4 of culture and the proliferation increased in all the extracts except on extract 1:2 where proliferation decreased. Besides this assay, a matrigel coating assay was performed which showed a network of cell attachments similar in both extracts, which could suggest a good influence on angiogenesis. An additional CAM assay was performed to assess angiogenesis when in contact with the MTA Plus extracts, and a thin layer of blood vessels surrounding the filter could be observed, which is perhaps suggestive of being an angiogenesis stimulator.

As a general rule, on all cell lines tested, the higher the concentration of the sealer, the lower the cell proliferation. Overall, MTA Plus extracts showed similar results to ProRoot MTA extracts which is expectable since, according to the manufacturer, they are similar in composition, with MTA Plus being ground finer and using a salt-free water-soluble polymer gel as the mixing vehicle to improve its washout resistance.

In a study aiming to evaluate the cellular viability, apoptosis and necrosis profiles, and oxidative stress levels exhibited by a rat odontoblast-like cell line (MDPC-23 cells) after their exposure to the grey (GMTAP) and white (WMTAP) versions of MTA Plus, they concluded that the cytotoxic effects are both time and concentration dependent, and that they possess negligible cytotoxic risks after the elution of their cytotoxic components. Because these risks are significantly lower than those imposed by ZnOE cement, a favourable *in vivo* tissue response is likely to occur. Finally, GMTAP may favour more cell growth and viability compared with WMTAP [146].

Judging by the apoptosis/necrosis profile, a recent study attributed the initial cytotoxicity of MTA Plus and ProRoot MTA to apoptosis rather than necrosis, as evidenced by detection of phosphatidylserine expression on the cell surfaces via the use of AnV. This suggests that the initial sites of irreversible damage by the cytotoxic agents are extracellular, whereas the nuclear membranes of those cells still remain intact [146].

In this study, MTA Fillapex extracts presented the cellular cytotoxicity on day 1 of the culture with L929 fibroblasts especially on extracts 1:5 and 1:2 which was even more severe on days 4 and 7. On extracts 1:20 and 1:10, an increase in proliferation was verified; however it was significantly lower than the control.

In hMSCs cultures, extracts from MTA Fillapex started presenting inhibition of proliferation more noticeable on extracts 1:5 and 1:2, which would get even lower on the next days of culture. Only extract 1:20 showed increase in cell proliferation, even that it was lower than control. Cell morphology, ALP activities, ALP and collagen staining results were also in agreement with proliferation results, being dose and time-dependent and also representative of a severe cytotoxic profile. Over all the tested sealers, it was the sealer with the worst behaviour of cell proliferation, morphology, ALP activity, ALP and collagen staining. Besides these assays, an *ex vivo* osteogenic assay was performed where defects of mouse parietal bone were put in contact with medium with the extracts which resulted in a faster regeneration with extract 1:20, which was almost closed, than with extract 1:5 where it did not even had closed half of the defect by the end of the culture time.

In HUVECs cultures, MTA Fillapex extracts presented cellular cytotoxicity on day 1 of the culture, which was higher with high concentration extracts. On the next days of culture, cell proliferation decreased, mostly on extracts 1:5 and 1:2 but also on extract 1:10. On extract 1:20, a small increase in proliferation was observed on day 4, even that it was a lot lower tha control. Besides this assay, a matrigel coating assay was performed which showed a good network of cell attachments on extract 1:20, but not on extract 1:5 where cells were not abundant. An additional CAM assay was performed to assess angiogenesis when in contact with the MTA Fillapex extracts, and no significant features were observed as the blood vessels were converging through the filter with the extract.

As a general rule, on all cell lines tested, the higher the concentration of the sealer, the lower the cell proliferation. According to the present study, MTA Fillapex displayed the highest cytotoxic rates in a dose-dependent manner, having less cell proliferation in all the tested cell lines, less ALP activity and less ALP and collagen staining than the other sealers.

The addition of certain components in MTA Fillapex, such as resins [147], bismuth oxide [148] and other pigments, may have increased the toxic effect of this sealer compared to its precursor, ProRoot MTA. A previous study that evaluated the effect of resin salicylate on human fibrosarcoma cell line (HT-1080) by using MTT assay showed 25% of cellular apoptosis after 24 hours of exposure and by histologic examination where clear signs of apoptosis such as cell rounding, shrinkage, presence of vacuoles and fragments of genetic material in the cytoplasm were observed. The mechanism by which this apoptosis occurs is still unclear, but this study showed that the higher the concentration of salicylate, the higher is the cell death rate [149]. This finding is consistent with the results of our study, because the most concentrated dilutions of MTA Fillapex levels have caused evident cytotoxicity and cell death.

