Newly formed cementum induced by the osteogenic proteins of the TGF-ß supergene family

A comparative histomorphometric study of newly formed cementum as induced by different applications of osteogenic proteins

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A research report submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in partial fulfilment of the requirements for the degree

Master of Dentistry (Periodontics and Oral Medicine)

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Declaration

I hereby declare that this research report is my own unaided work, except where due acknowledgment for assistance received has been made. It is being submitted for the degree of Master of Dentistry (Periodontology and Oral medicine) in the University of Witwatersrand, Johannesburg. It has not been presented before for any degree or examination at this or any other University.

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Safiyya Hassam Essa

Signed this ……………………………day of…………………………………..2015

The work reported in this research report was performed at the Bone Research Unit, School of Oral Health Sciences, Faculty of Health Sciences University of Witwatersrand, Johannesburg, South Africa

Abstract

Background and Objective: Osteogenic proteins/ Bone morphogenetic proteins are soluble signals that have potent and pleiotropic functions which have enticed researchers to explore their role in periodontal regeneration. The potential of these proteins to induce cementum formation in a periodontal healing context has been previously demonstrated. However, is the ability to induce cementum uniformly applicable to different BMPs? Do some BMPs have more of a cementogenic effect than others? This study was designed to measure newly formed cementum induced by different osteogenic proteins of the transforming growth factor-ß supergene family.

Material and Methods: Histological sections prepared from previous periodontal regeneration studies of surgically created furcation defects of 26 mandibular molar teeth of *Papio ursinus,* exposed to different applications of BMPs were assessed and compared. The BMPs used in these periodontal studies included recombinant human osteogenic protein-1 (hOP-1), recombinant human bone morphogenetic protein-2 (hBMP-2) , binary application of hOP-1 and hBMP-2, recombinant human transforming growth factor β_3 (hTGF-β_3), synergistic application of hTGF- β_3 and hOP-1 as well as naturally-derived BMPs. Histomorphometric measurements of the extent of cementum formation along the mesial and distal root surfaces were made. The thickness of newly formed cementum was measured in three regions along the root surface in apical, middle and coronal regions.

Results: The BMPs which yielded the most favourable cementogenic outcome included hOP-1, hTGF-β_3 and the synergistic application of hOP-1 and hTGF-β_3. Naturally-derived highly purified BMPs and hOP-1 applications showed greater cementogenic effects with regards to the extent of cementum formed. Applications of hOP-1, hTGF-β_3 and the synergistic application of hOP-1 and hTGF-β_3 showed positive cementogenic effects with regards to the thickness of cementum formed along root surfaces. The hBMP-2 applications were found to be the least inductive category of BMPs in forming new cementum along the root surfaces, both in extent and thickness measurements.

Conclusion: BMPs show variable inductive potential for cementogenesis along exposed root surfaces of *P. ursinus* possibly reflecting a structure-activity profile of each tested protein.

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Abbreviations

BMPs	Bone morphogenetic proteins
BSP	Bone sialoprotein
CAM	Cell adhesion molecules
ECM	Extracellular matrix
FGF	Fibroblast growth factor
Gdn HCL	Guanidinium hydrochlroide
hOP-1	Recombinant human osteogenic protein-1
hBMP-2	Recombinant human bone morphogenetic protein-2
hTGF-β_3	Recombinant human transforming growth factor-β_3
µm	Micrometer
OCN	Osteocalcin
P. ursinus	Papio ursinus (Chacma baboon)
Runx-2	Runt-related transcription factor-2
Shh	Sonic hedgehog proteins
Wnt	Wingless intergrated transcription factor

1. Introduction

The periodontium is defined as ‘the tissues that invest and support the teeth and include the gingiva, alveolar mucosa, cementum, periodontal ligament and alveolar bone’ (Glossary of periodontal terms, 2001). It is a collective term describing the tooth supporting structures and tissues that is, the root cementum, periodontal ligament, alveolar bone and gingiva (Page & Schroeder, 1982). Periodontal diseases lead to the loss or damage of the periodontal tissues (Page & Schroeder, 1982). Periodontal therapy involves two primary components. First, the elimination of the periodontal infection and second, the regeneration of damaged or lost components of the periodontal tissues (Garrett, 1996). Tissue regeneration in postnatal life recapitulates events which occur in the normal course of embryonic development (Ripamonti, 2007). Development of the periodontium is initiated with the process of root formation, as the apical proliferating mesenchyme forms a bi-layered epithelial root sheath. These epithelial root sheath cells are thought to produce basement membrane containing chemotactic proteins, which facilitate the migration and differentiation of pre-cementoblast cells, hence contributing to cementogenesis (Zeichner-David, 2006). Additionally, the cells of HERS undergo epithelial-mesenchymal transformation to become functional cementoblasts contributing to production of acellular and cellular cementum (Zeichner-David, 2006).

Several genes and their secreted products have been identified as crucial for regulating this process of tooth morphogenesis (Levander, 1945; Thesleff & Nieminen, 1996; Äberg, Wozney, & Thesleff, 1997; Thesleff & Sharpe, 1997). The pleiotropic activity of the soluble molecular signals of the transforming growth factor-ß (TGF-ß) supergene family including the bone morphogenetic proteins (BMPs), initiate cementogenesis as well as the assembly of the periodontal attachment apparatus (Ripamonti & Petit, 2009). Originally BMPs have been isolated and identified by the osteoinductive capacity of demineralised bone matrix (DBM) when implanted in heterotopic extraskeletal sites (Urist, 1965; Reddi and Huggins 1972).The BMPs are pleiotropic proteins with several different functions and activities in the context of different organs and tissues modulated by various extracellular matrix components (Ripamonti, 2007). BMPs are soluble molecular signals that are deployed during tooth development and morphogenesis (Hogan , 1996; Thesleff & Sharpe, 1997; Ripamonti, 2007). This is at the crux of therapeutic tissue induction and morphogenesis by recombinant human BMPs, which may additionally be deployed recapitulating events which occur in the normal course of embryonic development to induce periodontal tissue regeneration, specifically cementogenesis along exposed root surfaces.

1.1 Aim

To compare the effect of different osteogenic proteins, namely hOP-1, hBMP-2, hTGF-β_3, the binary application of hOP-1 and hBMP-2, the synergistic application of hOP-1 and hTGF-β_3, and naturally-derived bone morphogenetic proteins, on the induction of newly formed cementum on root surfaces of molar teeth in surgically created furcation defects of P.*ursinus.*

1.2 Objectives

1. Evaluate whether all the osteogenic proteins were capable of inducing cementum formation
2. To quantify the newly formed cementum induced by various osteogenic proteins by:
    a. Measuring the extent of newly formed cementum along mesial and distal roots of molar teeth
    b. Measuring the thickness of newly formed cementum along different regions of the roots of molar teeth to establish if any significant differences in cementum induction were present

2. Review of the literature

Untreated periodontal diseases may lead to tooth loss due to destruction of the tooth supporting structures. Regeneration of these lost structures is a strategy which is central to successful periodontal therapy, but yet remains an elusive outcome in clinical periodontal practice (AAP Position paper, 2005). Historical treatment approaches, such as flap debridement, have served as a reliable method for access to root surfaces to facilitate removal of infective aetiology. This treatment approach has however yielded limited, if any, potential for regeneration of lost components.

Regenerative therapies aimed at reconstitution of tooth supporting structures including alveolar bone, cementum and periodontal ligament have yielded more positive outcomes (Reynolds et al., 2003). These therapies such as osseous grafting and guided tissue regeneration (GTR) have been used in the management of infrabony periodontal defects and furcation defects, predominantly yielding bone fill, and not complete recapitulation of the periodontium i.e. cementum and PDL formation (Reynolds et al., 2003). It is on this premise, that developments in biology and material sciences could provide new regenerative materials and delivery systems for predictable regeneration of all components of the periodontium (Position paper AAP, 2005).

