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Remodelling compartment in root cementum

Running head: Dental remodelling compartment

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ABSTRACT

Bone remodelling represents the most remarkable bone response to mechanical stress and mineral homeostasis. It is the consequence of complex highly orchestrated and tightly regulated cellular processes taking place in a specialized entity - the Bone Remodelling Compartment (BRC).

Cementum is an understudied tissues that requires more research to understand its biology, pathology, and potential for regeneration. Although analogue to bone in structure and composition distinct structural and functional differences were ascribed to each of these mineralized tissues. The precise role of cementocytes in cementum turnover is unclear but they may work the same way as osteocytes in bone remodelling, regulating the full process. Although cementum is not liable to regular physiological remodelling as bone is, pathological cases triggered by orthodontic forces or large periapical periodontitis, those lesions can acutely induce cementum remodelling. Nevertheless, the cellular mechanisms behind this

particular remodelling process are yet to be identified, as its eventual involvement of specialized anatomic structures as the BRC.

Hypothesizing that similar cellular mechanisms underlie bone and cementum remodelling, the present work shows, for the first time, the histological evidence of a specialized remodelling compartment in dental hard tissues.

Key words: bone remodelling compartment, bone, cementum, cementocytes, root resorption

INTRODUCTION

The importance of a specified structure overlying the bone-remodelling unit named Bone-Remodelling Compartment (BRC) has been underlined by several authors (Hauge et al. 2001, Andersen et al. 2009, Jensen et al. 2012, Parfitt 2001, Kristensen et al. 2013). This structure unit is formed by a canopy of bone lining cells, under which the remodelling process takes place isolated from the remaining intact tissues (Hauge et al. 2001, Jensen et al. 2012, Parfitt 2001, Kristensen et al. 2013, Feng and Teitelbaum 2013). The specific and non-random localization of blood capillaries and sinusoids close to the BRC ensures a fast access and targeted delivery of systemic regulators to the remodelling compartment, enabling guidance of osteoclasts and osteoblasts progenitors to critical points on the bone surface (Hauge et al. 2001, Andersen et al. 2009, Lafage-Proust et al. 2015). By secreting critical factors and expressing specific receptors, canopy cells along with osteocytes are the main orchestrators of the overall remodelling process, being the canopy an important reservoir of osteoblast progenitors during remodelling. (Kristensen et al. 2014).

Bone resorption and formation is synchronized by direct cell contact between osteoblasts and osteoclasts and a variety of secreted factors (Sanchez-Fernandez et al. 2008). It has also been established that the tight equilibrium between these two cellular populations is fundamentally maintained by a triad of elements composed by osteoprotegerin (OPG), the receptor activator of NF-κβ (RANK) and the RANK ligand (RANKL) (Lafage-Proust et al. 2015, Sims and Gooi 2008, Martin et al. 2009, Boyce and Xing 2008). However, osteocytes prevailed as the main orchestrators of the overall remodelling process, as they are the only cells with the lacuno-canalicular network, endowed with the capability to detect load changes, and consequently activate cellular signalling cascades aimed at either bone reabsorption or remodelling (Parfitt 2001, Tatsumi et al., 2007). In fact, several studies had

demonstrated that osteocytes are the major source of RANKL in the bone tissue, and not the osteoblasts (Bellido 2014, Sapir-Koren and Livshits 2014, Nakashima et al. 2011).

Osteocytes act as mechanossensing cells and initiate the remodelling process predominantly when microfractures and loading are involved (Proff and Romer 2009, Verbogt et al. 2000, Goulet et al. 2008). In these situations, osteocytes' physiology is profoundly altered and apoptosis is triggered (Vebogt et al. 2000, Xing and Boyce 2005, Krishnan and Davidovitch 2009). As a consequence, osteoclastogenesis and bone reabsorption prevail, thus driving deep changes in bone tissue architecture (Kurata et al. 2006, Henriksen et al. 2009). It is reasonable to conclude that the osteocyte acts as the leading mechanosensor in bone, which has also been recently confirmed by targeted ablation of osteocytes in a mouse model (Tatsumi et al., 2007).

