



***BLOOD PARAMETER AND HISTOPATHOLOGICAL CHANGES IN  
OSTEOPOROTIC RAT MODEL TREATED WITH COCKLE SHELL -  
DERIVED CALCIUM CARBONATE NANOCRYSTALS***

**ALHAJI ZUBAIR JAJI**

**FPV 2016 2**



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By

**ALHAJI ZUBAIR JAJI**

**Thesis Submitted to the School of Graduate Studies, Universiti Putra  
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Philosophy**

**July 2016**

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Abstract of the thesis presented to the senate of Universiti Putra Malaysia in fulfillment of the requirement for the award of degree of Doctor of Philosophy

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**Chairman : MdZuki Bin Abu Bakar @ Zakaria, PhD**  
**Faculty : Veterinary Medicine**

Osteoporosis is a chronic systemic metabolic condition characterized by decreased density of normally mineralized bone due to faulty remodeling process. The success of age long studies on the prevention and treatment of osteoporosis have always been hindered by the ineffectiveness and hazards of the therapeutics and routes of administration used. Nanotechnology is poised to address these issues. This study was aimed at investigating the effectiveness of cockle shell-derived CaCO<sub>3</sub> nanocrystals aragonite polymorph as a therapeutic and a hormonal-carrier in the management of primary osteoporosis. Standard techniques were used in the synthesis and evaluations of physicochemical and in vitro/in vivo potentials and safety of ANC and the human recombinant parathyroid hormone (PTH 1-34) - loaded ANC (PTH-ANC) for the management of primary osteoporosis. Transmission Electron Microscopy (TEM) and Field Emission Scanning Electron Microscopy (FESEM) results demonstrated highly homogenized spherical-shaped aragonite nanocrystals of 30±5 nm in diameter. PTH-ANC had a Zeta potential of -27.6 ± 8.9 mV. The encapsulation efficiency of the formulation were found to be directly proportional to the concentrations of the drug fed. The X-ray diffraction (XRD) patterns revealed strong crystallizations with no positional change of peaks before and after PTH-ANC synthesis. Fourier Transform Infrared (FT-IR) spectroscopy demonstrated no detectable interactions between micron aragonite and surfactant at molecular level. PTH-ANC formulation was stabilized at pH 7.5, enabling sustained slow release of PTH 1-34 for 168 hours (one week). A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cytocompatibility assay in Human Foetal Osteoblast Cell Line hFOB 1.19 showed that ANC can safely support osteoblast proliferation up to 48 hours while PTH-ANC can safely support the proliferation at 72 hours and beyond due to the sustained slow release of PTH 1-34. A14 day acute toxicity and repeat dose 28 day trial studies of ANC in Sprague Dawley rats recorded no mortality. However, significant haematological anomalies, clinical signs and gross and histopathological lesions were recorded in the acute toxicity groups and the high dose (1g/kg body weight) and medium (0.1 g/kg/kg body weigh) toxicity groups of the repeat dose 28 day trial study. The low dose groups (0.01 g/kg

body weight) recorded mild. *In vivo* efficacy evaluation of antiosteoporotic and drug delivery efficacy of ANC and PTH-ANC demonstrated significant interactions between treatments and regimens. Daily administration of ANC or PTH 1-34, and weekly and fortnightly administrations of PTH-ANC, enabled best gains in bone mass density and strength in ovariectomized and orchidectomized rats. These were consistently demonstrated by results from proliferation and resorption proteomic analyses, bone morphometry and densitometry, flexural 3 point biomechanical bending test, serum calcium and phosphorus analyses, bone ash, calcium and phosphorus analyses and immunohistochemistry. It was concluded that due to its biogenic nature, ANC is a biocompatible antiosteoporotic agent with a cheap method of synthesis. It doubles as a nanocarrier for the enhancement of efficacy and safety of the bone anabolic PTH 1-34. ANC will reduce the cost, dosage and dose frequency associated with the use of PTH 1-34 management of primary form of osteoporosis and enable better compliance to its prescriptions.



Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

**PARAMETER DARAH DAN PERUBAHAN HISTOPATOLOGI PADA  
MODEL TIKUS OSTEOPOROSIS YANG DIRAWAT DENGAN NANO  
KRISTAL KALSIUM KARBONAT TERBITAN CENGERANG**

Oleh

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**Julai 2016**

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Osteoporosis adalah satu keadaan metabolic sistemik kronik yang dicirikan oleh pengurangan ketumpatan tulang disebabkan oleh kesilapan proses pembentukan semula. Kejayaan hasil kajian jangka yang lama mengenai pencegahandan rawatan osteoporosis sentiasa terhalang oleh ke tidak berkesan dan kemudaratan terapeutik dan laluan pemberian yang digunakan. Teknologi nano merupakan jawapan untuk menangani isu – isu tersebut. Kajian ini bertujuan untuk menyiasat keberkesanan Kristal nano polimorf Aragonit  $\text{CaCO}_3$  terbitanceng kerang kerang sebagai pembawa terapeutik dan nano dalam pengurusan utama osteoporosis. Teknik standard telah digunakan dalam sintesis dan penilaian fisio kimia dan potensi in vitro/ in vivo dan keselamatan ANC dan Hormon Paratiroid Manusia Rekombinan (PTH 1-34) yang dimuatkan dalam ANC (PTH-ANC) untuk pengurusan utama osteoporosis. Keputusan Mikroskop Pancaran Elektron (TEM) dan Mikroskop Elektron Imbasan Pancaran Medan (FESEM) menunjukkan penghomogenan kristal nano Aragonit berbentuk sfera berukuran diameter  $30 \pm 5$  nm. PTH-ANC mempunyai potensi Zeta iaitu  $-27.6 \pm 8.9$  mV. Keupayaan pengkapsulan formulasi tersebut didapati berkadar terus dengan kepekatan suapan drug. Corak belauan sinar-x (XRD) mendedahkan kekuatan penghabluran dan tiada pertukaran pos isi puncak pada sebelum dan selepas sintesis PTH-ANC. Spektro skopi inframerah trans formasi Fourier (FT-IR) menunjukkan tiada interaksi yang dapat dikesan antara micron Aragonit dan sufaktan pada aras molekular. Formulasi PTH-ANC telah menjadi stabil pada pH 7.5, membolehkan perlepasan perlahan yang berterusan PTH 1-34 yang berterusan untuk 168 jam (seminggu). Asai ke serasi sel 3- (4,5-dimethylthiazol-2-YL) -2,5-diphenyltetrazolium bromida (MTT) dalam pada titisan selosteoblas fetus manusia hFOB 1.19 menunjukkan bahawa ANC dapat menyokong proliferasi osteoblas dengan selamat sehingga 48 jam manakala PTH-ANC boleh menyokong proliferasi pada 72 jam dan lebih dengan selamat disebabkan pelepasan perlahan PTH 1-34 yang berterusan. Kajian ujian ketoksikan akut 14 hari dan pengulangan dos 28 hari ANC pada tikus Sprague Dawley mencatatkan tiada kematian. Walau bagaimanapun, perbezaan hematologic yang penting, tanda-tanda klinikal dan tanda – tanda serius dan lesi histopatologi telah direkodkan dalam kumpulan