The induction of newly formed cementum is an essential ingredient to engineer periodontal tissue regeneration (Ripamonti, 2006). In the past several years, tissue induction and regeneration has witnessed a rapid growth due to several discoveries in regenerative medicine but particularly in bone tissue induction. The essence of tissue engineering and regeneration is to use morphogenetic signals or morphogens first described by Turing (1952) as ‘forms generating substances’ (Turing, 1952). Morphogens, initiate the multi-step cascade of gene expression, protein synthesis, and secretions resulting in the induction of tissue formation or morphogenesis (Reddi, 1988; Reddi, 1994; Reddi, 1997; Ripamonti & Duneas, 1998; Reddi, 2000.)

The discovery of these regulatory morphogens with novel biological activities and therapeutic potential has been the subject of exciting research. The recognition of the extracellular matrix of bone as a multi-factorial repository of locally active pleiotropic morphogenetic proteins that initiate and modulate bone formation by induction, has been at the crux of continuously developing research (Ripamonti & Reddi, 1992; Reddi, 2000; Ripamonti, 2006). Progression in the isolation and characterization of regulatory morphogens within the bone matrix has been hampered by the fact that the extracellular matrix of bone is in the solid state (Reddi, 1997). The bulk of the extracellular matrix proteins are tightly bound to the collagenous bone matrix, further cemented by the mineralised component of the bone matrix. Reddi and co-workers were the first to unlock the problem of the bone matrix in the solid state (Reddi, 1997). Sampath and Reddi used chaotropic agents such as urea or guanidinium hydrochloride (Gdn HCL) to extract and solubilize the putative osteogenic proteins contained within the demineralised bone marix (Sampath & Reddi, 1981). Solubilized putative osteogenic proteins were then reconstituted with the insoluble collagenous matrix or residue obtained after the dissociative extraction of the bone matrix (Sampath & Reddi, 1981). This operational reconstitution restored the biological activity of the intact demineralised bone matrix (Sampath & Reddi, 1981; Reddi, 1997).

A futher contribution by Sampath and Reddi was an experiment which addressed the species specificity of the bone matrix, showing homology among the osteoinductive proteins from diverse species of mammals. The species specificity of matrix-induced bone formation is due to species-specific alloantigens or inhibitors (or both) present in the Gdn HCl-extracted allogenic insoluble collagenous bone matrix and the Gdn HCl-solubilized extracellular bone matrix components of >50,000 daltons (Sampath & Reddi, 1983).

The above study highlighted that the osteogenic proteins extracted from the bone matrix are homologous amongst mammalian species. On the contrary, the insoluble collagenous bone matrix carries the antigenic load across mammalian species, and the restoration of the biological activity *in vivo* is only achieved when using allogenic collagenous matrix components (Sampath & Reddi, 1983). These key experiments, showing the combination of an insoluble signal or substratum, with solubilised osteogenic soluble signals, propelled the bone induction principle into the pre-clinical and clinical arena culminating in the isolation and purification of an entirely new family of protein initiators, the BMPs which were found to be members of the TGF-β supergene family (Sampath & Reddi, 1981; Wozney et al., 1988; Celeste et al., 1990; Özkaynak et al., 1990; Ripamonti et al., 1996).

2.1 Bone morphogenetic proteins

The BMPs are a family of highly conserved secreted proteins that have potent and pleiotropic functions and activities in the context of different organs and tissues, further modulated by the biomimetism of the extracellular matrix (Thesleff, 1995; Hogan, 1996; Thomadakis et al., 1999, Reddi, 2000, Ripamonti, 2006). BMPs show sequence homologies with members of the transforming growth factor β (TGF-β) family (Wozney et al., 1988; Celeste et al., 1990; Özkaynak et al.1990).

A striking and discriminatory prerogative of highly purified naturally derived BMPs is the induction of *de novo* bone formation. Classic experiments by Urist , Reddi and Huggins have demontrated the ‘bone induction principle’(Huggins, 1931; Urist, 1965; Urist et al., 1967; Reddi & Huggins, 1972; Reddi , 1997).

Increasing purification schemes of large quantities of naturally derived bovine BMPs yielded protein extracts and final purification bands including electroendosmotic elution that provided amino acid sequencing information (Wang et al., 1988; Sampath, et al., 1992). This was followed by expression cloning of the recombinant human proteins (Wozney et al., 1988; Celeste et al., 1990; Özkaynak et al., 1990). More than 40 related proteins with BMP-like sequences and activities have been sequenced and cloned (Ripamonti, 2006).

In addition to bone induction in postnatal life, the BMPs are involved in inductive events that control pattern formation during morphogenesis and organogenesis in such disparate tissues and organs as the kidney, eye, nervous system, lung, teeth, skin and heart (Hogan, 1996; Äberg et al., 1997; Chinsembu, 2012). The impressive evolutionary conservation of the BMPs genes indicate that the secreted proteins are critical in development and are invovled in several unrelated events that control pattern formation during both embryonic development and postnatal tissue morphogenesis and regeneration (Hogan, 1996; Äberg et al., 1997; Chinsembu, 2012).

2.2 Tooth morphogenesis

Tooth morphogenesis is a classic example of epithelial-mesenchymal interaction involving reciprocal signalling events mediated by several proteins (Thesleff et al., 1995). Signalling between the epithelial and mesenchymal tissues regulates morphogenesis and cell determination from the very beginning of odontogenesis.

Soluble signals such as the fibroblast growth factors (FGFs) and the BMPs, and transcription factors such as the wingless intergrated (Wnt) and sonic hedgehog (Shh) proteins play a crucial role in tooth initiation, morphogenesis and differentiation (Äberg et al., 1997; Thesleff, 2006; Chinsembu, 2012). In the developing tooth BMPs secreted by the epithelium (BMP-2 and/or BMP-4) are suggested to be early signals that stimulate expression of the homeobox-containing genes Msx-1 and Msx-2, which have central regulatory roles in tooth morphogenesis as demonstrated in knockout mice experiments (Gao et al., 1998; Thesleff, 2006). A weak BMP signal, ensuing from a loss of BMP receptors or overexpression of BMP inhibitors, results in various defects in different cusps and teeth (Chinsembu, 2012). During the bud-to-cap stage transition, BMP-2 signalling from the condensing mesenchyme plays a critical role in the induction of the enamel knot (Chinsembu, 2012). Synchronously, BMP-4 within the oral epithelium leads to an up-regulation of enamel knot markers such as *p21* (Caton & Tucker, 2009). Loss of BMP-4 signalling by knockout of the receptor Bmpr1a gene leads to an arrest of tooth development at the bud stage (Chinsembu, 2012).

The expression of BMPs is not confined to early tooth development since its expression is sustained during root morphogenesis (Thomadakis et al., 1999). As mantle dentine is secreted, the epithelial root sheath disintegrates, followed by migration of mesenchymal cells of the dental follicle towards the root surface and differentiation into cementoblasts, secreting cementoid which later mineralises. The outer cells of the dental follicle differentiate into osteoblasts lining the alveolar bone, whilst the more centrally located cells differentiate into fibroblasts that produce collagen that become embedded in both bone and cementum as Sharpey's fibres (Zeichner-David, 2006). In this context, the localisation of BMP-3 and OP-1 during morphogenesis of the murine root, suggests that the secreted proteins play a role during cementogenesis and the assembly of the periodontal ligament fibres (Thomadakis et al., 1999). The localisation of BMP-2 in alveolar bone alone and BMP-3 and OP-1 in all three components of the periodontium suggests that the morphogenesis of periodontal tissues may involve a composite pattern of co-ordinated expression of different signalling BMPs, each endowed with a specific pleiotropic biological activity (Thomadakis et al., 1999).

