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CORRELATION BETWEEN SYSTEMIC AND PERIODONTAL BONE LOSS IN NON-HUMAN PRIMATES *PAPIO URSINUS*

A HISTOLOGICAL STUDY ON THE CHACMA BABOON *PAPIO URSINUS*

Khudaija Suliman

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

Johannesburg, 2014

DECLARATION

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

.....

Khudaija Suliman

Signed this ..... day of 2014

The work described in this research report has been performed at the Bone Research Laboratory, School of Physiology, Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, South Africa.

To my mother

Fatima Ismail

ABSTRACT

Oestrogens deficient state in women either due to hypogonadism, ovariectomy (OVX) or menopause, may result in systemic bone loss leading to osteoporosis. The disease is manifested by microtrabecular vertebral and neck of femur fractures with episodes of severe pain. The disease is diagnosed by dual energy x-ray absorptiometry (DXA). Presently, the general management of osteoporosis consists of one or of several of the following therapies: (1) additional calcium (dairy products and green vegetables) and 1 alpha-hydroxy vitamin D (with sun exposure), or (2) sex hormones therapy (oestrogens in females, testosterone in males), or (3) selective oestrogen-receptor modulators (Raloxifene), or (4) antiresorption medicaments (calcitonin and bisphosphonates).

In OVX baboons demonstrating a decreased bone mineral density measured by DXA (Dal Mas *et al.*, 2007), Ripamonti *et al.* (2002b) conducted an innovative therapy by injecting recombinant human osteogenic proteins (hOPs) directly into the trabecular bone of lumbar vertebrae and neck of femora.

Digital histomorphometric measurements were done on undocumented specimens from the Dal Mas *et al.* (2007) and Ripamonti *et al.* (2002b) studies to investigate any possible correlation between alveolar bone loss and systemic bone loss as previously described in the above-mentioned studies. The aim was to measure the bone fractions composition on undecalcified histological sections of the furcation alveolar bone harvested from the first and second mandibular molar regions.

This investigation was performed on two experimental groups:
(1) 18 adult OVX baboons injected with hOPs;
(2) 7 old baboons non-OVX but injected with hOPs.
The two control groups are:
(1) 1 adult OVX baboon non-injected (the second animal was euthanized subsequent to a severe chronic nasal infection not responding to treatment)
(2) 2 adult baboons which have neither been OVX nor treated with hOPs.

The quantitative histomorphometric analyses failed to show a correlation between systemic and mandibular alveolar bone loss in non-human primates *Papio ursinus*. Furthermore, the data did not show both in OVX and in non-OVX old baboons that local injections of hOPs into the lumbar vertebrae and neck of femora have a systemic effect on the mandibular alveolar bone.

Key words: non-human primates, induced systemic bone loss, intra-osseous local injection of recombinant human osteogenic proteins.

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◆ Although it is uncommon to include personal comments in a research report, I however feel the need to express the benefit I have gained from a study that I started with enthusiasm. Soon in this study, difficulties arose leading to an inconclusive end. However, I have scientifically benefited from the interactions with the caring staff of the Bone Research Laboratory. I have also developed confidence in the microscopic examination of the histological material not only in the identification of the various periodontal anatomical structures through multiple discussions with my supervisors, but in the complex techniques of digital measurements as well. Furthermore, I feel that my writing skills have improved considerably and, finally, I am looking forward to starting another research project in the near future.

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ABBREVIATIONS

A	apex
AM	apex of the furcation to the mesial aspect of bone recession
AD	apex of the furcation to the distal aspect of bone recession
BMD	bone mineral density
BMMSCs	bone marrow mesenchymal stem cells
BPs	bisphosphonates
DXA	dual energy X-ray absorptiometry
D	distal
FGF-21	fibroblast growth factor-21
Fv	fibrovascular
hBMPs	human bone morphogenetic proteins
hOPs	recombinant human osteogenic proteins
hTGF-β_3	human transforming growth factor-β_3
HTR	hormone replacement therapy
ICBM	insoluble collagen bone matrix
M	mesial
Md	mineralized bone
N°or n°	number
ND	not done
nr	not readable
NR	not recorded
Pi	inorganic phosphate
PO	*per os* (by mouth)

Oe	osteoid
OP-1	recombinant human osteogenic protein-1
ORT	oestrogen replacement therapy
OVX	ovariectomized
PTH	parathyroid hormone
R	re-cut
RAMCs	*rectus abdominis* mesenchymal stem cells
RANKL	Receptor activator of nuclear kappa-B ligand
SORMs	selective oestrogen-receptor modulators
SR	strontium ranelate

Statistical abbreviations

ANOVA	analysis of variance
C or c	control
DF	degree of freedom
DV	dependant variable
Ex	experimental
F	F-ratio
ICC	intraclass correlation coefficient
IQR	interquantile range
MS	mean square
p	*p*-value (statistical significance)
medul	medullary spaces
propn	proportion
RSD	relative standard deviation
SS	sum of squares
Std Dev/SD	standard deviation
Std Err	standard error

1 INTRODUCTION

Osteoporosis is a common disorder of the skeleton in which low bone mass and deterioration of bone micro-architecture lead to increased bone fragility and fracture risk in the elderly, most particularly in post-menopausal women. Biopsies of bone reveal thinning and diminished numbers of trabeculae (Binte Anwar *et al.*, 2007).

Systemic bone loss may or may not be accompanied by alveolar bone loss (Orrico *et al.*, 2007). Alveolar bone loss may be mild or absent particularly when the exposed dental surfaces are regularly devoid from bacterial plaque and are surrounded by healthy periodontal tissues. Studies are however needed, particularly in non-human primate species, to determine whether a correlation exists between systemic bone loss and periodontal bone loss as the other studies, referred to later, were done on other experimental animals and are therefore not necessarily applicable to humans (Binte Anwar *et al.*, 2007).

1.1 Review of the literature

Yang *et al.* (2005) ovariectomized (OVX) rats to investigate the effects of long-term oestrogen deficiency on the mandibular cortical thickness. OVX resulted only in minor mandibular cortical bone loss after one year. The authors concluded that the low susceptibility of the mandible to cortical bone loss may be due to functional masticatory forces maintaining the bone mass.

While Yang *et al.* (2005) studied mandibular cortical thickness, Mavropoulos *et al.* (2007) analysed the microstructure of mandibular alveolar trabecular and proximal tibial sites by comparing after 112 days the effect of (1) OVX, (2) isocaloric protein undernutrition, and (3) OVX combined with isocaloric protein undernutrition on bone mineral density (BMD) in adult rats. The loss of mandibular alveolar trabecular BMD was less than in the proximal tibial spongiosa in all experiments. The mechanical loading of the alveolar process during mastication may protect the alveolar trabecular bone from the detrimental effects observed in other skeletal sites, such as the proximal tibia. These results confirm the findings of Yang *et al.* (2005) and it appears that mandibular and systemic losses of BMD are poorly correlated.