Cementum prevails as the least-known mineralized tissue and is often referred to as a bone-like tissue. It is, however, avascular and non-innervated, it lacks marrow and the typical lamellar organization of the bone, and it does not undergo dynamic remodelling or increased thickness throughout life (Yamamoto et al. 2016). On contrary, both cementocytes and osteocytes are sensitive to mechanical features, mainly pressure and trauma (Nanci and Ten Cate 2003, Huang et al. 2009). Finally, cementocytes express RANKL and sclerostin, thus underlying their particular importance on triggering cementum resorption, remodelling and repair, which eventually occurs in a similar way as bone remodelling (Jäger et al. 2010, Van Bezooijen et al. 2009, Lehnen et al. 2012). Although cementum is not exposed to regular physiologic remodelling, recent evidence showed that cementum resorption might be triggered under specific circumstances as, for example, orthodontic forces or large apical periodontitis lesions.

The purpose of this work was to evaluate the effect of a distraction protocol on bone remodelling and root surface of the anchoring teeth.

The results hereby presented describe the identification of a specialized remodelling structure, which we refer to as the cementum remodelling compartment (CRC). It is associated with cementum at the dental root surfaces, and this work provides a description on its light microscopic morphology and cellular phenotype in comparison to the BRC.

MATERIALS AND METHODS

Animals

Four skeletal mature conditioned male Beagle dogs, 1 year old and weighing 15 to 18 kg, were selected for the study. Animals were kept in adequate standard structures, precisely

identified and with the appropriate conditions of light and temperature. The water, administered *ad libitum* to the animals, was originated from the municipal supply network. Food was provided once daily, as commercial dry feed (Canine Adult Lamg & Rice, Chicken Advanced Fitness, HillsTM) with concentrations of contaminants analysed and monitored by the manufacturer. After surgical intervention and during latency and mandibular stretching phases, the dry feed was softened in warm water (ratio 1:3). From the consolidation period until euthanasia, the softened food was mixed with water in a ratio 1:1. All animal procedures were conducted according to the EU Directive 2010/63/EU for animal experiments and reviewed and approved by DGAV (no 0420/000/000/2012) and the animal facility ethics committee.

Anaesthesia protocol

Dogs were anesthetized by intravenous administration of 0.2 mg/kg Diazepam (Diazepam Labesfal, Portugal) and 2 mg/kg Propofol (Propofol Lipuro 2%, Braun Medical, Portugal), and maintained alive with inhalation of oxygen and 1-2 % isoflurane (Isoflo, Esteve Farma, Portugal).

Experimental group

Three animals underwent mandibular distraction following bilateral midbody osteotomy (between the third and fourth premolar), to preserve the integrity of the mandibular nerve and lingual periosteum. After ensuring bone mobility, tooth distractor was placed in each hemimandible, using the third and the fourth premolars as anchorage teeth for the distraction apparatus. The 6 hemimandibles were subjected to a repeated daily activation of 1 mm, with moderate to severe pressure applied to the anchorage teeth for 10 days (10 mm in total), followed by a consolidation period of 12 weeks.

Control group

Two hemimandibles underwent no treatment. The animal was kept under the same conditions as its experimental counterparts, for the same period of time.

Euthanasia protocol

Animals were euthanized by anaesthetic overdose (Pentobarbital 100 mg/kg intravenously, Pentotal, Abbot, Portugal), followed by bilateral perfusion with 10 % phosphate buffered formalin (PRS Panreac, Spain).