ketoksikan akut dan kumpulan dos tinggi (1g / kg berat badan) dan sederhana (0.1 g / kg / badan kg berat) kumpulan ketoksikan ulangan dos 28 hari kajian perbicaraan. Kumpulan-kumpulan dos rendah (0.01 g / kg berat badan) mencatatkan tanda sederhana. Penilaian keberkesanan *in vivo* anti-osteoporosis dan keberkesanan penyampaian drug ANC dan PTH-ANC menunjukkan interaksi yang signifikan antara rawatan dan regimen. Pemberian harian ANC atau PTH 1-34, dan pemberian mingguan dan setiap dua minggu PTH-ANC, membolehkan gandaan terbaik dalam ketumpatan jisim dan kekuatan tulang pada tikus jantan dan tikus betina yang telah dikembirikan. Ini telah ditunjukkan secara konsisten oleh hasil daripada proliferasi dan analisis resorpsi proteomik, morfometri dan densitometri tulang, ujian biomekanikal takat lentur Flexural 3, analisis serum kalsium dan fosforus, abu tulang, analisis kalsium dan fosforus dan immunohisto kimia. Ini dapat disimpulkan bahawa oleh kerana sifat semulajadi biogenic bahan tersebut, ANC adalah ejen anti-osteoporosis yang mempunyai keserasian bio. Iadigandakan sebagai pembawa nano untuk meningkatkan keberkesanan dan keselamatan anabolic tulang PTH 1-34. ANC akan mengurangkan kos, dos dan kekerapan dos yang dikaitkan dengan penggunaan PTH 1-34 sebagai bentuk pengurusan utama osteoporosis dan membolehkan pematuhan yang lebih baik terhadap preskripsi tersebut.

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I certify that a Thesis Examination Committee has met on 22 July 2016 to conduct the final examination of Alhaji Zubair Jaji on his thesis entitled "Blood Parameter and Histopathological Changes in Osteoporotic Rat Model Treated with Cockle Shell-Derived Calcium Carbonate Nanocrystals" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Doctor of Philosophy.

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## LIST OF ABBREVIATIONS

ACC	Amorphous Calcium Carbonate
ALP	Alkaline Phosphatase
ANC	Cockle Shell derived Aragonite CaCO <sub>3</sub> Nanocrystals
ANOVA	Analysis Of Variance
BMD	Bone Mineral Density
BMU	Basic Multicellular Unit
BMUs	Bone Remodeling Units
BS-12	Dodecyl Dimethyl Betaine (RN+(CH <sub>3</sub> ) <sub>2</sub> CH <sub>2</sub> COO <sup>-</sup> )
CTX-1	Carboxyl Terminal Collagen 1
d,w,m	Daily, Weekly, Monthly
DEW	Distal Epiphyseal Width
DHBCs	Double-Hydrophilic Block Copolymers
DMSO	Dimethyl Sulphur Oxide
DW	Mediolateral Mid-Diaphyseal Widths
DXA	Dual Energy X-Ray Absorptiometry
ECM	Extracellular Matrix
EG	Ethylene Glycol
FDA	Food And Drug Agency
FESEM	Field Emission Scanning Electron Microscope
FL	Femoral Length
FT-IR	Fourier Transform Infrared
GBR	Guided Bone Regeneration
GCE	Glassy Carbon Electrode
Hb	Haemoglobin

hMSCs	Human Mesenchymal Stem Cells
HRP	Horseradish Peroxidase
HW	Widest Widths Of The Femoral Head
IACUC	Institutional Animal Care And Use Committee
IGF-1	Insulin-Like Growth Factor-1
KNN	K-Nearest Neighbour
MAC	Micron Sized Aragonite Caco3
MALT	Mucosa Associated Lymphoid Tissue
M-CSF	Macrophage Colony-Stimulating Factor
M-CSF	Macrophage Colony-Stimulating Factor
MCV	Mean Corpuscular Volume
MCHC	Mean Corpuscular Haemoglobin Concentration
MHC	Major Histocompatibility Complex
MSCs	Mesenchymal Stromal Cells
MTT	3-(4, 5-Dimethylthiazol-2-Yl)-2, 5-Diphenyltetrazolium Bromide
NW	Widest Widths Of The Femoral Neck
OCX	Orchidectomized
OCX/OVX 1	Untreated Orchidectomized/Ovariectomized rat (Positive control)
OCX/OVX 2	PTH Treated Orchidectomized/Ovariectomized rat
OCX/OVX 3	ANC Treated Orchidectomized/Ovariectomized rat
OCX/OVX 4	PTH-ANC Treated Orchidectomized/Ovariectomized rat
OCX/OVX S	Untreated Sham Orchidectomized/Ovariectomized rat (Negative control)
OECD	Organisation For Economic Co-Operation and Development
OPG	Osteoprotegerin