To verify the conclusions of Yang *et al.* (2005) and Mavropoulos *et al.* (2007), Elsubeihi *et al.* (2009) investigated the effect of OVX on bone changes in the edentulous and dentate mandibles of rats and compared these to changes in tibiae and femora using dual energy x-ray absorptiometry (DXA) and histomorphometrical measurements. At 6 weeks, 6 and 9 months, results showed that loss of bone mass in the edentulous mandible of OVX animals is similar to that occurring in the tibiae and femora, while OVX has little effect on bone mass in the dentate mandible. These results suggest that functional loading related to biting force prevents bone loss in the dentate mandible and confirm the findings of Yang *et al.* (2005) and Mavropoulos *et al.* (2007).

As the above three experiments were performed on OVX rats with a healthy periodontium, Orrico *et al.* (2007) assessed the effect of time and of an additional

variable, namely periodontitis. The authors studied the influence of the period after OVX on femoral and mandibular BMD and on induced periodontal disease in rats. Periodontitis was induced by placing ligatures on the mandibular first molars during the last 30 days of the 51-day and 150-day periods. BMD was measured by DXA. Alveolar bone loss was measured from the crestal bone to the cemento-enamel junction on the mesial side of the mandibular first molar. Statistical analyses revealed that the BMD of the femoral proximal epiphyses was significantly lower in the OVX groups than in the control groups of each period. There was no difference in mandibular BMD between the experimental and control groups of each period. This is another study showing poor correlation between systemic and mandibular bone loss.

To better correlate animal and human studies on effects of OVX, Binte Anwar *et al.* (2007) measured and compared the microstructure of the alveolar bone with the lumbar BMD at baseline in OVX monkeys (*macaca cynomolgus*) and in a control group. From the baseline to 76 weeks later, the lumbar BMD decreased significantly in the OVX group when compared to the control group. The inter-radicular septum of second mandibular molars showed a decreased nodes number and an increase in porosity at the apex of the septa in OVX monkeys when compared to the control group, but no significant differences were found between groups. Therefore, oestrogen deficiency led to some fragility of the trabecular structure of the molar alveolar bone, but this should be considered coincidental when correlated with lumbar BMD.

Baboons and humans exhibit a high degree of similarity in relation to their bone

physiology and trabecular architecture as confirmed by histomorphometric studies of iliac crests (Schnitzler *et al.*, 1993). Baboons are thus ideally suited for comparative studies. Additional comparative studies were was done by conducting an experiment to develop a non-human primate model of systemic bone loss after OVX in 24 adult baboons over a period of 48 months (Dal Mas *et al.* (2007). Eight non-OVX baboons were used as controls. OVX animals demonstrated a decline in trabecular number and an increase in trabecular spacing with significant bone loss. Those animals were later used by Ripamonti *et al.* (2002b) [note: the Dal Mas *et al.* (2007) OVX study started in 1999, was completed in 2004 and published in 2007; in the meantime, the Ripamonti *et al.* (2002b) experiment on the same baboons started in 2001 and was published in 2002] to develop a novel anabolic therapy aimed to locally induce bone formation as opposed to systemic administration of pharmacological compounds inhibiting osteoclastogenesis [note: strontium ranelate (Protos) has an osteoformative effect but needs to be administered twice daily, which makes it unsuitable for long-term animal experiments]. The experimental anabolic local therapy of systemic loss of bone mass (see point **1.6** p. 11 below) consisted in injecting into the lumbar vertebrae a matrix composed of extracellular matrix components (Matrigel™) reconstituted with recombinant human osteogenic proteins (hOPs). Findings revealed that vertebral BMD, measured by DXA, increased in all animals but not uniformly.

From the above review, it appears that the correlation between systemic bone loss and periodontal bone loss is not yet clearly demonstrated in dentate human and non-human primates.

1.2 A brief history of osteoporosis

Osteoporosis may have haunted women since antiquity when they survived long enough to reach menopause. Egyptian mummies from 4,000 years ago have been found with the dowager's hump (popular name for the dorsal kyphosis caused by multiple wedge fractures of the thoracic vertebrae) (Stride *et al.*, 2013). Today, most women can expect to spend their old age almost standing as straight and tall as they ever were, thanks to recent improvements in the prevention, diagnosis and treatment of osteoporosis.

The physicians listed below in chronological order have been pioneers in the study of osteoporosis.

John Hunter (Scottish anatomist and surgeon, 1728-1793), discovered that as new bone is laid down in the skeleton, old bone is resorbed. This process, known as remodelling, was later shown to play a critical role in osteoporosis, though it was not even a recognized disease for more than 100 years after his death (Paget, 1897).

Sir Astley Paston Cooper (English surgeon, 1768-1841) mentioned osteoporosis when he discovered the relation between fractures and bone density reduction due to aging and, consequently, laid the base for studying the aetiology of osteoporosis (Doganay, 2014).

Johann Friedrich Lobstein (German surgeon, 1777-1835) described the appearance and the pathological condition of bone fragility. Today, the disease is known as osteogenesis imperfecta type I (autosomal dominant), which is sometimes called Lobstein's disease

(Kissel, 1953).

Fuller Albright (American physician and endocrinologist, 1900-1969) observed in the 1930s that most of his patients with osteoporosis were postmenopausal women. Around the same time, anatomists noticed that bones of ovulating female pigeons were more massive than those of male pigeons. Researchers found that by injecting oestrogen, a hormone secreted primarily by the ovaries, the bone mass of male pigeons increased dramatically. Given this, Albright proposed that oestrogen triggers the build-up of calcium reserves in bone. After menopause, the reduction in circulating oestrogen leads to bone loss (Reifenstein *et al.*, 1947). Oestrogen replacement therapy has proven to be effective at staving off osteoporosis in both women and men.

1.3 Osteoporosis in arts

The image of old women bent with age is ingrained in the classical literature and other arts.

And Athena lavished a marvellous splendour on the prince so that all the people gazed in wonder as he came forward. The elders making way as he took his father's seat. The first to speak was an old lord, Aegyptius, stooped with age, who knew the world by heart. (In: "The Odyssey", poem by Homer in the Greek mythology; translation by Robert Fagles)

Ask an actress to portray an old woman and chances are she'll start by stooping over so she appears to have a curved spine, or "dowager's hump." Until recently, such a posture

1.4 Brief synopsis of the present therapy for osteoporosis

Each therapy listed below has specific indications and contra-indications which cannot be entirely described in this short synopsis. In a review paper on the diagnosis, prevention and therapy of osteoporosis, Cianferotti *et al.* (2012) revealed that the prevalence of osteoporosis in women rises markedly with age rising from 2 % at 50 years to more than 25 % at 80 years. In Italy (population of 61 million inhabitants), it is estimated that 3.5 million women and 1 million men suffer from osteoporosis, with more than 90,000 femoral neck fractures each year in the population older than 50 years. Risk of fracture is also increased by factors related to lifestyle, medical therapies, family history, and other conditions that cause secondary osteoporosis (Cianferotti *et al.*, 2012).

Presently, pharmacologic treatments are as follows:

Bisphosphonates (BPs) (Cianferotti *et al.*, 2012)

BPs (alendronate, risedronate, ibandronate, zoledronate, etidronate, clodronate)

Alendronate (10 mg PO/day) and *risedronate* reduce vertebral, non-vertebral and femoral fractures by 40 to 50 % in the course of a three-year therapy.

Ibandronate reduce vertebral, non-vertebral fractures when administered orally (150 mg PO/month or 3 mg IV/3 months).