Experimental studies in non-human primates have also shown that BMPs in addition to the induction of bone formation also induce cementogenesis (Ripamonti et al., 1994). Naturally-derived highly purified BMPs implanted in furcation defects of the non-human primate Chacma baboon *Papio ursinus* showed tissue induction of the three essential components of the periodontium, that is cementum, periodontal ligament and alveolar bone (Ripamonti et al., 1994). Short term studies in *Papio ursinus* showed that furcation defects implanted with human recombinant osteogenic protein-1 (hOP-1) primarily induces cementogenesis, with minimal, if any, bone formation (Ripamonti et al 1996). Importantly, within the context of the periodontal wound and in contact with the dentine extracellular matrix, hOP-1 is primarily cementogenic, whilst hBMP-2, when implanted in identical periodontal defects of *P. ursinus* is primarily osteogenic (Ripamonti et al., 2001; Ripamonti, 2007).

2.3 Structure-activity profile

Morphological differences in periodontal tissue induction, as demonstrated by experimental studies, highlight the pleiotropic functions of members of the BMPs family (Ripamonti, 2006; Ripamonti, 2007). Ripamonti (2007) has stated "The mosaicism of BMPs' expression, synthesis and localization during periodontal tissue development in embryogenesis indicates the presense of several BMP isoforms synchronously expressed" (Ripamonti, 2007). The expression of multiple forms of BMPs may reflect different functions *in vivo* which could have therapeutic significance. *In vivo* studies, highlighted in primate studies, have indicated that amino acid sequence variations in the active carboxy-terminal domain of a morphogenetic protein confer specialised pleiotropic properties to each BMP isoform. The amino acid sequence variations in the carboxy-terminal domain form the molecular basis that determine the structure-activity profile of each morphogenetic protein (Ripamonti, 2006; Ripamonti, 2007).

In vivo studies in *P. ursinus*, have demonstrated this novel concept, that there is a structure-activity profile of each BMP that results in the induction of different tissue morphologies when evaluated in periodontal regenerative procedures (Ripamonti et al., 2001; Ripamonti et al., 2009). These experiments focused on the efficacy of highly purified naturally derived and different recombinant human BMPs for periodontal tissue regeneration after implantation in mandibular furcation defects (Ripamonti, 2007). Experimental application of recombinant hOP-1 in contact with dentine extracellular matrix, shows a specific preferential cementogenic function, with newly induced cementum and with inserted Sharpey's fibres visible on morphologic examination of treated sites (Ripamonti et al., 1996; Ripamonti et al., 2001). The predominant cementogenic effect of hOP-1 is further highlighted in a study which compares the induction of periodontal regeneration by doses of recombinant hOP-1 and hBMP-2 applied singly or in combination (Ripamonti et al., 2001). Histomorphometric analysis showed hOP-1 to be strongly cementogenic in its single application. On the other hand hBMP-2 showed limited cementum formation. However, hBMP-2 induced greater amounts of bone formation when applied alone or when combined morphogens were applied (Ripamonti et al., 2001). The data supports the notion of an existing structure-activity profile, as tissue morphogenesis induced by hOP-1 and hBMP-2 is qualitatively different when the morphogens are applied singly to furcation defects (Ripamonti et al., 2001). Choi et al (2002) using a canine experimental model investigated the effects of hBMP-2 in 3-wall intrabony periodontal defects. The study showed positive healing outcomes for hBMP-2 with regards to alveolar bone, where accelerated and enhanced bone formation was noted. However, cementum regeneration was minimal, with the authors concluding that hBMP-2 does not appear to have a significant effect on cementum regeneration (Choi et al., 2002).

2.4 Mechanistic insights

In vitro studies have attempted to provide mechanistic insights which support the concept of a structure-activity profile of BMPs. Hakki et al (2010) studied the effects of hOP-1 on murine cementoblast cells *in vitro*. The study focused on the effect of hOP-1 on regulating mineralised tissue-associated genes in cementoblasts and the expression profile of cementoblasts, extracellular matrix (ECM) components and cell adhesion molecules (CAM) (Hakki et al., 2010). The findings of the study, demonstrated that hOP-1 at a concentration of 50ng/ml upregulated the expression of mineralised tissue associated genes such as osteocalcin (OCN), bone sialoprotein (BSP) and runt-related transcription factor-2 (Runx-2) mRNA expression on the cementoblasts. These effects of hOP-1 on gene regulation possibly modulate the response of cementoblasts during tissue induction, mineralisation and tissue turnover, highlighting the role of hOP-1 during cementogenesis (Hakki et al., 2010). The effect of hBMP-2 on periodontal regeneration using *in vitro* experimental models has also been explored (Zhao et al., 2003). The study by Zhao et al. (2003) examining the effects of hBMP-2 on murine cementoblasts *in vitro* showed that hBMP-2 inhibited cementoblast-mediated mineral nodule formation at concentrations of 10 ng/mL, with a downregulation of gene and protein expression. hBMP-2 does however play a promotive role in the process of osteoblastic differentiation as demonstrated by osteoblastic differentiation of preogenitor cells upon exposure to hBMP-2 (Gorri et al., 1999; Zhao et al., 2003).

3. Materials and Methods

This was a retrospective study which used different tissue sections from six previous studies of periodontal regeneration in furcation defects of *P. ursinus* (Ripamonti et al., 1994; Ripamonti et al., 1996; Ripamonti et al., 2001; Ripamonti et al., 2009b; Teare et al., 2012). In these studies, furcation defects of mandibular molars of *P. ursinus* were exposed to different applications and concentrations of morphogenetic proteins, the effect of which was examined histomorphometrically on undecalcified serial sections. The surgical methodology of the furcation defect model of tissue morphogenesis by bone derived and recombinant BMPs in the adult baboon has been described in detail in Addendum A (Ripamonti et al., 1994; Ripamonti et al., 1996; Ripamonti, 2001). The surgical methodology was the same for all six studies with the exception of the application used in each study. The same operator cut all the furcation defects within the reported studies providing a highly reproducible furcation defect model for sequential morphological and morphometric analyses (Ripamonti et al., 1994; Ripamonti et al., 1996). All animals were killed 60 days after soluble morphogen application. Preparation of serial histological sections from these experiments for histomorphometric analysis have also been described in detail in Addendum A (Ripamonti et al., 2001).

In summary, the applications used in the previous studies were:

1. **hOP-1**

   100 µg recombinant human osteogenic protein-1 combined with a bovine insoluble collagenous bone matrix as a carrier.

2. **hBMP-2**

100 µg recombinant human bone morphogenetic protein-2 combined with a bovine insoluble collagenous bone matrix as a carrier.

3. Binary application of hOP-1 and hBMP-2

100 µg recombinant human osteogenic protein-1 and 100 µg recombinant human bone morphogenetic protein 2 combined with a bovine insoluble collagenous bone matrix as a carrier.

4. hTGF-β_3

75 µg recombinant human transforming growth factor-β_3 combined with Matrigel® matrix as a carrier.

75 µg recombinant human transforming growth factor-β_3 combined with Matrigel® matrix and morcellated fragments of *rectus abdominis* muscle.

5. Synergistic binary application of hOP-1 and hTGF-β_3

25µg recombinant human osteogenic protein-1 and 1.25 µg recombinant human transforming growth factor-β_3 combined with Matrigel® matrix as a carrier

25µg recombinant human osteogenic protein-1 and 1.25 µg recombinant human transforming growth factor-β_3 combined with Matrigel® matrix and morcellated fragments of *rectus abdominis* muscle

6. Naturally-derived highly purified bone morphogenetic protein

250 µg naturally derived highly purified bone morphogenetic protein from bovine bone matrix combined with allogenic insoluble collagenous bone matrix as a carrier.