Other studies have been performed. However the results related to the biological response of MTA Fillapex are conflicting.

- A study with chinese hamster fibroblasts (V79) showed that freshly mixed MTA Fillapex was the most cytotoxic in comparison with the intermediate cytotoxic AH Plus and the less cytotoxic white MTA Angelus, however after 48h it developed the best behaviour even at less diluted concentrations of 1:2 and 1:4 where an increasing level of cell viability could be seen [119].

- Other study with human osteoblasts, showed that all tested materials (Sealapex, Pulp Canal Sealer EWT, Real Seal and MTA Fillapex), prepared 1 or 7 days after the sealer manipulation, had high cytotoxic levels for human primary cells, mostly on a time-dependent basis, as shown by three different cell viability tests: mitochondrial activity (XTT), membrane integrity (neutral red test) and total cell density (crystal dye exclusion test). MTA Fillapex extracts displayed a significant cytotoxicity [121].
- Another study with human tooth germ stem cells (hTGSCs) showed that MTA Fillapex had the highest cytotoxicity analysed using the MTS test compared with iRoot SP and AH Plus Jet after all the four experimental periods (1, 3, 7 and 14 days) [150].
- Other study with periodontal ligament fibroblasts showed that MTA Fillapex presented the highest cytotoxicity rates in a dose-dependent manner than WMTA and PC [125]. In this study the fibroblasts were in contact with the extracts for 24h only and the culture medium was in contact with materials after 24, 48 or 72h of setting time.
- In another cytotoxicity evaluation study of eight root canal sealers with Balb/c 3T3 fibroblasts over long periods of analysis, MTA Fillapex showed a severe cytotoxicity when cells were exposed to the fresh extract of the sealer that did not decrease much over the time of five weeks in comparison to the other root canal sealers: AH Plus, Epiphany, Endomethasone N, Endo REZ, Pulp Canal Sealer EWT, Roeko Seal and Sealapex [151].
- Another study showed that when this sealer was implanted in subcutaneous tissues in rats, it remained toxic even after 90 days [152]. However, a recent study showed that despite these initial toxic effects during setting, the cytotoxicity of MTA Fillapex decreases, and the sealer presents suitable bioactivity to stimulate nucleation sites for the formation of apatite crystals in human osteoblast-like cell culture [153].

Some subcutaneous studies showed that MTA Fillapex produced a moderate chronic inflammatory reaction evident on day 7 that resolved over a short period of time (15 days) with many collagen fibers indicating normal tissue healing [154], similar to that induced by MTA Angelus and faster than that induced by Sealapex [155]. Comparing MTA Fillapex with a zinc-oxide based sealer (EndoFill) and an epoxy resin-based material (AH Plus), AH Plus promoted more favorable results showing a moderate inflammatory response after 7 days, which became mild and well limited for fibrous condensation after 60 days [156]. In an application model study aimed to evaluate the healing of chronic apical lesions in canine teeth, it was concluded that the endodontic treatment performed in a single session using MTA Fillapex and Sealapex cannot support complete healing of the periapical tissues of canine teeth [157]. An antibacterial activity study concluded that MTA Fillapex along with Endofill had an effect against *E. faecalis* before setting, however they did not maintain the antibacterial activity 7 days after mixture [158].

Biodentine is a new bioactive cement based on calcium silicate for pulp capping, derivation of bioengineering, with anti-inflammatory behaviour, different from the classic materials based on calcium silicate, such as MTA. The technology behind the manufacturing process of the active bio-silicate, the main constituent of Biodentine, removes the metallic impurities which are present in other cements. The setting reaction involves the hydration of tricalcium silicate, the production of a calcium silicate-based gel and calcium hydroxide, which in contact with phosphate ions, it is able to create precipitated similar to hydroxyapatite.

In this study, Biodentine extracts also presented some cellular cytotoxicity on day 1 of the culture with L929 fibroblasts, however on days 4 and 7, the cultures recovered and the proliferation increased considerably, with the exception of extract 1:2 where the increase was minimal.

In hMSCs cultures, cell proliferation with Biodentine extracts was similar to control on day 1 with the exception of extract 1:2 which was significantly lower. Cell proliferation increased on day 7, but decreased on days 14 and 21. Cell morphology, ALP activities, ALP and collagen staining results were similar with proliferation results, being also dose and time-dependent. Besides these assays, an *ex vivo* osteogenic assay was performed where defects of mouse parietal bone were put in contact with medium with the extracts which resulted in a faster regeneration with extract 1:20, which was almost closed, than with extract 1:5 where it did not even had closed half of the defect by the end of the culture time.