Zoledronate (5 mg IV/year) significantly reduces the risk vertebral, non-vertebral and femoral fractures, and reduces further fractures when administered two weeks after femoral fractures.

Etidronate (5 mg PO/kg/day initially) and *clodronate* (800 mg PO/day) increase vertebral BMD and maintain BMD at the femoral neck. However, these two medicaments are

second choice therapies in osteoporosis.

These above medicaments, when administered IV, may cause chemo-osteonecrosis of the jaws. However, the risk is greatly reduced with a good plaque control and the administration of antibiotics before oral surgery.

Denosumab (Prolia®) (Cianferotti *et al.*, 2012)

RANKL (receptor activator of nuclear kappa-B ligand) is a surface-bound molecule on osteoblasts that activates osteoclasts. The inhibition of RANKL by Denosumab (60 mg SC/6 months) results in an antiresorption effect similar to the effect of BPs.

Parathyroid hormone (PTH) (Cianferotti *et al.*, 2012)

For more severe forms of osteoporosis, PTH is administered subcutaneously daily up to 24 months. This anabolic therapy results in major increments in vertebral BMD up to 9.7 %.

Strontium ranelate (SR) (Cianferotti *et al.*, 2012)

The antiresorption-osteoformative SR (2 g/daily) reduces the risk of vertebral and non-vertebral fractures in the short and long term as proven with 5 years controlled clinical evaluation.

Oestrogen replacement therapy (ORT) (Cianferotti *et al.*, 2012)

ORT reduces the risk of osteoporotic fractures at any site along with a reduction in the risk of colon-rectal cancer. The counterpart is an increase in the rate of breast cancer, cardiovascular events and venous thromboembolic events.

Selective oestrogen-receptor modulators (SORMs) (Cianferotti *et al.*, 2012)

Raloxifene (60 mg/daily) prevents postmenopausal bone loss resulting in an increase of 2 to 3 % in BMD of osteoporotic women. Raloxifene reduces by 30 % the risk of vertebral

fractures in women with pre-existing vertebral fractures and by 60 % in women without previous fractures along with a significant decrease in the risk of invasive breast cancer. A reduction in non-vertebral fractures has not been demonstrated.

Bazedoxifene reduces the risk for vertebral fractures by 42 and 32 % at 3 and 5 years respectively. A reduction in non-vertebral fractures at 3 and 5 years has been demonstrated in patients with a high risk of fractures.

1.5 Innovative therapies for osteoporosis

Phyto-oestrogens (Bedell *et al.*, 2014)

Concerns about the risk of oestrogen exposure through hormone replacement therapy (HRT) have resulted in an increase of natural therapy alternatives. Current research demonstrates that phyto-oestrogens (isoflavones, lignans, coumestans) are effective in reducing the intensity of hot flushes, and some combinations result in a decrease frequency. Moreover, there is decreased vaginal atrophy, improved sleep, cognition and bone health. Trials, although not specifically designed to find neoplasia, have shown neither an increase in breast cancer risk or in endometrial hyperplasia. Furthermore, unlike HRT, lignans may not increase clotting risk in postmenopausal women. However, as research regarding phyto-oestrogens is relatively new, pharmaco-vigilance is still required.

Bone marrow mesenchymal stem cells (Wan, 2012)

Bone marrow mesenchymal stem cells (BMMSCs) are multipotent stromal cells with the ability to differentiate into a variety of cell types, including osteoblasts and chondrocytes, required for tissue regeneration. The differentiation of BMMSCs into osteoblasts is

regulated by various environmental cues and intrinsic signalling pathways. Recent advances in the understanding of BMMSC function and regulation include the interaction between BMMSCs with the haematopoietic and immune systems and the identification of novel modulators of BMMSC differentiation such as the metabolic hormone fibroblast growth factor-21 (FGF-21). Thus, BMMSCs may be included in therapeutic strategies to prevent and treat degenerative disorders such as osteoporosis and osteoarthritis.

1.6 Innovative therapies for bone induction

Local injections of recombinant human osteogenic proteins (hOPs) in *Papio ursinus*, with or without autogenous morcellated rectus abdominis muscle cells (RAMCs), have shown that the application of RAMCs did improve the induction of the recombinant human transforming growth factor-β_3 (hTGF-β_3) isoform when implanted in calvarial bone defects treated by the morphogens combined with insoluble collagenous bone matrix (ICBM) as carrier (Ripamonti *et al.*, 2009a). Moreover, the implantation of morcellated RAMCs induced greater amounts of newly formed cementum and alveolar bone in Class II and III furcation defects in *Papio ursinus* (Ripamonti *et al.*, 2009b; Ripamonti *et al.*, 2009c).

Soluble molecular signals of hTGF-β supergene family need to be combined, or reconstituted with insoluble signals or substrata. The reconstitution triggers the ripple-like cascade of bone formation by induction (Sampath *et al.*, 1981; Reddi, 2000). Ripamonti reported that naturally-derived hBMPs and hOPs when reconstituted with Matrigel™ matrix induce bone formation in subcutaneous tissues of rats (Ripamonti, 1993,

2002b). The use of Matrigel™ matrix combined with osteogenic members of hTGF-β supergene family suggested a novel anabolic treatment of systemic bone loss in non-human primates *Papio ursinus*. When kept on ice, Matrigel™ matrix stays in a liquid state without undergoing gelation. This knowledge prompted the use of liquid Matrigel™ reconstituted with hOPs as an effective delivery system for direct and local injections into lumbar vertebrae and neck of femora in non-human primates affected by systemic bone loss (Ripamonti *et al.*, 2002b).

2 AIM

To establish whether there is a possible correlation between systemic and periodontal bone loss does exist in the non-human primate *Papio ursinus*. All the biochemical, clinical and histological procedures related to the study of the induction of systemic bone loss have been previously performed from 1999 to 2004 (Dal Mas *et al.* 2002, 2007). However, the putative bone loss within the furcation of the mandibular molars has neither been examined nor evaluated.

Study objectives

As a complementary investigation on the animals studied by Dal Mas *et al.* (2002, 2007), this study was designed to evaluate, by histomorphometry, bone fraction composition of the mandibular alveolar bone of OVX baboons and compare the measurements with the non-OVX control animals. The bone fraction composition consists of mineralized bone (Md), osteoid (Oe) and medullary space (bone marrow) or fibrovascular space (Fv). The study further evaluated the distant effects on mandibular alveolar bone of the local injections of hOPs within lumbar vertebrae and femora. The animal cohorts published by Dal Mas *et al.* (2007) were additionally used in a pilot study for local injections of OPs in the lumbar vertebrae and neck of the femora (Ripamonti *et al.*, 2002b). Based on the harvested specimens chronologically listed in the laboratory record book from 2003 to 2004 (Table 3.5 p. 21 and Table 7.2 pp. 71-74.), the following experimental groups were:

1 OVX - hTGF-β_3 (13 animals)

2 OVX - hTGF-β_3 with RAMCs (1 animal)

3 OVX - recombinant human osteogenic protein-1 (hOP-1) (2 animals)

4 OVX - hOP-1 with RAMCs (2 animals)

5 non-OVX with hTGF-β_3 (6 animals)

6 non-OVX with hOP-1 (1 animal)

The control groups were:

1 OVX - non-injected (1 animal)†

2 non-OVX - non-injected (2 animals)

The reasons for using six experimental and two control groups, composed of 19 OVX and 9 non-OVX animals, were that these expensive experiments on osteoporosis required a large number of animals over a four-year period of observation, demanding husbandry and clinical and laboratory procedures which cannot be repeated frequently. Therefore, it had to be a once-off experiment within the existing budget and clinical constraints.