3.1 Study sample

Prepared histological sections of periodontal tissues of treated mandibular teeth from the six studies were accessed from the archives at the Bone Research Laboratory of the University of Witwatersrand. For inclusion into the study the slides had to display the following:

- Good staining properties to allow for discrimination between the different components of the periodontium
- Complete view of both roots for measurement purposes
- Identifiable apical notches on each root for measurement reference point
- Identifiable furcation region between the two roots of selected teeth

All histological sections with poor staining preservation and resultant unclear distinction between the cementum and other hard tissue were excluded from the study. Histological sections displaying anatomical distortion of roots, apical notch and furcation regions were also not considered for evaluation in this study.

The total number of teeth analysed in this study were dictated by these inclusion and exclusion criteria. Hence for each study the number of teeth analysed did differ as reflected in Table B.

3.2 Study Design

Histometric measurements of the extent and thickness of cementum formed along individual mesial and distal roots of each tooth were taken (Figure 1). Selected sections were analysed at objective 10x (cementum extent measurements) and objective 4x (cementum thickness measurements) with a Provis AX70 research microscope (Olympus

Optical Tokyo, Japan). Histometric measurements (in µm) were made and recorded using Stream Essentials software (Shinjuku Monolith, 3-1 Nishi-Shinjuku 2-chrome, Tokyo, Japan).

a. Apico-coronal extent of cementum formation from the apical border of the radicular notch to the most coronal extent of cementum. Measurements were recorded for mesial and distal roots independently. The measurements were recorded as a proportion of the total root length.

b. Measurement of the thickness of cementum formed along the root surfaces. Three points along each root (mesial and distal root), were selected for measurement. Mesial and distal root measurements for each region were combined and a mean value was calculated. The reference points along the root surface for each measurement were equidistant and described as follows:

    1. Apical reference point: Thickness of newly formed cementum was measured coronal to the apical notch

    2. Middle reference point: Thickness of newly formed cementum was measured from a measured halfway reference point between the apical and coronal aspect of the root surface

    3. Coronal reference point: Thickness of newly formed cementum was measured corresponding to the most coronal extension of cementum along the root surface

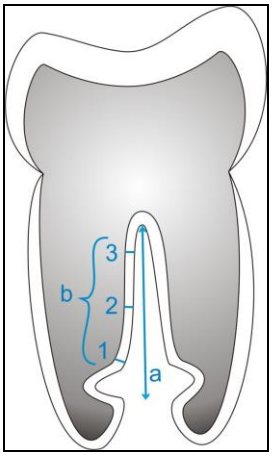

Figure 1: Schematic diagram of measurements

3.3 Data Collection and Statistical analysis

Histometric measurements were recorded using Stream Essentials Software (Shinjuku Monolith, 3-1 Nishi-Shinjuku 2-chrome, Tokyo, Japan). The data was recorded and saved on an electronic excel spreadsheet. Measurements were done by analysing sections more than once and comparing measurements from different measurement sittings. If discrepancies in measurements occurred, this was cross checked by a consulting supervisor. All the final measurements selected for the statistical analysis were checked and verified by the consulting supervisor. The use of Stream Essentials data capturing and measurement program did ensure a high level of standardized data recording and decreased the risk of manual measurement errors. Other information captured on the data collection sheet included covariates such as study ID, Baboon ID, molar location (Right or Left) and molar type (1st/2nd).

The data file was restructured and cleaned in collaboration with the statistician. The number of sections varied from 1-3 depending on the availability of appropriate histological sections per group/tooth. The sections analyzed per tooth were selected on an applicability basis for measurements. Where multiple slides were evaluated for the same tooth, fractional weights were applied so that each tooth had a total weight of 1 in the data set.

Differences between treatments were assessed using a General Linear Model (GLM) procedure for each dependent variable (DV), with treatment and the covariates as independent variables. Post-hoc tests were conducted using the Tukey-Kramer test. Effect sizes were calculated using Cohen's d, which were interpreted according to Table A. The 5% significance level was used throughout, unless specified otherwise. *In other words, p-values <0.05 indicate significant results.*

***Cohen's d**	**Effect sizes**
0.80 and above	large effect
0.50 to 0.79	moderate effect
0.20 to 0.39	small effect
below 0.20	near zero effect

*Table A: *Cohen's d (effect size used to indicate standardised difference between two means)*

4. Results

There were 27 teeth in the study, spread over six treatments, with 1-9 teeth per treatment. It must be noted at the outset that the sample size per treatment is small, and this affects the power of the study that is the ability to detect a significant difference.

	Treatment	**Frequency**	**Percent**
1.	**hBMP-2**	4	14.81
2.	**hBMP-2 and hOP-1**	1	3.70
3.	**Naturally-derived BMPs**	4	14.81
4.	**hOP-1**	9	33.33
5.	**Synergistic hOP-1 and hTGF-β_3**	4	14.81
6.	**hTGF-β_3**	5	18.52

Table B: Distribution of the number of teeth per treatment group

The data were taken from different studies (Ripamonti et al., 1994; Ripamonti et al., 1996; Ripamonti et al., 2001; Ripamonti et al., 2009; Teare et al., 2012), with treatment 2 only contributing one tooth. The data were obtained from 14 baboons, each contributing between 1 and 4 (the maximum) teeth. The study teeth were reasonably well-balanced between the 1st and 2nd molars (59% and 41% respectively). The teeth were predominantly from the right mandible (67%).

Between-group analysis for the dependent variables

A one-way analysis of variance (ANOVA) was conducted for each dependent variable with Treatment as factor. Baboon ID (nested in Treatment), molar type (1st/2nd) and molar location (L/R) were used as blocking variables. Study ID (nested in Treatment) could not be used as an additional blocking variable due to the confounding between Treatment, Study ID and Baboon ID. A natural log transformation of the dependent variable was used in order to meet the assumptions of the ANOVA technique.

Extent of cementum	**Source**	**DF**	**Type III SS**	**Mean Square**	**F Value**	**Pr > F**
Mesial root	**Treatment**	5	0.93	0.19	3.97	0.013
Distal root	**Treatment**	5	0.19	0.04	2.31	0.087

Table C: Source table for extent of cementum formation

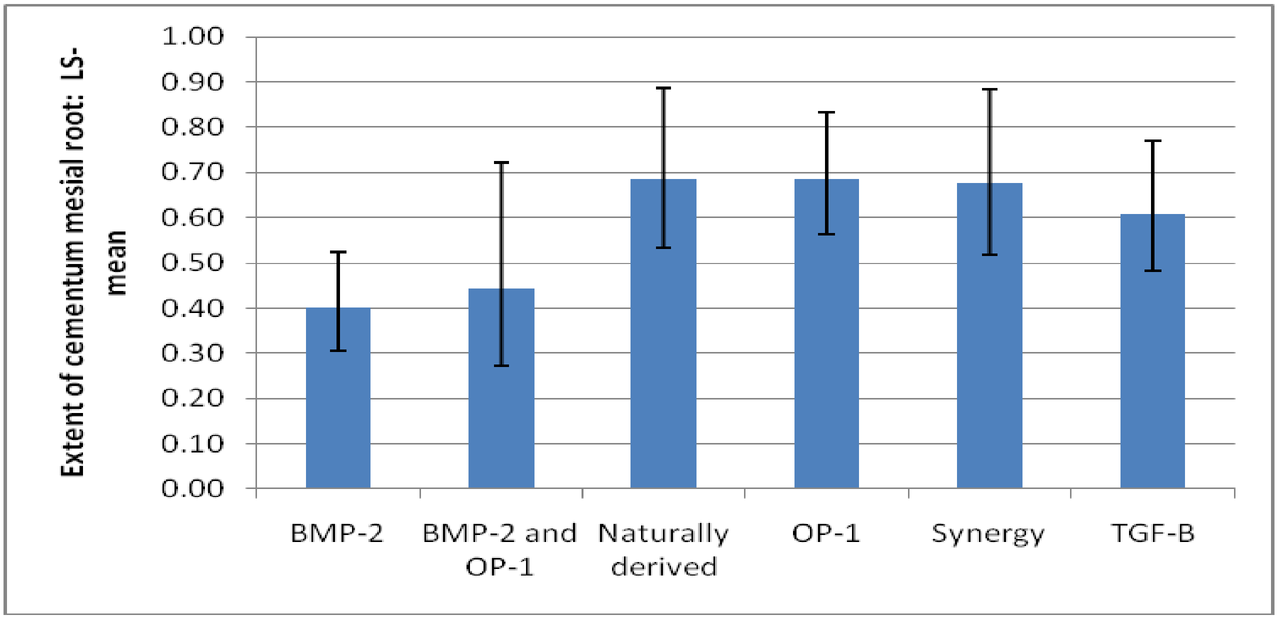

Figure 2: Extent of cementum along the mesial root (mean values)