Histological analysis

Histological processing was carried out at the Hard Tissues Laboratory of the Faculty of Medicine of the University of Coimbra. Half of the samples were prepared for histological evaluation in non-decalcified material and the other half for decalcified material. Mandibular blocks were dissected. Half of the samples from the anchorage teeth were processed and embedded in methylmethacrylate, sectioned and ground to a thickness of 20 μm on Exact Cutting-Grinding System (Exakt® Apparatebau, GmbH & CO, Norderstedt, Hamburg, Germany). Mandibular blocks were dissected, post-fixed with 10 % phosphate buffered formalin and subsequently decalcified with Morse's solution for 8 weeks. The specimens were then trimmed and embedded in paraffin wax, and thirty serially sections with 6 μm each were subsequently prepared. Toluidine blue, hematoxylin-eosin and Masson trichromic were used to stain the specimens for bone tissue, periodontal tissue and the radicular structure. Histological assessment was performed under a light microscope (Nikon® SMZ 1500, Tokyo, Japan), attached to digital cameras (Optronics® DEI 750D CE, Goleta, California, USA and Nikon® Digital Camera DXM-1200 C, Tokyo, Japan) connected to a computer (Intel® Pentium™ III and Intel®Core™ 2 Duo Core™) with an image software analysis program (Nikon® ACT-1C, Tokyo, Japan) for the evaluation of the bone tissue and root surfaces. One observer blinded to all group's allocation examined all specimens. The images were prepared and edited with Adobe Illustrator CC 2017, Adobe Systems.

RESULTS

Following the experimental protocol, dogs were euthanised and the mandibles collected and processed for histological analysis. Not surprisingly, hemimandibles from the control group displayed normal radicular architecture and bone histology (Figure 1).

In the experimental group, however, although the distraction protocol did not induce alterations in either the routine or the alimentary habits of the animals, bone remodelling was observed inside the BRC in both groups (Figures 2 and 3). The first evidence of bone reabsorption was indicated by the detachment of the bone lining cells from the bone surface, and the invasion of clastic cells to attach to the denuded bone matrix surface (Figure 2.1). Reversal cells were subsequently recruited to the BRC and repopulated the bone surface, and osteoblasts were embedded by mineralized bone matrix (Figure 2.2, black arrow). Figure 2.3 displays what is believed to be a pericyte (red arrow) lying just above the canopy. During bone formation phase, osteoblasts (OsB) populated the bone surface, layering osteoid, while

flattened cells with thin and flat nucleus bordering the marrow side and forming the canopy of the BRC (Figure 3).

Remodelling was also observed in discreet zones along the dental root (Figure 4). Surprisingly, however, it also implicated the formation of a special remodelling compartment, delineated by a canopy of flat cells in close proximity to the blood vessels (Figure 4.1). In fact, an Howship lacuna reaching dentine was observed, along with a layer of flattened cells (Figure 4.1, black arrow) that formed the equivalent to the canopy. Even though no clastic cells were identified, the ones close to the cementum and dentin surfaces displayed characteristics of reversal cells, and they also repopulated the resorption area during the formation phase. Because it was difficult to distinguish the canopy from the endothelial cells, a complete remodelling compartment could not be perfectly delineated. However, a singular cell-rich space between the mineralized tissues and a blood vessel was evident, resembling the remodelling progression in the characteristic cutting cones of the compact bone tissue (Figure 4.2). Moreover, by morphologic comparison to the bone tissue, the cell population adjacent to the dentin and cementum surface most probably represented the beginning of the reversal phase (Figure 4.2). Finally, the tissue apposition phase was also recognized in the cement remodelling process by the identification of a remodelling compartment next to a newly formed cementum area, covered by cementoid tissue and cementoblasts (Figures 4.3 and 4.4). Figure 4.3 illustrates in detail canopy cells and a layer of cuboid cementoblasts, morphologically similar to osteoblasts, adjacently placed to a region of non-mineralized cement. It also identifies a band of mineralized matrix of cementum repairing an area of resorbed dentin matrix, as well as a region of dentin portraying its typical tubular structure.

DISCUSSION

The present work provides evidence of organized cementum remodelling taking place inside specific structures along the dental root of distracted tooth, similarly to bone remodelling inside BRCs. These structures, named CRCs, are formed to enclose local factors and potentiate the remodelling process. They are also delineated by a flattened canopy of cells, and always localize in close proximity to blood vessels, thus allowing the trade of cells and/or systemic factors from the bloodstream.