1 To investigate whether the alveolar bone volume within the furcations of the first and second mandibular molars differs between experimental and control animals.

2 To investigate whether the alveolar bone volume within the furcations differs between animals injected with either hOP-1 with or without RAMCs or recombinant human hTGF-β_3 with or without RAMCs.

3 To investigate whether the alveolar bone composition within the furcations differs between OVX injected and control animals versus OVX non-injected and control animals.

4 To evaluate the distant effects of the local injections of hOPs within lumbar vertebrae and femora on the alveolar bone composition within the furcations of the first and second mandibular molars.

† In the course of the Dal Mas *et al.* (2007) study, the second animal developed a chronic staphylococcal infection of the nasal cavities. Despite antibiotic therapy based on an antibiogram, the treatment failed and the animal was euthanized.

3 MATERIALS AND METHODS

The biochemical, clinical and histological procedures under this heading were completed in 2004 and published by Dal Mas *et al.* in 2002 and 2007. However, the mandibular specimens, i.e. two blocks per animal each containing the first and second molars from the 3rd or 4th quadrants, which were the object of this study, had neither been examined or evaluated.

3.1 Summary of the experimental procedure

In the Dal Mas *et al.* (2007) study, research protocols were approved by the Animal Ethics Screening Committee of the University (Clearance certificate 94/01/5) and the study was conducted according to the *Guidelines for Care and Use of Experimental Animals* prepared by the University, and in compliance with the *National Code for Animal Use in Research and Diagnosis in South Africa* (Public Service Department, 1990). Thirty-two adult female Chacma baboons (*Papio ursinus*), 24 OVX and 8 non-OVX controls, were studied while housed at the Central Animal Service of the University of the Witwatersrand, Johannesburg. [Note: in the Dal Mas *et al.* (2007) study, 24 OVX animals were simply compared to 8 non-OVX controls. Then, when systemic bone loss was documented, the same animals were either injected or not with hOPs in the Ripamonti *et al.* (2002b) study. Criteria for inclusion in the study were normal haematological values (Melton *et al.*, 1982) and skeletal maturity as confirmed by closure of the distal epiphyseal growth plates of the radius and ulna (Ripamonti, 1991; Schnitzler *et al.*, 1993). All animals had normal regular menstrual cycles as shown by regular perineal sex skin swelling. Twenty-four baboons were matched by weight with eight controls and ovariectomized. Eight adult animals were used as non-OVX controls (see above Note). DXA was used to evaluate the BMD of the lumbar spine, total hip

and radius at 0, 6, 12, 18, 24, 36 and 48 months. Annual bone biopsies consisting of both cortices and intervening cancellous bone were taken from the iliac crest. Histomorphometric analyses were confined to trabecular bone. Microarchitectural deterioration of trabecular bone in OVX animals was demonstrated by a decline in trabecular number and an increase in trabecular spacing (Dal Mas *et al.*, 2007). Subsequently, the experimental animals underwent various therapies as summarized in Table 3.1 below.

Table 3.1 Systemic bone loss in each category of experimental and control animals at the end of the experiment†

Bone percentage by the Least Squares Means method

Treatment	N° of animals	Femoral head	Femoral trochanter	Femoral neck	Iliac crest	Vertebra
OVX						
hTGF-β_3	13	5.69	5.04	5.15	3.88	4.18
hTGF-β_3 + RAMCs	1	5.21	4.56	4.76	3.40	3.70
hOP-1	2	5.38	4.74	4.84	5.58	3.88
hOP-1 + RAMCs	2	5.66	5.01	5.12	ND*	4.15
OVX control						
Non-injected	1††	ND*	ND*	ND*	ND*	ND*
Non-OVX						
hTGF-β_3	6	5.87	5.22	5.33	4.06	4.36
hOP-1	1	ND*	ND*	ND*	ND*	ND*
Non-OVX control						
Non-injected	2	5.06	4.41	4.52	3.25	3.55
Total	28					

* not done

† Data requested by the Post-graduate Research Protocol Committee and provided by the Bone Research Laboratory.

†† After two years of observation, one OVX control non-injected animal was euthanized subsequent to a staphylococcal infection of the nasal cavities unresponsive to treatment. This animal could not be replaced.

In the present Suliman's study, the above 28 animals were investigated as the specimens from the euthanized animal and 3 other baboons were not available for evaluation.

Data obtained from the Dal Mas et al. (2007) study†

Table 3.2 Serum values at 48 months to assess bone metabolism

	OVX (N=23)††	Non-OVX (N=8)	*p* value OVX vs non-OVX
Oestradiol (pg/mL)	28 ± 6	138 ± 88	<0.001
PTH (pg/mL)	82 ± 50	67 ± 33.7	NS*
Calcitonin (pg/mL)	10.7 ± 7.2	11.8 ± 7.3	NS*
1,25 $(OH)_2$ D (ng/L)	69.3 ± 26.2	61.9 ± 17.19	NS*
Ca (mM/L)	2.44 ± 0.11	2.35 ± 0.15	NS*
Pi (mM/L)	1.32 ± 0.3	1.32 ± 0.32	NS*

* not significant

As a result of OVX, serum oestradiol levels initially decreased rapidly but were detectable at each subsequent evaluation period with little variation. All the other biochemical variables did not differ between OVX and non-OVX animals.

† Data requested by the Post-graduate Research Protocol Committee]

†† After two years of observation, one OVX control animal was euthanized subsequent to a staphylococcal infection of the nasal cavities unresponsive to treatment.

Histometrical assessments were confined to the trabecular bone and done on two non-consecutive sections (250 μm apart) from each of the following bones: head, trochanter and neck of femora; iliac crest; lumbar vertebra (see Table 3.3 below).

Number of histological slides used for measurements: two slides for each of the five anatomical sites in 24 experimental and 8 control animals, i.e. 320 slides.

Table 3.3 Average trabecular bone thickness in μm by the Least Squares Means method†

	N° of animals	Femoral head	Femoral trochanter	Femoral neck	Iliac crest	Vertebra
OVX						
hTGF-β_3	13	17.51	17.97	18.41	15.65	16.20
hTGF-β_3 + RAMCs	1	16.40	16.86	17.30	14.54	15.08
hOP-1	2	17.87	18.33	18.77	16.00	16.55
hOP-1 + RAMCs	2	19.46	19.92	20.36	ND*	18.14
OVX control						
Non-injected	1	ND*	ND*	ND*	ND*	ND*
Non-OVX						
hTGF-β_3	6	16.47	16.93	17.37	14.60	15.15
hOP-1	1	ND*	ND*	ND*	ND*	ND*
Non-OVX control						
Non-injected	2	14.85	15.31	15.75	12.99	13.54
Total	28					

* not done

† Data requested by the Post-graduate Research Protocol Committee and provided by the Bone Research Laboratory.