The effect of treatment was significant (p=0.013) for the mesial root between treatment groups. Post-hoc tests showed that the mean extent of cementum on the mesial root was significantly higher for Naturally-Derived, hOP-1 and synergistic binary application of hTGF-β_3 and hOP-1, compared to hBMP-2 (Figure 7 A-E; Figure 8 E). The effect sizes were large (Cohen's d=1.9, 1.0, and 2.4 respectively). The Least-Squares (LS) Means are illustrated below (the error bars denote the 95% confidence intervals for the means).

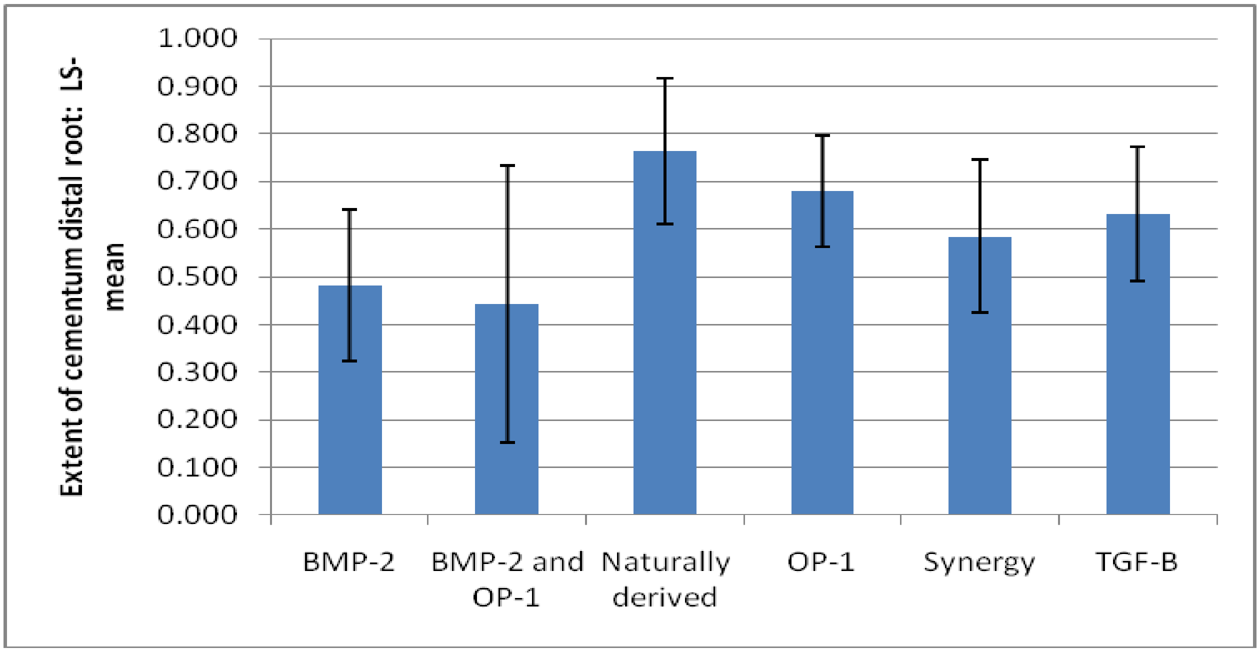

Figure 3: Extent of cementum along the distal root (mean values)

The effect of treatment for the distal root was not significant (p=0.087).

Thickness of cementum	**Source**	**DF**	**Type III SS**	**Mean Square**	**F Value**	**Pr > F**
Apical	**Treatment**	5	1969.41	393.88	1.31	0.31
Middle	**Treatment**	5	1.42	0.28	5.10	0.005
Coronal	**Treatment**	5	2.67	0.53	22.77	<.0001

Table D: Source table for thickness of cementum formation

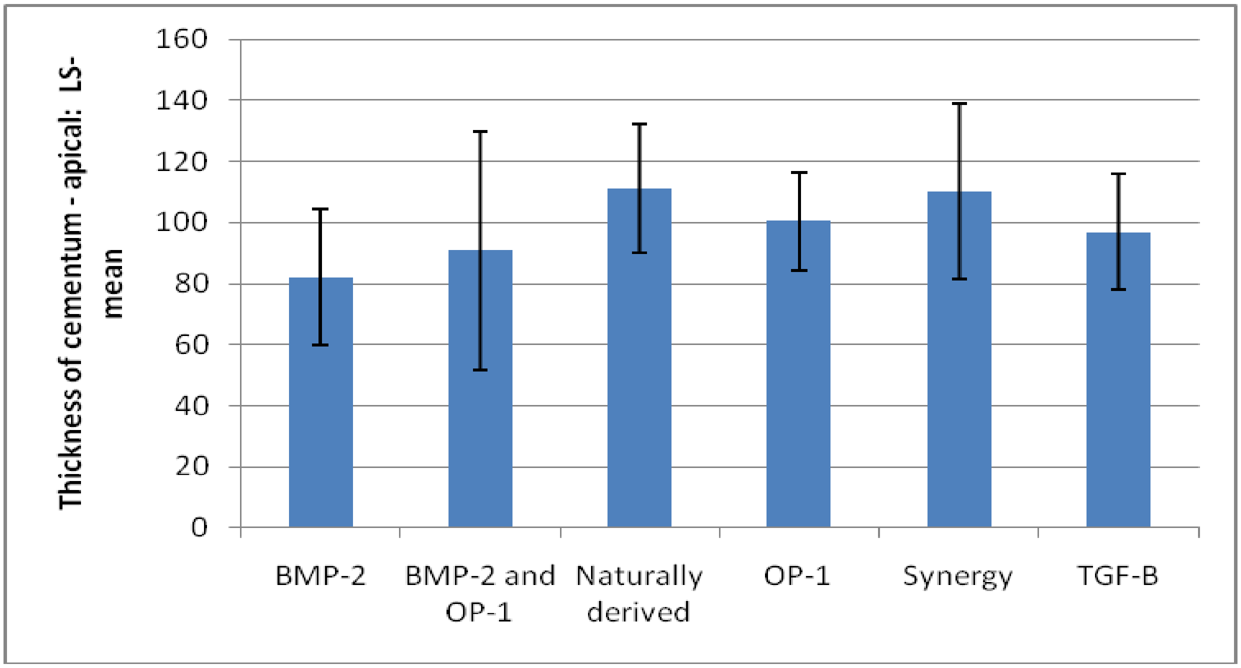

Figure 4: Thickness of cementum in the apical region (mean values calculated for combined mesial and distal root measurements)

Three outliers were removed (this did not change the conclusions). The effect of treatment was not significant (p=0.31).