Cementum is a highly responsive mineralized tissue, being its biological activity of extreme importance to maintain the integrity of the root, namely its adequate position. It shares morphological, functional and biochemical similarities with the bone tissue; however,

cementum is avascular and consequently depicts higher levels of hypoxia (Zhao et al. 2016, Van Bezooijen et al. 2009). There are two main types of cementum fibbers, acellular extrinsic fibbers (AEFCs) and cellular intrinsic cementum fibbers (CIFCs). According to Schroeder and collaborators, AEFCs are mostly involved in tooth support, and thus are the ones recruited following tooth distraction; while CIFCs, on the other hand, appear to be more associated with tooth adaptation, *i.e.*, the reshaping of the root surface during tooth' movement. CIFCs are also recruited as reparative cementum to fulfil the resorbed root surfaces (Schroeder HE 1992). The results attained in the present work come to support others in the literature postulating that given the similarities between the bone and cementum, it is reasonable to hypothesise that cementocytes may play a central regulatory role in the homeostasis and remodelling of cementum, not only following extrinsic stimulation, but also during the normal orthodontic movement and endodontic infections (Yamamoto et al. 2016, Rossi et al. 2016, Zhao et al. 2016, Schroeder 1992, Krishnan and Davidovitch 2006).

Cementum remodelling, as the bone, also depends upon a mechanic stimulus to be transmitted to the mineralized tissue matrix, and the consequent triggering of the reabsorption-formation cascade. In agreement, the application of a severe pressure to the anchored teeth of the animals in study, triggered a radicular cementum remodelling, along with alveolar bone remodelling and redistribution of the principal fibbers, thus driving dental repositioning. In fact, even though cementum is more resistance to resorption than bone, when orthodontic forces are applied, they not only disrupt cementum's structure, but proceed invading the root dentin (Krishnan and Davidovitch 2006). Studies in orthodontic patients to whom extrinsic forces were applied, showed that the application of an external pressure to the dental root activated cementoblasts, and consequently drove the removal of the cementum from the root surface, followed by a cementoblast-mediated restoration (Yamamoto et al. 2016, Kumasako-Haga et al. 2009, Tyrovola et al. 2008, Mullally 2010).

Root repair is constant during orthodontic tooth movement, and permanent total root loss only occurs if the tooth structure is not fully repaired (Hartsfield 2009). Using a mice endodontic infection model, Rossi and collaborators proved that in healthier mature teeth, cementocytes and osteocytes did not express RANKL. In response to infection, however, RANKL was strongly expressed by cementocytes and its expression increased along the lesion and time progression, an effect that was not observed in osteocytes (Rossi et al. 2016). The detection of a CRC emphasizes the fact that hard tissues remodelling is a demanding and controlled process that might not occur with external interferences; thus, a remodelling compartment is created to allow local regulation of the process. Simultaneously, the canopy

may act as a diffusion barrier for cytokines, growth factors and chemoattractants, thus allowing systemic factors to act over the cells inside the remodelling compartment (Martin et al. 2009, Owen and Reilly 2018). Moreover, its presence seems to have a crucial impact on the overall remodelling cycle success, as highlighted by several studies on diverse pathologic situations where the absence of BRC canopies beyond eroded surfaces was correlated with a greater amount of arrested reversal surfaces and a reduced amount of bone formation, thus reflecting both disrupted and arrested remodelling cycles (Wesseling-Perry 2014). Besides, the absence of BRC canopies above formative surfaces leads to a shift in the osteoblastic phenotype, which causes an arrest or even the non-progression of the formative phase. Altogether, these facts suggest that BRC canopies had a critical role for longevity, function and the control and coordination of the underlying cells activity (Wesseling-Perry 2014), and the same may be envisioned for the CRC. In agreement, remodelling areas with fragmented canopies were also observed in the animals under study, and were also associated to the interruption or arrest of the formative or inversion phases, driving cementolytic lesions that the authors hypothesise that may also occur in humans.

Limitations of this study

Limitations of this study include the fact the it is merely an observational study meant to report the existence and identification of the CRC. To determine the cellular ultrastructure as well as the nature and function of each type of cells involved, immunohistochemistry and electron microscopy would be needed. Additionally, more studies are needed to clarify the interactions of the multiple signal transduction pathways. It is also likely that the future holds the discovery of novel molecules that will lead to further insights into the mechanisms responsible for cementum formation and resorption.