The mandibular specimens harvested from OVX and non-OVX animals included in the Dal Mas *et al.* (2007) and Ripamonti *et al.* (2002b) studies were processed to cut undecalcified 5-µm thick sections at the Bone Research Laboratory. Histological sections were thus available to correlate systemic and alveolar bone loss in experimental and control animals.

This present investigation was performed on two experimental groups:
(1) 18 adult OVX baboons injected with hOPs;
(2) 7 old baboons non-OVX but injected with hOPs.
The two control groups are:
(1) 1 adult OVX baboon non-injected (the second animal was euthanized subsequent to a severe chronic nasal infection not responding to treatment)
(2) 2 adult baboons which have neither been OVX nor treated with hOPs.

3.2 Data collection

The undecalcified sections of the Dal Mas *et al.* (2007) and Ripamonti *et al.* (2002b) studies consisted of the left and right first and second mandibular molars with surrounding alveolar bone obtained from 28 baboons. Based on the record books for the years 2002 to 2004, 28 female baboons, involved in the osteoporosis experiments, were recorded as having had histological sections of the bilateral mandibular first and second molars. The laboratory data (i.e. Slide nb, Animal's ear tag, Treatment, Quadrant 3 or 4, Molar 1st or 2nd, Furcation apex to mesial, mm, Furcation apex to distal, mm, Furcation surface area, mm^2, Furcation bone area, mm^2, Furcation bone + osteoid, mm^2, Furcation osteoid area, mm^2, Furcation bone %, Furcation osteoid %, Furcation medul %) from those 28 animals were transferred onto an initial Microsoft Excel spreadsheet (Addenda Table 7.2 Initial spreadsheet pp. 71-74).

The slides of baboon wearing the ear tag 1591, OVX-control non-injected, could not be retrieved; therefore, the animal was not given an identification number and was subtracted from the list of the original 29 baboons† (Table 4.1 p. 43; Addenda Table 7.2 pp. 71-74). Furthermore, six histological slides representing 12 furcations were missing in four different animal groups (see Table 3.4 below).

Table 3.4 Missing histological slides

N°	Slide n°	Animal's tag	Treatment	Quadrant
19	262-04	1661	OVX-25 µg TGF-β_3	Q 4
24	274-04	1681	OVX-25 µg TGF-β_3	Q 4
50	18-04	1706	OVX-25 µg OP-1	Q 3
54	31-04	1588	OVX-25 µg OP-1 + RAMCs	Q 3
-	142-02	1591	OVX-control non-injected	Q 3
-	143-02	1591	OVX-control non-injected	Q 4

For easy identification purpose, all six slides are listed in the initial spreadsheet (Addenda Table 7.2 pp. 71-74) and marked with a green square at the site of the identification number. Rows of white and light yellow alternating colours identify each animal in sequence. A single bright yellow row separates each experimental and control group.

Each harvested mandibular specimen, both left (quadrant 3) and right (quadrant 4) blocks, provided one histological section of two furcations. However, due a delicate and newly acquired laboratory process, some specimens had to be re-cut (R) several times to obtain better sections.

† The histological slides of 3 other baboons were not available thus reducing the initial number of animals from 32 to 29 and then to 28.

When additional sections were cut, 250 µm apart from each other, measurements were pooled to calculate the average values for each furcation.

Regretfully, 45 furcations could not be evaluated due to inadequate processing. The reasons were folds and dilacerations in the sections preventing precise measurements. For easy recognition, their identification numbers were marked with a blue square on the same initial spreadsheet (Addenda Table 7.2 pp. 71-74). All missing slides, and furcations inadequate for histomorphometry, were not included in the working spreadsheet which was used for the statistical analysis (Addenda Table 7.3 pp. 75-78). The histological material examined in this study originated from six experimental and two control groups listed in Tables 3.5 and 3.6 below.

Table 3.5 Experimental groups and independent variables

N° of animals	OVX	Injection
13	OVX	25 µg hTGF-β_3
1	OVX	25 µg hTGF-β_3 + RAMCs
2	OVX	25 µg hOP-1
2	OVX	25 µg hOP-1 + RAMCs
6	Non-OVX	25 µg hTGF-β_3
1	Non-OVX	25 µg hOP-1
Total 25		

Table 3.6 Control groups

N° of animals	OVX	Injection
1	OVX	Non- injected
2	Non-OVX	Non-injected
Total 3		

[Note: after two years of observation, one OVX control non-injected animal was euthanized subsequent to a staphylococcal infection of the nasal cavities unresponsive to treatment]

3.3 Histomorphometry

Hardware

The system for histomorphometry consisted of four separated units: Olympus UPS, microscope AX70, freestanding control unit, and a camera with associated software loaded on a PC for visualisation on a computer screen. The software "Stream Essentials" (Olympus Corporation, Tokyo) was used for all histomorphometric linear and area measurements. The measurements were recorded on an Olympus spreadsheet compatible with Microsoft Excel for easy transfer to an Excel worksheet. Before starting any measurements, enlarged colour photographs were taken by scanning each furcation to study the morphology and analyse whether the furcation was suitable for histometry or had to be rejected.

The undecalcified histological sections were stained by the Goldner's Trichrome method and then digitalized at a 20-time magnification, 20 pixels, to take the measurements on a computer screen using the programme Olivia (Olympus). As the initial step, the intraobserver and interobserver reliability data for the measurements of the furcation areas

were gathered by taking measurements of furcation areas for ten randomly selected histological slides on ten different occasions and viewed each time in a random order. This was done by both the applicant and the supervisor. Then, the composition of the alveolar bone (mineralized bone, osteoid, medullary spaces) within the furcation of the first and second mandibular molars was measured together with the level of bone recession at the apex of the furcations. The eight experimental procedures in 28 animals are listed in Table 3.7 below.

Table 3.7 Treatments groups and number of animals

N°	Treatment	N° of animals
1	OVX-25 µg hTGF-β_3	13
2	OVX-25 µg hTGF-β_3 + RAMCs	1
3	OVX-25 µg hOP-1	2
4	OVX-25 µg hOP-1 + RAMCs	2
5	OVX *control non-injected*	1
6	non-OVX-25 µg hTGF- β_3	6
7	non-OVX-25 µg hOP-1	1
8	non-OVX *control non-injected*	2
	Total	28

The following measurements were done:

1 Linear measurements by using an arbitrary straight line to evaluate the distance between the apex of a furcation to the mesial and distal aspects of the inter-radicular crestal bone in instances of bone loss (straight green lines in Fig. 3.1). When there was along the full periphery of the furcation a uniform and continuous periodontal

4.2 Statistical analysis

Brief review of the overall investigation

As the initial step, the intraobserver and interobserver reliability data for the measurements of the furcation areas were gathered by taking measurements of furcation surfaces for ten randomly selected histological slides. The same ten slides were randomly remeasured on ten different occasions. This was done by both the researcher and the supervisor. Then, the composition of the alveolar bone (mineralized bone, osteoid, medullary spaces) within the furcation of the first and second mandibular molars was measured together with the level of bone recession at the apex of the furcations. The eight experimental procedures in 28 animals are listed in Table 3.7 p. 23.