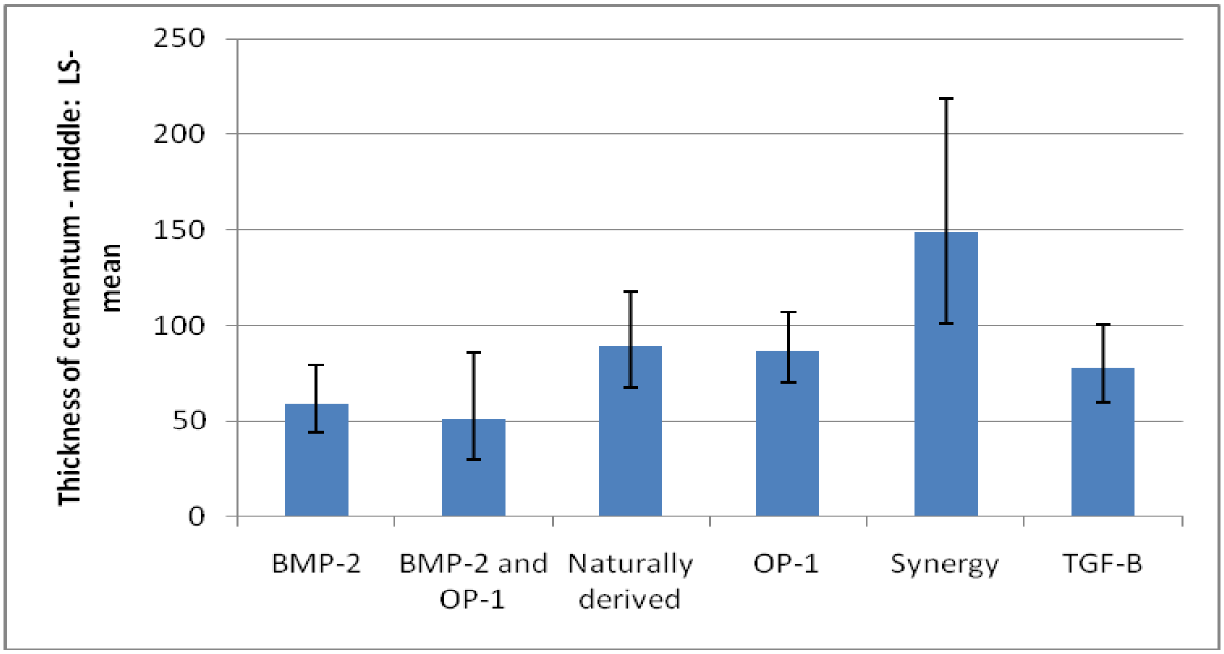

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Figure 5: Thickness of cementum in the middle region (mean values calculated for combined mesial and distal root measurements)

The effect of treatment was significant (p=0.005). Post-hoc tests showed that the thickness of cementum in the middle region of the root was significantly higher for the synergistic application of TGF-β_3 and hOP-1, compared to hBMP-2 and hBMP-2 with hOP-1 (Figure 8 A-E). The effect sizes were large (Cohen's d=2.7 and 1.7 respectively). The Least-Squares (LS) Means are illustrated in Figure 5 above.

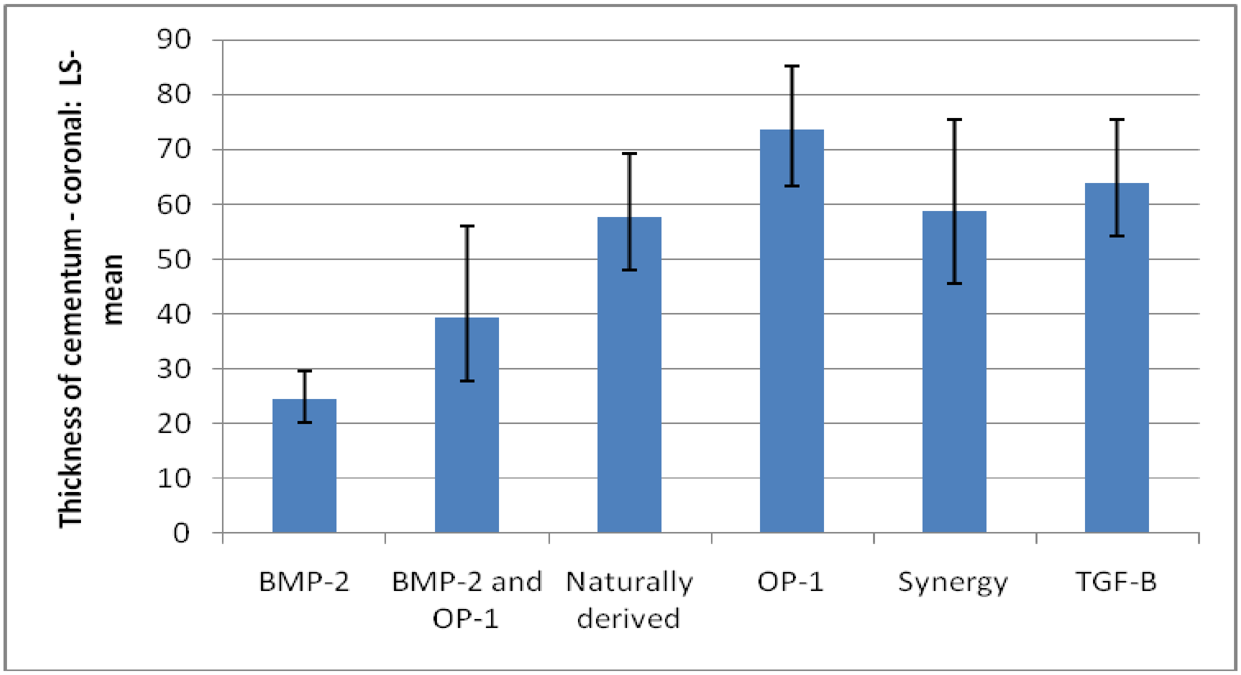

Figure 6: Thickness of cementum in the coronal region (mean values calculated for combined mesial and distal root measurements)

Three outliers were removed. The effect of treatment was significant (p<0.0001). Post-hoc tests showed that the thickness of cementum in the coronal region of the root was significantly higher for hOP-1, compared to hBMP-2 and hBMP-2 with hOP-1, and also higher for Naturally Derived, synergistic application of TGF-β_3 and hOP-1 and hTGF-β_3 compared to hBMP-2 (Figure

8). The effect sizes were large (Cohen’s d=3.3, 1.9, 5.4, 1.9, 2.5 respectively). The Least-Squares (LS) Means are illustrated in Figure 6 above.