OVX	Ovariectomized
P1NP	Procollagen Type 1 N Terminal Protein
PBS	Phosphate Buffered Saline
PC10	PCNA Antibody
PCC	Precipitated Calcium Carbonate
PCL	Polycaprolactone
PEG	Poly (Ethylene Glycol)
PET	Poly (Ethylene Terephthalate)
PEW	Proximal Epiphyseal Width
PGE2	Prostaglandin E2
PLA	Poly lactide
PPO	Polyphenol Oxidase
PSS-ACC	Poly (4-Sodium Styrene Sulfonate)-Stabilized Amorphous Calcium Carbonate
PTH	Parathyroid Hormone
PTH 1-34 & PTH 1.84	Human Recombinant Parathyroid Hormones
PTH-ANC	PTH 1-34, Loaded Cockle Shell derived Aragonite CaCO <sub>3</sub> Nanocrystals
RANKL	Receptor Activator Of Nuclear Factor-Kb Ligand
RANK-L	Receptor For Activation of Nuclear Factor Kappa B Ligand
SD	Standard Deviation
SD rat	Sprague Dawley Rat
TEM	Transmission Electron Microscope
TGF	Transforming Growth Factor
TNF	Tumor Necrosis Factor
UPM	Universiti Putra Malaysia

WHO

World Health Organization

XRD

X-Ray Powder Diffraction



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## CHAPTER 1

### INTRODUCTION

#### 1.1 Study Background

Bone undergoes continuous adjustments (remodelling) by resorption of old bones by osteoclast and formation of new bones by osteoblast (Cosman et al., 2014). These activities are normally stable and are under physical and hormonal control. Imbalance between osteoclast and osteoblast activities characterised by bone resorption at a level that exceeds the rate of bone formation have been found to be responsible for osteoporosis and its associated bone loss and skeletal fragility (Kamel, 2006). Osteoporosis and its associated fractures has been predicted as a disease with potentials of increased public health importance in future generations, possibly due to increase in life expectancy (Holroyd et al., 2008).

The recombinant Human parathyroid hormone 1-34 (PTH 1-34), commercially known as Teriparatide, Forteo is a 4117.8 Da. peptide approved by FDA as a therapeutic agent for osteoporosis (Matthew et al., 2009). PTH 1-34 is a potent bone anabolic that treats osteoporosis by prompting remodelling cascades in osteogenic cells through stimulation of bone matrix production and suppression of osteoblast apoptosis (Lotinum et al., 2002, Jilka, 2007). PTH 1-34 has a high absolute bioavailability, rapid absorption and disposition after a single subcutaneous administration in healthy rats. It displayed a characteristic mono-exponential swift and substantial absorption and decline, with respective absolute bioavailability and elimination half-life of 65% and 3.4-4.1 hours. About 6% of the administered PTH 1-34 was excreted into bile for up to 24 hours after administration, while about 91% were recovered in urine and 2% were recovered from faeces after 72 hours of administration (Hu et al., 2006). Based on available data from 10, 20 and 40  $\mu\text{g}$  subcutaneous doses of PTH 1-34 in humans, it was concluded that its absolute bioavailability is approximately 95%. PTH 1-34 has a rapid rate of absorption and elimination. It reaches peak serum concentrations in 30 minutes after subcutaneous administration of a 20  $\mu\text{g}$  dose and drops to an undetectable concentration within 3 hours. It has a distribution of about 0.12 L/kg after intravenous injection and a half-life in serum of approximately 5 minutes when injected intravenously, and roughly 1 hour when administered by subcutaneous injection (Lily, 2010). Due to its short life and fast clearance, PTH 1-34 peptide is presently injected subcutaneously daily for a two year. Thus, there is a pressing need to improve patient compliance through reduction of frequency of injection or sorting for a feasible alternate route of administration that will prolong the time taken for the peptide to be exposed to serum. A PTH 1-34 loaded nanoformulation can enable sustained slow release of its anabolic dose and rule out possible side effects associated with its sudden exposure into patient's blood (Naka et al., 2006). Moreover, studies have shown that blood clearance of the smaller nanoparticles was twice as slow as those of larger nanoparticles formulations (Frank et al., 2008).