There were thus three essential variables (treatments) with the experiments representing an incomplete 2 x 3 x 2 design, namely:

- OVX (present or absent)
- hOPs (hTGF-β_3 or hOP-1 or none)
- RAMCs (present or absent)

The following measurements were taken for each of the five outcome variables:

- furcation (apex to mesial) (mm)
- furcation (apex to distal) (mm)
- area of bone (%)
- area of osteoid (%)
- area of medullary spaces (%)

The recorded covariates were:

- baboon
- quadrant (3^{rd} or 4^{th})
- molar (1^{st} or 2^{nd})

The research investigations were:

1. Determine the intraobserver and interobserver reliability, and for the five outcome variables:
2. Compare all treatments to their respective OVX and non-OVX controls
3. Compare all treatments with hTGF-β_3 or hOP-1 or none, within the OVX and non-OVX groups
4. Compare corresponding treatments with hTGF-β_3 or hOP-1 with and without RAMCs
5. Compare corresponding OVX and non-OVX hTGF-β_3 or hOP-1 treatments

Intraobserver and interobserver reliability to measure furcation surfaces

Intraobserver reliability was assessed by calculating the relative standard deviation (RSD) for each of the ten slides measured ten times on different days by each observer (Table 4.1 p. 43; Addenda: Table 7.1 pp. 68-70). Examination of the results indicated that the RSDs for the measurements made by Observer 1 (researcher) were slightly larger than those for the measurements made by Observer 2 (supervisor) but they were of the same order of magnitude. Also, the RSDs for both observers were all below 2 %.

Table 4.1 Intra-observer reliability in histological measurements

	Mean (μm^2)		RSD (%)	
Slide n°	Observer 1	Observer 2	Observer 1	Observer 2
1582-4	19 594 620	19 217 155	1.32%	0.88%
1613-L1	22 580 096	22 986 669	0.59%	0.82%
1624	22 825 488	23 084 065	0.98%	0.67%
1661	23 822 010	23 201 122	0.57%	0.31%
1672	26 414 789	27 329 155	1.24%	0.63%
1674	28 862 481	28 434 586	1.00%	0.51%
1677	31 195 639	30 798 439	0.47%	0.58%
1680	37 367 549	36 679 853	0.67%	0.38%
1681	31 823 865	31 767 387	0.53%	0.36%
1682	30 991 655	30 828 994	1.47%	0.48%

Comparing the mean measurements obtained by the two observers for each of the 10 slides, it appears that Observer 1 (researcher) tended to obtain slightly higher results than Observer 2 (supervisor) in most cases. The Bland-Altman plot (below) showed that the bias did not increase with the average magnitude of the measurements (Altman and Bland, 1983; Bland and Altman, 1986).

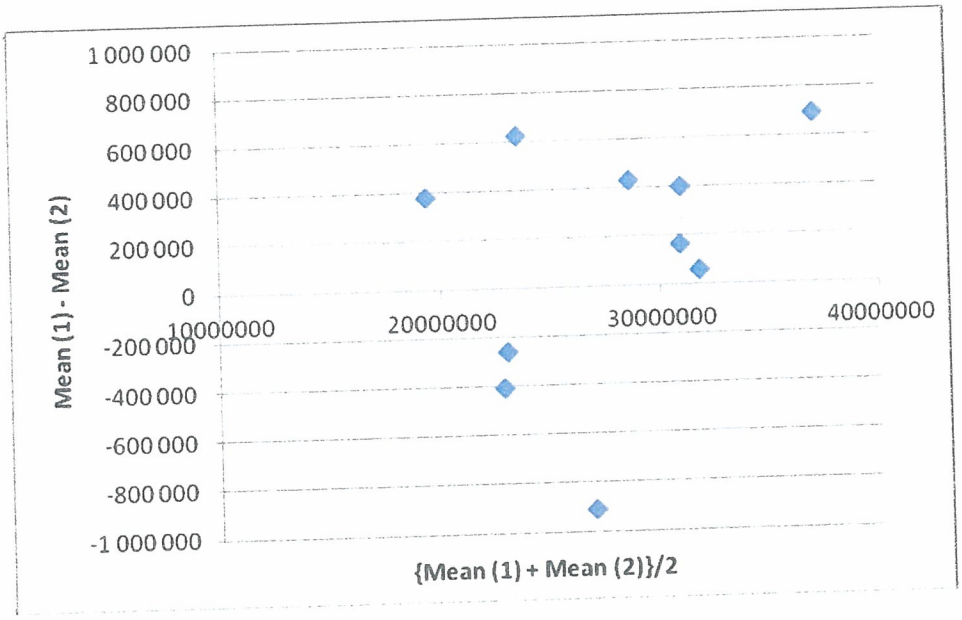


Figure 4.15 Bland-Altman plot

The interobserver reliability was assessed by means of the intraclass correlation coefficient (ICC), derived from a two-way Analysis of Variance (ANOVA) (with interaction), with slides as a random effect and observers as a fixed effect. The ICC is defined as the proportion of the overall variance in the measurements attributable to the objects of the measurements (the histological slides). In this analysis, the ICC = 0.99 which indicates an excellent interobserver agreement for the measurement of the furcation surfaces (Shoukri, 2004).

Analysis of the full data set

Structure of the data set (Addenda: Table 7.3 Working spreadsheet 2 pp. 75-78).

The tabulations and cross-tabulations of the frequencies of experimental units (furcations) with respect to the different covariates are given in Table 7.3. In the study, it was noted the following:

- There were 78 experimental furcations spread over eight treatments, with 1 to 32 experimental sites per treatment.

Table 4.2 Frequency and percentage of experimental sites per treatment

Treatment	Frequency	Percentage
OVX-25 µg hTGF-β_3	32	41
OVX-25 µg hTGF- β_3 + RAMCs	4	5.1
OVX-25 µg hOP-1	5	6.4
OVX-25 µg hOP-1 + RAMCs	3	3.9
OVX control non-injected	4	5.1
non-OVX-25 µg hTGF- β_3	23	29.5
non-OVX-25 µg hOP-1	1	1.3
non-OVX control non-injected	6	7.7
Total	78	100

From Table 4.2, it appears that the sample size is very small for most treatments, and this affects the power of the study, i.e. the ability to detect a significant difference between outcomes of treatments†. To illustrate this point, a sample size calculation for an ANOVA with 8 groups and a treatment main effect (significance level (α) adjusted from 0.05 to 0.037 to allow for 14 multiple comparisons (see later, power = 0.80, and a large effect size) would require 112 experimental units (14 per group); a higher sample size is needed for unbalanced groups. The sample sizes in this study fall far short of these requirements.

† In the Dal Mas *et al.* (2007) study, the results were reported comparing OVX and non-OVX animals without taking the type of treatment, or its absence, into consideration.

- The data were obtained from 28 baboons, each contributing between 1 and 4 teeth. The study teeth were reasonably well-balanced between the 1st and 2nd molars (56 and 44 % respectively). The teeth were well-balanced between quadrants (50 % each).
- Each baboon received only one treatment, thus baboon is nested within treatments.
- Each treatment was applied to between 1 and 13 baboons.
- Treatments were reasonably well distributed between quadrants.