In HUVECs cultures, cell proliferation with Biodentine extracts was similar to control on day 1 of the culture, except with extract 1:2 that was inhibitory to cell proliferation. Cell proliferation increased in the cultured time on all extracts, except on extract 1:2 where proliferation decreased. Besides this assay, a matrigel coating assay was performed which showed a network of cell attachments similar in both extracts, which could suggest a good influence on angiogenesis. An additional CAM assay was performed to assess angiogenesis when in contact with the Biodentine extracts, and no significant features were observed as the blood vessels were converging through the filter with the extract.

As a general rule, on all the cell lines tested, the higher the concentration of the sealer, the lower the cell proliferation. Comparing to ProRoot MTA and MTA Plus, cell proliferation with Biodentine extracts was lower with L929 fibroblasts, similar with hMSCs and higher with endothelial cells. On all cell lines, cell proliferation was higher than MTA Fillapex.

Though the information accumulated so far regarding the biocompatilibility of Biodentine is rather limited, the available data generally is in favour of the material in terms of its lack of cytotoxicity and tissue acceptability.

Cytotoxicity effects of Biodentine and MTA have been tested on:

- Human pulp cell cultures, concluding an absence of cytotoxicity for Biodentine compared with MTA [110] and murine pulp cell cultures, observing an initial inhibitory effect on proliferation during the first 2 days when cells were exposed to Biodentine followed by an increase in proliferation when compared with UT cells [111];
- Human dental pulp stem cells (hDPSCs) to examine the influence of various concentrations of Biodentine (BD 0.02, BD 0.2, BD 2, BD 20) on its proliferation, migratory and adhesion effect *in vitro* during four experimental periods (1, 3, 5 and 7 days) [159]. It was observed that BD 0.2 and BD 2 concentrations significantly increased proliferation of stem cells and BD 20 significantly decreased the proliferation. Biodentine favourably affected healing when placed directly in contact with the pulp by enhancing the

proliferation, migration, and adhesion of hDPSCs, confirming the bioactive and biocompatible characteristics of the material [159].

- Human gingival fibroblasts, observing a similar nonsignificant result with MTA [160]. The viabilities of cells exposed to extracts derived from all the materials tested were highly dependent on extract concentration, and the viabilities of cells exposed to extracts from Biodentine and MTA at low extract concentration (1:8) showed no significant difference between each other and compared with the DMEM control [160]. Extracts from GIC Fuji IX caused significantly more cell death at all extract concentrations than extracts from Biodentine, MTA, and the DMEM control after culture for 1 day, probably due to small amounts of aluminium and/or iron ions present in GIC extracts [160];
- Mouse embryonic fibroblast cells (fibroblast 3T3 cells), observing similar behaviours with MTA and UT controls at all-time points, with the exception of 6 hours when cell viability was decreased for both compounds [161]. GIC was included as a control because it has been previously shown to be more cytotoxic than MTA [162] and in the study showed consistently lower levels of cell viability throughout the time course compared with control cells and both MTA and Biodentine treatments.
- MG63 osteoblast-like cells, observing similar behaviours with MTA, where cell viability was inversely correlated with incubation time [163]. After 24h, the two materials revealed high levels of cell viability and no cytotoxicity was observed. However, after 3 days of incubation, both materials induced a slight reduction in viability and following 5 days of incubation, the materials were also rated as slightly cytotoxicity [163].
- Mouse odontoblasts cell line (MDPC-23), observed that Biodentine proved to be the more biocompatible material compared with ProRoot MTA, MTA Angelus and Dycal. Biodentine reported percentage of vitality measured by Alamar blue test and MTT assay above the negative control on measures from the 24, 48 and 72 hours [164]. Other study by the same author and with the same cell line showed that Biodentine and MTA Angelus exhibited the highest percentage of cell cytocompatibility measured by the MTT assay if compared with the other pulp-capping materials (Calcitur, Calcimol LC, Dycal and Theracal LC) [165].

It has a positive effect on vital pulp cells and stimulates tertiary dentin formation [109] and when in direct contact with vital pulp tissue, it also promotes formation of reparative dentin [166]. This material is reported to induce pulp cell differentiation and biomineralization *in vitro*, suggesting its ability to stimulate reparative dentinogenesis after direct pulp capping [109][166]. A study demonstrated that Biodentine showed more prominent Ca and Si incorporation by adjacent human root canal dentine when immersed in PBS for up to 90 days compared with WMTA [167] and BC sealer [168] due to the lower ion-releasing abilities of these materials.

A biocompatibility study aimed at assessing the biological effects of Biodentine for use in pulp-capping treatment on pseudo-odontoblastic (MDPC-23) and pulp (Od-21) cells indicate that media conditioned with Biodentine and MTA may slightly modify the proliferation of these cells which vary over time and differ between cell lines [169]. This study uses a spheroid model to study cytotoxicity and validates, in terms of the behaviour of pulp cells, indications for Biodentine similar to those of MTA [169].