CONCLUSIONS

This is the first study to identify and histologically describe a histologic remodelling structure in dental mineralized tissues, which the authors named CRC. The existence of such a remodelling compartment in cementum, and the fact that it shares so many defining characteristics with may, may hide the existence of a common underlying. Understanding physiology underlying the dynamics of cementum modelling and remodelling will be essential for the comprehension of its kinetics in orthodontics and trauma.

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Figure 1. Histologic section of an hemimandible attained from the control group dog depicting normal bone tissue architecture (Toluidine Blue, 7,5x in the original). Higher magnification of the periodontal apparatus (100x) showing the alveolar bone (**AB**), the space of the periodontal ligament (**PL**), cementum (**C**) and dentin (**D**).

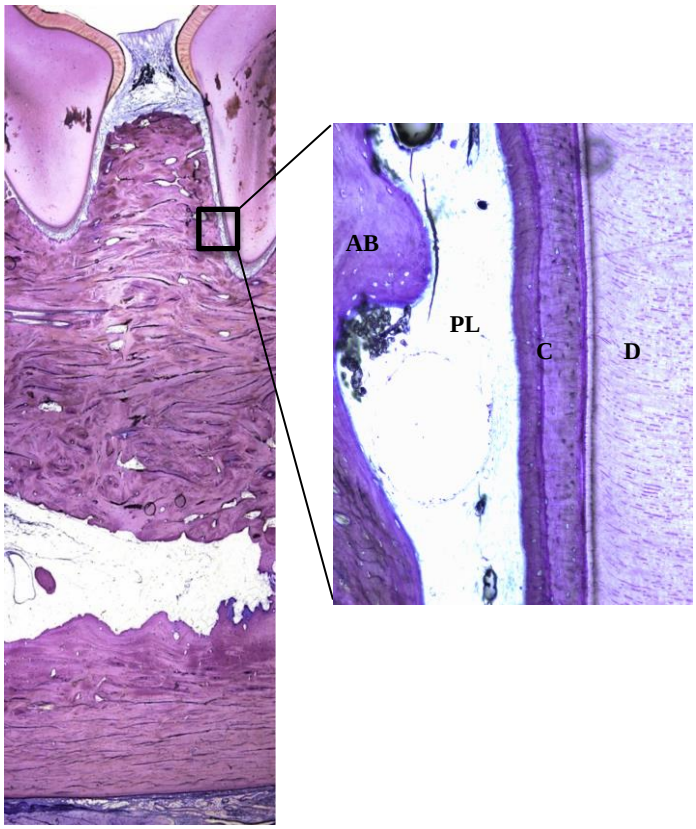

Figure 2. The bone remodelling compartment. 2.1. Microphotography of a bone remodelling compartment, in resorption phase, observed in the compact bone tissue. Howship's lacuna and several osteoclasts (*) are observed on the bone surface, in close proximity to a blood vessel (Toluidine blue, 400x in the original). **2.2.** Reversal phase. It is also observed of a blood vessel in close proximity to BRC as well as an osteoblast being embedded by the extracellular matrix (*black arrow*) (Toluidine blue, 400x in the original). **2.3.** Reversal phase of bone remodelling compartment, in compact bone tissue containing in its interior a great density of elongated cells, the reversal cells (*black arrows*). Note the presence a pericyte (*red arrow*) (Toluidine blue, 400x in the original). **2.4.** Morphological appearance of the initial formation of the BRC canopy in a trabecula of alveolar woven bone. (Hematoxylin-eosin, 1000x in the original).