Table 4.3 Number of furcations examined per quadrant

Treatment	Quadrant 3	Quadrant 4	Total
OVX-25 µg hTGF-β_3	18	14	32
OVX-25 µg hTGF-β_3 + RAMCs	2	2	4
OVX-25 µg hOP-1	2	3	5
OVX-25 µg hOP-1 + RAMCs	0	3	3
OVX control non-injected	2	2	4
non-OVX-25 µg hTGF-β_3	11	12	23
non-OVX-25 µg hOP-1	0	1	1
non-OVX control non-injected	4	2	6
Total	39	39	78

- Treatments were well distributed between 1st and 2nd molars.

Descriptive statistics for the dependent variables (Univariate)

The univariate statistics (mean, standard deviation, median, interquartile range) for the dependent variables are given in the working spreadsheet (Addenda: Table 7.3 Working spreadsheet 2 pp. 75-78) overall and by treatment group.

Histograms plots for the (unweighed) dependent variables are also given in Table 7.3 Working spreadsheet. The following is noted:

1. Bone: slightly negatively skewed.
2. Osteoid: highly positively skewed, with a large proportion of zero values.
3. Medullary spaces: positively skewed.
4. Furcation: Apex to Mesial
5. and Apex to Distal: very positively skewed with a large proportion of zero values. It appears that there are two populations in the sample, one with values of zero, and one with a fairly normal distribution of non-zero values.

Between-group analysis for each of the five dependent variables (separate table for each dependant variable)

A linear mixed model was used with treatment (at 8 levels) as main effect. Baboon (nested in treatment) (random effect), molar and quadrant (both fixed effects) were included as blocking variables. Post-hoc comparisons were carried out using the Bonferroni correction for multiple testing. Fourteen comparisons reduced the significance level from 0.05 to 0.037.

5 DISCUSSION

Non-human primates *Macaca fascicularis* and *Macaca mulatta* have been often used in laboratory research. However, about fifty years ago, and mainly in South Africa, the Chacma baboon of the genus *Papio*, a much larger primate, became a favourite experimental animal to design new surgical protocols for organ transplantation, tissue induction and morphogenesis (Groenewald *et al.*, 1973; Jonck, 1975; Kalter, 1977; Barnard *et al.*, 1981; Ripamonti, 2013).

For over two decades, the Bone Research Laboratory of the University of the Witwatersrand, Johannesburg, has successfully created experimental animal models using the adult Chacma baboon *Papio ursinus* for regenerative procedures and efficacy tests in preclinical contexts before clinical applications in humans. Baboons and humans exhibit a high degree of similarity in relation to their bone physiology and wound repair processes as confirmed by histomorphometric studies conducted on iliac crest biopsies (Schnitzler *et al.*, 1993).

Thirty-two healthy adult baboons were included in the study designed to induce systemic bone loss by ovariectomy in 24 animals, the remaining 8 animals being used as controls. At the end of a 48-month period, mandibular blocks with left and right first and second molars were harvested with their surrounding soft tissues. Undecalcified sections prepared from the undecalcified mandibular blocks were subjected to histomorphometric

analysis to compare furcations of first and second mandibular molars of OVX and non-OVX baboons which had undergone novel therapies for osteoporosis by local injections of OPs within lumbar vertebrae and neck of femora to locally increase systemic anabolic bone mass (Ripamonti *et al.*, 2002b).

The initiation of systemic bone loss by ovariectomy in 24 baboons with 8 additional non-ovariectomized animals being used as controls was experimentally sound for the power of the statistical analysis. In the long-term ovariectomized baboons, histological data on the morphology and structure of biopsies harvested from the iliac crest disclosed that bone architecture was permanently lost 36 months post-ovariectomy. Furthermore, BMD measurements of the lumbar spine revealed that lumbar vertebrae were more affected by oestrogen depletion than other skeletal structures (Dal Mas *et al.*, 2002; Ripamonti *et al.*, 2002b).

The composition of both OVX and non-OVX groups changed considerably when subgroups were formed to treat systemic bone loss by injecting OPs with a carrier into affected vertebrae for bone induction and recovery of the original trabecular morphology (Ripamonti *et al.*, 2002b). The composition of groups and subgroups became more focussed on the therapeutic potential hTGF-β_3 on 13 OVX and 6 non-OVX baboons which are highlighted in Table 5.1 p. 57.

The groups and subgroups were as follows:

Table 5.1 modified from Table 3.3 p.18

Treatment	N° of animals
OVX	
hTGF-β_3	13
hTGF-β_3 + RAMCs	1
hOP-1	2
hOP-1 + RAMCs	2
OVX control	
Non-injected	1
Non-OVX	
hTGF-β_3	6
hOP-1	1
Non-OVX control	
Non-injected	2
Total	28

The above table can easily be modified by replacing the numbers of animals per treatment by the percentage of experimental sites per treatment as seen in Table 5.2 below. The highlights are still on both hTGF-β_3 groups.

Table 5.2 first modified from Table 4.3 p. 46 and then modified from Table 5.1 p. 57

Percentage of experimental sites per treatment

Treatment	Percentage
OVX-25 µg hTGF-β_3	41
OVX-25 µg hTGF- β_3 + RAMCs	5.1
OVX-25 µg hOP-1	6.4
OVX-25 µg hOP-1 + RAMCs	3.9
OVX control non-injected	5.1
non-OVX-25 µg hTGF- β_3	29.5
non-OVX-25 µg hOP-1	1.3
non-OVX control non-injected	7.7
Total	100

The percentage column clearly shows that the sample size was very small for most treatments except for OVX-25 µg hTGF-β_3 and non-OVX-25 µg hTGF- β_3 groups, and this affected the power of the study, i.e. the ability to detect a significant difference between outcomes of treatments. For instance, a sample size calculation for an ANOVA with 8 groups and a treatment main effect (significance level (α) adjusted from 0.05 to 0.037 to allow for 14 multiple comparisons (power = 0.80, and a large effect size) would require 112 experimental units (14 per group); a higher sample size is needed for unbalanced groups. The sample sizes in this study felt far short of these requirements and constituted a major shortcoming of the study. The only meaningful comparative study could be done between groups OVX-25 µg hTGF-β_3 and non-OVX-25 µg hTGF-β_3 representing 41 % and 29.5 % of all measured sites respectively, although the non-OVX group has lost its control status by undergoing therapy with 25 µg hTGF-β_3.

Missing histological slides, together with the technical hitches in processing undecalcified large blocks, contributed to the difficulty of this study. The collection of data revealed six missing, or lost, histological slides (12 furcations), and a substantial number of furcations which could not be evaluated due to inadequate processing (45 furcations). Consequently, the expected 112 furcations in 28 baboons were reduced to 55 furcations, i.e. hardly half of the expected number of furcation to be evaluated.

As the histological evaluation was done blindly, the statistical analysis was required to identify whether there were differences between alveolar bone histomorphologies which could have resulted from the systemic effects of localized therapies with hOPs. However, due to the small sample of evaluable furcations (55), the number of experimental groups (2) and independent variables (6) limited to 25 animals and two control groups composed of 3 animals, it became clear that the statistical analysis would reveal that the effect of treatment would be insignificant for any of the measurements, namely: mineralized bone, osteoid, fibrovascular spaces, apex to mesial and apex to distal.