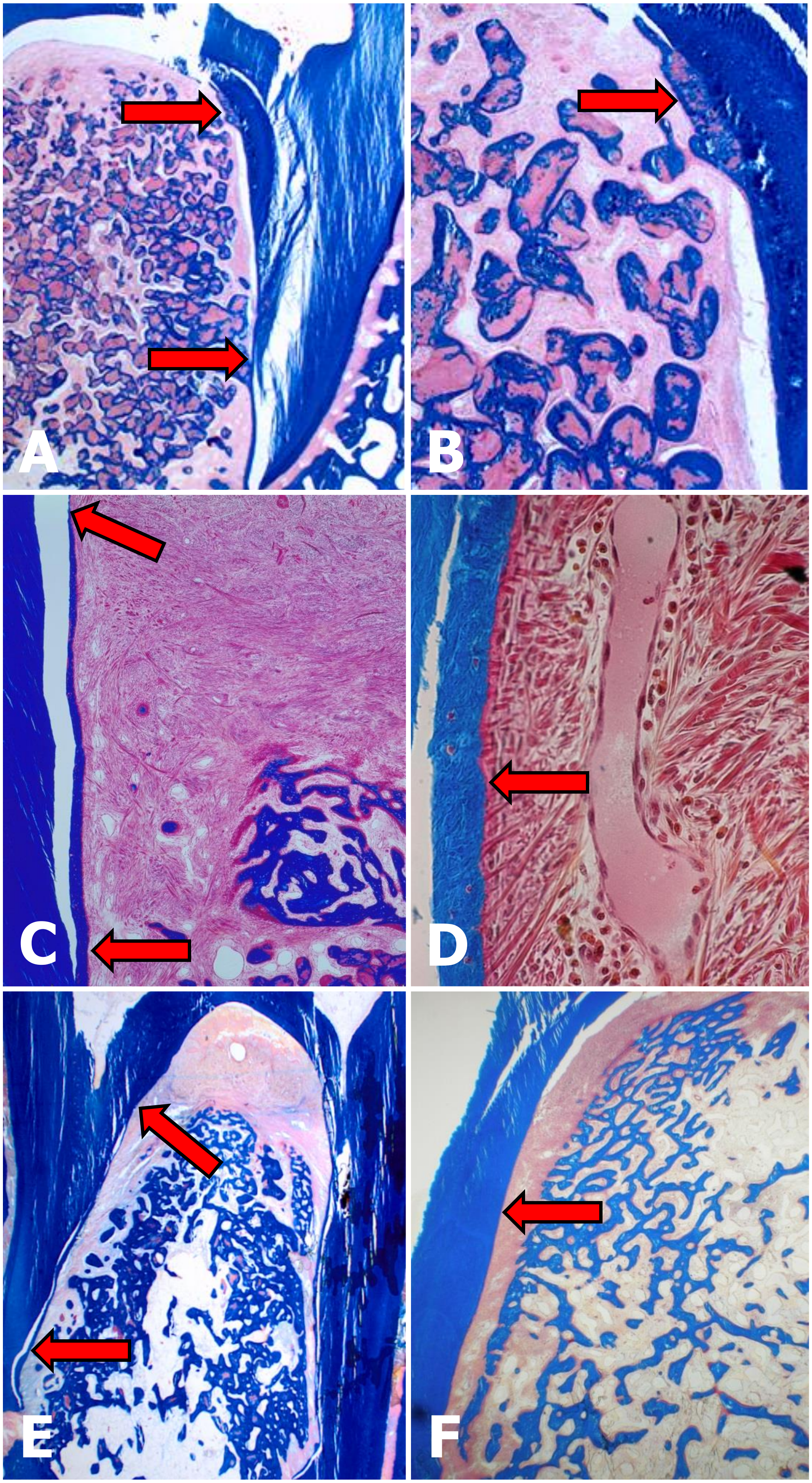
A
B
C
D
E
F

Figure 7: Extent of cementum formation along root surfaces. (A) hOP-1 application showing extensive newly formed cementum along distal root surface from the apical notch region continuing coronally into furcation roof region (red arrows). Magnification x25. (B) hOP-1 application into furcation roof region. Magnification X45. (C) hOP-1 application showing extensive cementum formation along mesial root. Magnification x75. (D) Naturally-derived BMPs application demonstrating new cementum formation mesial root. Magnification x125. (E) Naturally-derived BMPs application showing cementum formation along mesial root surface from apical notch region extending coronally magnification x25. (F) Naturally-derived BMP application showing extensive cementum formation along mesial root. Magnification x125.

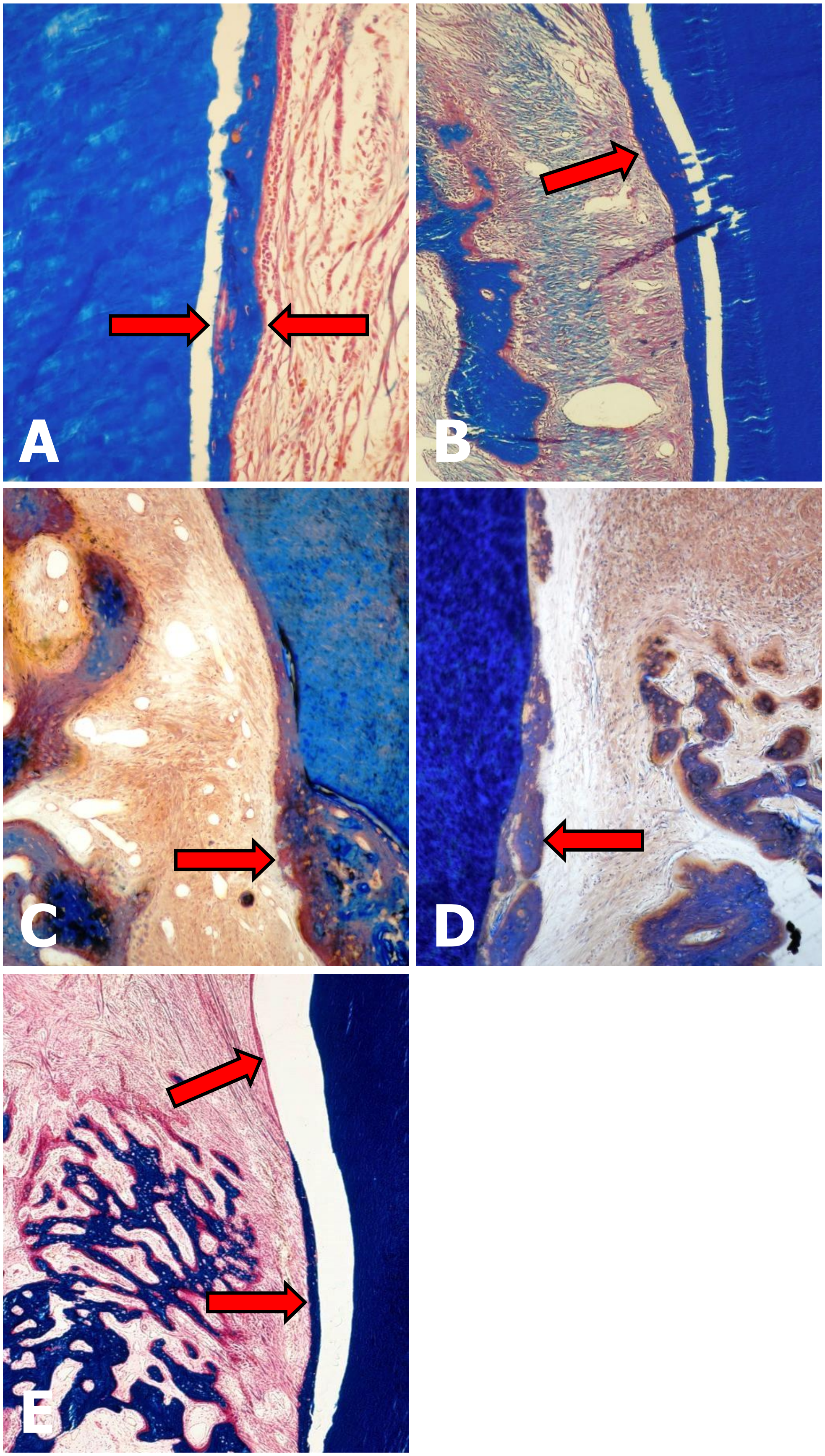
A
B
C
D
E

Figure 8: Thickness of cementum along different regions of root surfaces. (A) hTGF-β_3 application showing thickness of newly formed cementum along mesial root surface in the middle region with an appreciable "bulge" observed (red arrows). Magnification x150. (B) hTGF-β_3 application showing thickness of newly formed cementum along distal root surface in the coronal region. Magnification x125. (C) High-power detail of synergistic binary application of hOP-1 and hTGF-β_3 showing thickened areas of newly formed cementum along distal root surface in the middle region (red arrows). Magnification x300. (D) Synergistic binary application of hOP-1 and hTGF-β_3 showing irregular thickened areas of newly formed cementum along mesial root surface in the middle region. Magnification x150. (E) hBMP-2 application with limited new cementum formation along distal root surface confined to apical region of root surface not extending coronally (red arrows). Extent and thickness of new cementum are minimal. Magnification x75.