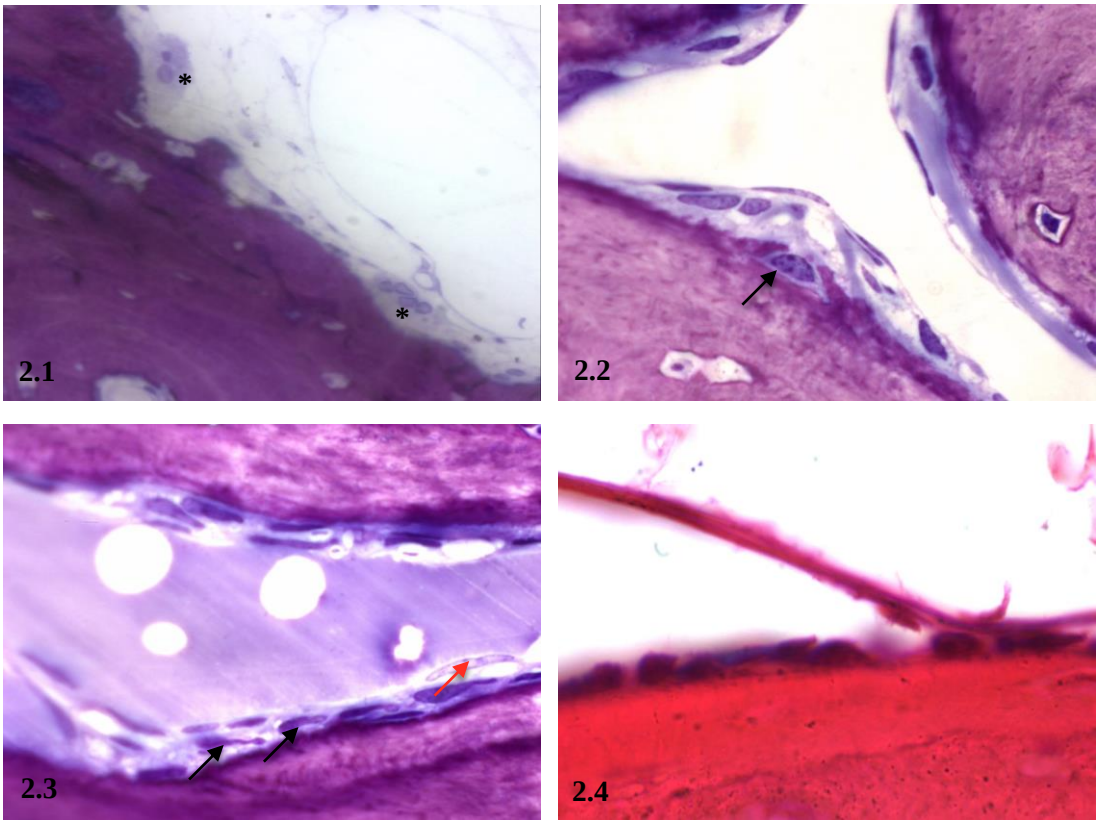

Figure 3. Osteo-angiogenic platform. 3.1. Histologic section of the capillary network located next to the canopy of a BRC, running along in a parallel direction in almost all its extension. Close proximity between endothelial cells (*) and lining cells (*black arrow*). Bone (**B**), Osteoblasts (**OsB**) (Hematoxylin-eosin, 200x in the original). **3.2.** Histological section of a capillary following a parallel path to the canopy of a BRC, in formative phase. Close proximity between endothelial cells (*) and lining cells (*black arrow*). Bone (**B**), osteoid (**Ost**), Osteoblasts (**OsB**), canopy (*black arrow*) (Masson's trichromic, 400x in the original). **3.3.** Microphotograph of a blood capillary following in close proximity the remodeling compartment. Cell intimately adjacent to the endothelial wall (*black arrow*) is seen as a pericyte (*red arrow*); Toluidine blue, 1000x in the original. **3.4.** Histological aspect of the constitution of a BRC in bone formation phase, located on the surface of a trabecula. Osteoblasts (**OsB**), coating cells that form the canopy (*black arrow*), the proximity of a blood vessel (*blue arrow*) that runs parallel path to the canopy. Bone (**B**), osteoid (**Ost**), Osteoblasts (**OsB**). Toluidine blue, 1000x in the original.