Even though systemic treatments were not effective in increasing alveolar bone fractional volumes when comparing OVX, non-OVX, treated and untreated animals, ovariectomy did not cause further alveolar bone loss when compared to non-OVX animals. However, although no apical radiographs of the mandibular molars were taken before the start of the experiments, it was assumed that animals had not been susceptible to cribriform bone loss as this bone was uniform, rather thick and extended to the full periphery of most furcations. Presumably, the functional loading related to biting force prevented bone loss

in the dentate mandible and confirm the findings of Yang *et al.* (2005) and Mavropoulos *et al.* (2007).

Though the fractional volumes of mineralized alveolar bone in injected OVX and non-OVX baboons showed no significant difference among treatments and base line controls, histomorphometric histogram of osteoid volume fractions (Fig. 4.17 p.50) showed that morphogens injection resulted in greater amount of osteoid particularly in non-OVX animals injected with doses of hOP-1 as well as hTGF-β_3 with *rectus abdominis* morcellated muscle cells. This trend indicates that further studies, if at all possible, should be implemented to show the anabolic effect of local injection of recombinant human morphogens on systemic bone loss.

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Table 7.1 Intraobserver and interobserver reliability to measure furcation surfaces (see summary on pages 41-42)

	Furcation area between roots (μm^2)			
	Rater 1 researcher	**Rater 2 supervisor**	**Rater 1 researcher**	**Rater 2 supervisor**
Slide n°	1582-4		1613-L1	
	19 177 248.2	18 990 975.5	22 679 579.6	22 781 502.4
	19 890 707.7	19 063 929.6	22 446 339.3	22 939 445.4
	19 442 865.1	19 356 385.2	22 531 434.7	23 274 395.5
	19 942 893.9	19 224 002.7	22 581 064.9	22 789 916.1
	19 555 651.2	19 191 732.5	22 563 279.0	23 163 313.5
	19 557 461.7	19 018 453.1	22 492 348.4	22 687 780.3
	19 391 957.0	19 389 720.4	22 867 023.8	22 966 816.6
	19 386 738.4	19 472 685.8	22 693 211.9	23 091 743.9
	19 688 459.7	19 115 476.8	22 513 755.3	23 137 113.9
	19 912 221.2	19 348 184.5	22 432 920.0	23 034 658.6
Mean (µm)	19 594 620.4	19 217 154.6	22 580 095.7	22 986 668.6
SD (µm)	258855.2	168910.9	133051.3	189364.7
RSD (%)	1.32%	0.88%	0.59%	0.82%

	Furcation area between roots (μm^2)			
	Rater 1 researcher	**Rater 2 supervisor**	**Rater 1 researcher**	**Rater 2 supervisor**
Slide n°	1624		1661	
	22 915 908.4	22 869 899.4	23 979 334.9	23 209 748.5
	23 002 920.9	22 878 845.6	23 715 847.9	23 298 678.0
	23 145 314.6	23 061 497.2	23 929 811.3	23 205 701.4
	22 979 703.3	23 089 613.8	23 724 155.1	23 209 535.5
	22 720 157.0	23 184 081.5	23 795 618.2	23 245 000.8
	22 526 748.6	23 113 896.4	23 695 080.0	23 274 395.5
	22 523 021.1	23 266 407.8	23 884 760.7	23 139 883.0
	22 735 812.8	22 896 205.5	23 772 613.7	23 062 881.7
	22 646 989.8	23 261 934.7	23 660 253.7	23 234 350.6
	23 058 302.1	23 218 268.7	24 062 619.7	23 131 043.3
Mean (µm)	22 825 487.9	23 084 065.0	23 822 009.5	23 201 121.8
SD (µm)	224019.2	155493.3	135179.6	71378.1
RSD (%)	0.98%	0.67%	0.57%	0.31%

Furcation area between roots (μm^2)

	Rater 1 researcher	Rater 2 supervisor	Rater 1 researcher	Rater 2 supervisor
Slide n°	1672		1674	
	26 707 287.3	26 880 353.7	28 437 418.8	28 200 557.5
	26 509 405.9	27 317 120.1	29 128 512.9	28 482 256.3
	26 419 943.9	27 308 067.4	29 104 017.4	28 348 382.8
	26 855 219.2	27 497 641.7	29 129 045.4	28 572 463.9
	25 888 603.4	27 411 161.7	29 009 975.7	28 289 380.5
	26 219 506.4	27 286 553.9	28 807 621.2	28 434 862.8
	25 926 305.2	27 396 464.4	28 585 031.2	28 314 834.6
	26 444 013.4	27 364 513.7	29 207 537.7	28 629 762.2
	26 767 993.7	27 473 465.6	28 519 212.7	28 462 127.4
	26 409 613.1	27 356 206.5	28 696 432.7	28 611 230.8
Mean (µm)	26 414 789.1	27 329 154.9	28 862 480.6	28 434 585.9
SD (µm)	327917.7	172007.1	288108.6	144899.9
RSD (%)	1.24%	0.63%	1.00%	0.51%

Furcation area between roots (μm^2)

	Rater 1 researcher	Rater 2 supervisor	Rater 1 researcher	Rater 2 supervisor
Slide n°	1677		1680	
	31 112 439.6	31 116 060.7	36 864 847.2	36 554 712.3
	31 357 288.6	30 627 534.2	37 196 602.2	36 594 863.7
	31 166 116.8	30 921 693.8	37 240 694.2	36 883 059.2
	31 141 195.3	30 782 495.2	37 634 646.6	36 688 585.8
	31 291 683.2	30 570 874.9	37 668 301.3	36 599 230.3
	31 251 212.2	30 701 872.9	37 549 125.2	36 823 630.8
	30 915 729.7	30 800 813.6	37 158 687.4	36 747 162.1
	31 292 428.7	30 738 616.2	37 509 612.8	36 557 587.8
	31 049 816.2	30 673 969.2	37 483 519.7	36 499 437.5
	31 378 482.6	31 050 455.2	37 369 455.6	36 850 256.4
Mean (µm)	31 195 639.3	30 798 438.6	37 367 549.2	36 679 852.6
SD (µm)	145912.8	179269.4	251671.1	138440.1
RSD (%)	0.47%	0.58%	0.67%	0.38%

	Furcation area between roots (μm^2)			
	Rater 1 researcher	**Rater 2 supervisor**	**Rater 1 researcher**	**Rater 2 supervisor**
Slide n°	1681		1682	
	31 862 536.0	31 712 048.1	31 056 206.3	30 686 004.0
	31 848 371.2	31 682 866.5	30 236 776.9	30 890 488.6
	31 915 787.2	31 594 576.0	31 027 237.7	31 007 108.7
	31 804 705.2	31 663 163.5	31 233 958.9	30 670 028.6
	31 804 705.2	31 764 340.8	31 183 050.7	30 901 671.4
	31 380 399.7	31 921 964.4	31 403 936.7	30 645 320.1
	31 967 334.4	31 826 325.2	31 344 934.4	31 074 311.8
	31 864 559.6	31 950 294.0	31 543 561.3	30 891 127.6
	31 972 233.5	31 831 543.8	30 390 672.8	30 702 511.9
	31 818 018.0	31 726 745.5	30 496 216.7	30 821 368.6
Mean (µm)	31 823 865.0	31 767 386.8	30 991 655.2	30 828 994.1
SD (µm)	167407.8	114276.6	456634.9	149197.1
RSD (%)	0.53%	0.36%	1.47%	0.48%