This material was tested *in vivo* in animals to evaluate the its capacity to induce pulp healing in a rat pulp injury model [170]. The researchers observed formation of a dentin bridge

at the injury site after 30 days that was secreted by cells displaying an odontoblastic phenotype in all evaluated materials: Biodentine and MTA induced homogenous reparative structures and in continuity with primary dentin. Dentin tubules could be clearly observed in Biodentine, and the cells secreting this structure displayed odontoblastic characteristics; calcium hydroxide ($Ca(OH)_2$) sealer induced reparative tissue with a porous organization, suggesting a different reparative process [170].

The studies are generally in favour of this product in terms of physical and clinical aspects despite a few contradictory reports. Though accumulation of further data is necessary, Biodentine holds promise for clinical dental procedures as a biocompatible and easily handled product with short setting time. As more research is performed regarding this interesting alternative to MTA, more reliable data will be provided and more confidently Biodentine will be implemented into routine clinical applications.

Other tests that could be performed are:

- other cytotoxicity assays such as metabolic activity of the cells assessed by the MTT assay, which is based on the reduction of tetrazolium salt, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium) by mitochondrial dehydrogenases which are expressed only in vital cells and are inactivated shortly after cell death. As a result, it occurs the formation of purple crystals, designated formazan crystals and the spectophotometric determination of the colored product is conducted. Accordingly, formation of highly colored formazan dyes is indicative of a metabolically active cell population.
- genotoxicity tests, which are essential for assessing the risks caused by toxic materials on human genetic material (30). One example is MNT, which is based on loss of entire chromosomes or their fragments during cell mitosis that are not reinstated by the nucleus after cell division and therefore are transformed into smaller nuclei or micronuclei.
- analysis of the mode of cell death (apoptosis/necrosis) via flow cytometry and fluorescent microscopy is helpful in better understanding the cytotoxic effects of the test materials on cell permeability.

The conclusion that MTA-based pulp-capping material does not show cytotoxic effects *in vitro* should be taken with caution because the experimental design *in vitro* has some inevitable limitations with respect to the *in vivo* situation, where cellular responses and inflammatory and/or reparative reactions may differently influence the effects.

Within the limits of the present study, it may be concluded that the initial cytotoxic effects imposed by ProRoot MTA, MTA Plus and Biodentine may be attributable to the high pH value of the components of each material and is both time and concentration dependent. However, among the tested materials, MTA Fillapex displayed even higher cytotoxic levels along the time of culture to L929 fibroblasts, HUVECs and hMSCs *in vitro*. Based on the results of this study, ProRoot MTA and MTA Plus appear to be more suitable as root canal sealers, and further studies should be performed in order to investigate its biological effects. Nonetheless, the possible clinical effects of the sealers should be investigated *in vivo*, as the cytotoxicity levels of pulp capping materials may not be the indication of their clinical success.

VI: CONCLUSION

Root canal sealers are intended to be contained within the root canal space, but they may extrude through the apical constriction, or eluents from the sealers may come into contact with periradicular tissues. This can affect the normal bone metabolism and regeneration. Regarding this, it is known that angiogenesis is critical in the bone environment for the normal bone remodeling and regenerative events. Accordingly, biological evaluation of endodontic sealers benefit from the biocompatibility profile concerning bone formation and angiogenesis.

This work compared four calcium silicate-based sealers, namely ProRoot MTA, MTA Plus, MTA Fillapex and Biodentine, regarding the effects of the sealers' extracts on osteoblastic cell behaviour, by using human bone-marrow derived mesenchymal stem cell cultures and an *ex vivo* osteogenic assay, and on endothelial cell behaviour, by using human endothelial cell cultures and an *in vivo* angiogenesis assay.

The effects of the sealers' extracts were dependent on the concentration and the time of exposure. Regarding the *in vitro* studies, the toxicity profile was similar for endothelial and mesenchymal stem cells regarding proliferation and functional parameters, however, endothelial cells were more sensitive to the toxic effects observed at the higher extract concentrations. ProRoot MTA and MTA Plus presented the best citocompatibility profile, followed by Biodentine and MTA Fillapex. This pattern of toxicity was also observed in the *ex vivo* regeneration of the rat parietal bone defects. However, on the CAM assay, no significant differences were observed on the angiogenic response elicited by the extracts of the four sealers.

Overall, this study corroborates the low toxicity of calcium silicate-based sealers, but with ProRoot MTA and MTA Plus presenting the best biocompatibility profile.

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