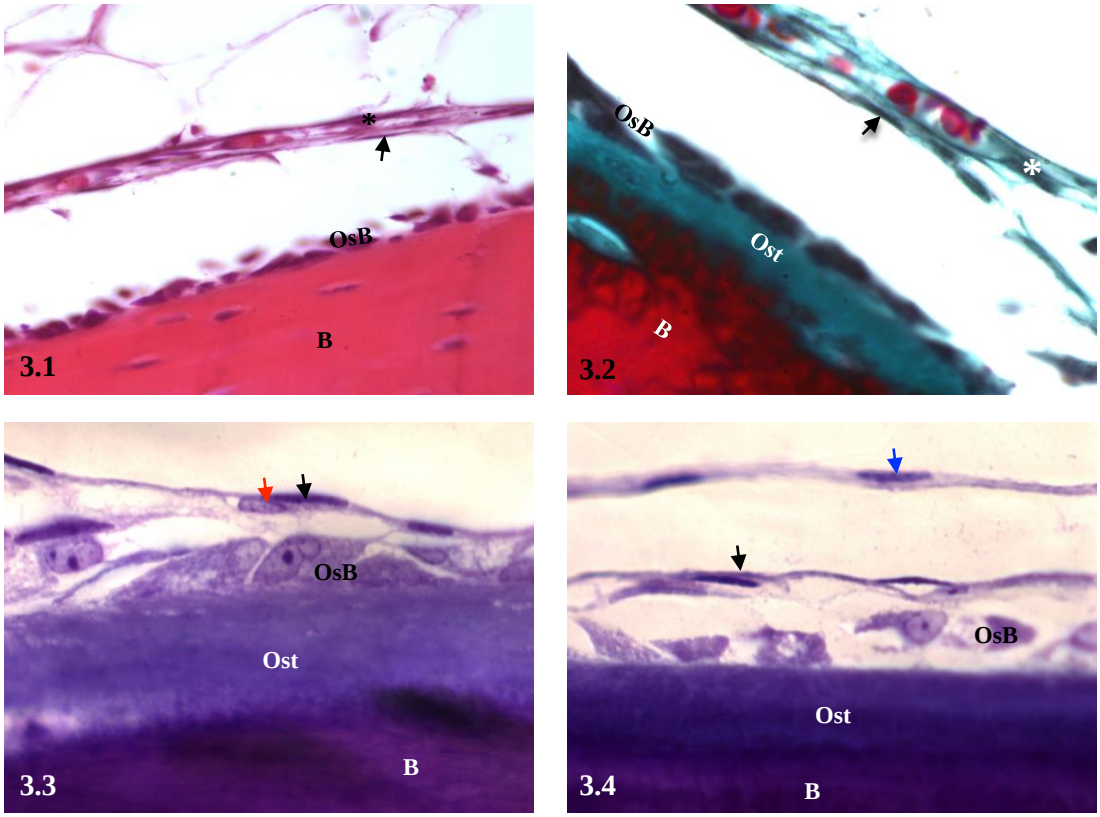

Figure 4. Dental Hard tissue Remodelling compartment. 4.1. Resorption phase observed in the cement reaching the dentin, showing a Howship lacunae (**HL**) even without the presence of odontoclasts. It is possible to observe some canopy cells (*red arrows*). Dentin (**D**), Cement (**C**). (Toluidine blue, 400x in the original). **4.2.** Dentine resorption area aspect (**D**) with the reversal cells (*black arrows*) close to resorption areas. It is obvious the resemblance to a cutting cone observed in compact bone tissue including the presence of a central blood vessel. (Toluidine blue, 1000x in the original). **4.3.** Appearance of a remodeling compartment in the formation phase located in the cementum adjacent to the dentin showing a cementum area (**C**) covered by cementoid (**Ct**) and cementoblasts (**Cb**), remodelling compartment (**CR**) and the canopy (*red arrows*). (Toluidine blue, 400x in the original). **4.4.** Analogous image to the previous, showing a dentine area (**D**), mineralized cementum (**C**), cementoid (**Ct**), a cementoblasts layer (**Cb**), a space corresponding to the remodelling compartment (**CR**) and the canopy (*red arrows*). (Toluidine blue, 1000x in the original).