5. Discussion

Experiments in the non human primate P.*ursinus* have given insight into the morphogenetic potential of BMPs in periodontal tissue regeneration (Ripamonti et al, 1994). The ability of BMPs to induce cementogenesis has been highlighted by a number of studies (Ripamonti et al., 1994; Ripamonti et al., 1996; Ripamonti et al., 2001; Ripamonti et al., 2009; Teare et al., 2012). The presence of multiple forms of BMPs and their varying potential to induce cementogenesis has therapeutic significance (Ripamonti et al., 2006). The molecular basis for the specialised and pleiotropic activities of BMPs is the result of the amino acid sequence variations in the carboxy terminal domain. This study, in assessing the magnitude of cementum formation (extent and thickness) in periodontal defects reinforces the concept of a structure-activity profile amongst BMPs (Ripamonti et al.,2006).

In this study, newly formed cementum can be observed on all the histological sections assessed, but variations in the magnitude of newly formed cementum can be appreciated between the different BMPs applications. The BMPs that show the most cementogenic effect in terms of extent of cementum formed along the root surfaces are hOP-1, the synergistic application of hOP-1 and hTGF-β_3 and naturally-derived highly purified BMPs. A comparison of the quantity of newly formed cementum measured amongst the different BMPs applications does demonstrate these specific groups to be favourable in terms of cementum deposition along the connective tissue root interface, albeit in relation to a small sample size.

The positive cementogenic effect of hOP-1 confirmed in this study, could be associated with its ability to directly influence the expression levels of other morphogens, thus enhancing its regenerative potential. A previous study in the primate *P. ursinus* has provided evidence that hOP-1 directly influences the expression levels of OP-1, type IV collagen, BMP-3 and TGF-β_1 mRNAs, demonstrating a transcriptional cascade during regeneration (Ripamonti, 2005). *In vitro* studies suggesting that hOP-1 can modulate the expression profile of cementoblasts in relation to ECM components and related genes, provide mechanistic insights into the cementogenic potential of hOP-1 (Hakki et al., 2010). The upregulation of adhesion molecules and proliferating genes as a result of hOP-1 applications suggest a positive contributory role to cementum regeneration (Hakki et al., 2010). A previous experimental study has also alluded to the role of naturally-derived highly purified BMPs contributing positively to periodontal regeneration, specifically cementogenesis (Ripamonti et al., 1994) . The findings of this study are consistent with the proposed notion about naturally-derived BMPs being highly cementogenic in relation to other BMPs.

In this study, evaluation and measurement of the thickness of newly formed cementum along the root surfaces, showed that the apical regions do not show a high level of variation between BMPs groups. This observation could be as a result of the defect configuration of the apical notch area which would favour formation of cementum by virtue of the process of cementogenesis being initiated in the apical region, regardless of the BMPs applied.

Differences in cementum thickness were however appreciated, as cementogenesis continued coronally along the root surfaces. The middle regions and coronal regions demonstrated differences in cementum thickness between BMP groups with the synergistic binary application of hOP-1 and TGF-β_3 yielding the most promising potential to induce cementogenesis. The binary application of these morphogens as well as the addition of morcellated autogenous *rectus abdominis* muscle fragments containing myoblastic stem cells could have accounted for this positive effect. However, a study by Teare et al. (2012) failed to show greater periodontal regeneration with the addition of morcellated muscle fragments highlighting that their precise role in periodontal regeneration is still unclear (Teare et al., 2012).

Thickness measurements in the coronal extent of the root surfaces did not differ significantly between the synergistic binary application of hOP-1 and hTGF-β_3 group and the hTGF-β_3 applied singly, although an appreciable difference relative to hBMP-2 group is observed. hBMP-2 applications yielded the least amount of cementum in the coronal extent suggestive of its poor inductive capacity for cementogenesis, although due to a small sample size, concrete conclusions cannot be made.

Previous studies highlighted minimal cementum formation when applications without BMPs and TGF-βs were used (Ripamonti et al., 1994). For this reason, measurements of cementum formation in the control sections where insoluble bone matrix without BMPs and TGF-βs were applied were not done and thus, were not accounted for in the results of this study, .This however, in retrospect, is a significant limitation within this study.

The comparative applications of different BMPs highlight the possibililty that different morphogens would initiate a variable periodontal regeneration outcome when in initial contact with the tooth root surface. The suggestion that multiple forms of BMPs have biologically different effects, modulated and selectively potentiated by different extracellular matrix components, underscores the critical regulatory role of the extracellular matrix substrata (Ripamonti et al., 1994).

The results of this study would categorise hOP-1 and hTGF-β_3, used singly or in synergistic binary application as being favourable for cementum formation whilst hBMP-2 showed the least capacity to induce cementogenesis. However, the observation should be interpreted in the context of a small sample size.

6. Conclusion

This study comparing the effects of different BMPs on cementum formation, gives insights into favourable therapeutic BMP applications which would facilitate periodontal regeneration. Variation in the inductive capacity of different BMP applications in forming new cementum can be appreciated and understood in context of an existing structure-activity profile of each BMP and the influences of the extracellular matrix on their functions. Future research should focus on the complex molecular and cellular cascades of regenerating periodontal tissues induced by BMPs to achieve predictable periodontal regenerative outcomes.

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Appendices

Addendum A

- Surgical methodology of furcation defect model as described previously by Ripamonti et al, 1994; Ripamonti et al, 2001.

Healthy adult Chacma baboons (*Papio ursinus)* with intact dentitions were selected for the experiments. Class II furcation defects were surgically prepared bilaterally in the first and second mandibular molars. Exposed roots were curetted to remove periodontal ligament fibers and cementum, and notched with small burs at the level of the residual bone housing. The depth of each furcation defect extended for at least 10 to 12 mm buccolingually as measured from the buccal entrance of the exposed furcations of the 1st and 2nd molars measured respectively. Applications of BMPs were selected and applied as per study objective. BMPs either naturally derived or in recombinant form were recombined with insoluble collagenous bone matrix as a carrier, prepared after dissociative extraction of demineralised bone matrix and lypholyzed, to form a pellet suitable for implantation into the furcation defect. Insoluble bone matrix without BMPs and TGF-βs were used as controls during these experiments. The surgically raised flaps were closely readapted and sutured. Sutures were removed 10 days after surgical intervention with a plaque control regimen instituted weekly until 60 days after the operation when animals were killed with an intravenous overdose of sodium pentobarbitone.

- Histological slide preparation model as described previously by Ripamonti et al, 1994; Ripamonti et al, 2001.

Specimen blocks including 1st and 2nd molars with surrounding bone and soft tissues were embedded, undecalcified in methylmethacrylate and trimmed along the buccal aspects until apical notches along the root surfaces could be detected. Serial sections, including dentine and associated periodontal tissues, were then cut at 3 to 7 µm in the mesiodistal plane throughout the entire buccolingual extension of each furcation defect using tungsten-carbide knives on a Reichert-Jung Polycut-S microtome (Reichert, Heidelberg, Germany). Every 14th section, approximately 100 µm apart, was stained using the free-floating method with Goldner’s trichome stain for undecalcified bone. Three-step serial sections, each 600 µm apart from each other, representing the buccal, internal and central regions of the buccal half of the defects were selected for histomophometry and histometry. The buccal half of the defect was evaluated by cutting the specimen blocks until the mesio-distal fossa was reached. At this level, serial sections were representative of the central region of the defects.