Calcium is the fifth most abundant element in the body. Calcium is an important structural component of bone and teeth and also is necessary for the normal function of all muscles (skeletal, heart, and smooth muscles) and nerves as well as the normal clotting of blood. Prolonged, inadequate intake of calcium causes weak bones (osteoporosis). Products containing calcium carbonate ( $\text{CaCO}_3$ ) are used to increase the intake of calcium in individuals whose diets are low in calcium. The National Institutes of Health recommend 1000 to 1500 mg of calcium per day as part of a regimen to prevent the loss of bone associated with aging.  $\text{CaCO}_3$  products contains 40% absorbable calcium. Therefore, a 1500 mg tablet of  $\text{CaCO}_3$  provides 600 mg of calcium.  $\text{CaCO}_3$  is also used as an antacid for treating stomach distress (Deborah and Straub, 2007).  $\text{CaCO}_3$  is a calcium salt found in limestone, chalk, marble, plant ashes, bones, many shelled Mollusks and Coccoliths. It is also obtained as a white precipitate by passing carbon dioxide into a suspension of calcium hydroxide in water. It is used in dentifrices and in pharmaceuticals as an antacid and to supplement bodily calcium stores (Sugawara et al., 2003; Merriam-Webster, online Medical Dictionary, 2016).  $\text{CaCO}_3$  has presently become popular in the field of nanotechnology as a highly biocompatible–porous compound with pH dependent degradability. It has an easily manipulative physicochemical properties, surface chemistry (shape and size) and method of production at a large scale (Rodríguez et al., 2013).  $\text{CaCO}_3$  is pH sensitive, its solubility is exponentially and inversely proportional to its pH (Ming-Jium et al., 2011). These make it a novel inorganic material with a huge potential in biomedical applications and controlled drug delivery (Render et al., 2014).

The cockle shell (*Anadara granosa*) derived aragonite  $\text{CaCO}_3$  nanocrystals (ANC) is the chosen nanocarrier for this study. ANC is an inorganic nanocrystal synthesized using the top down approach of nanoparticle preparation. Cockles are dominant faunal bivalves present, sometimes comprising the entire bivalve fauna in deep shells beds on sandy mud flats in the upper parts of estuaries and harbors. They live in super abundance in the low tidal and shallow subtidal zones of most of our present-day estuaries and enclosed bays and harbours (Haywardl 1990). In Malaysia, the cockle (*Anadara granosa*) are cultivated in a large scale in the area of intertidal coastal bordering mudfield regions and in many part of South East Asian countries, mainly Thailand and Indonesia. They are by far, the most vital species cultured in Malaysia (Ibrahim, 1995). The cockle shells contain more than 98%  $\text{CaCO}_3$  and thus, has the potential for the development of biomaterials for orthopedic applications (Awang-Hazmi et al., 2007).

Aragonite  $\text{CaCO}_3$  polymorph is a thermodynamically less stable and less available form of crystalline  $\text{CaCO}_3$  synthesized in laboratory. The size and shape of aragonite is strongly dependent on the preparation methods and conditions (Wang et al., 1999). Due to the huge striking properties of aragonite nanoparticles as a material of biomedical importance, researchers have paid huge attention on invention of methods for its controlled and facile synthesis at appropriate sizes and shapes using bottom up methods (Wang et al., 2006; Guo et al., 2007). Yet, none of these methods can promise production of pure aragonite nanoparticles of suitable sizes and shapes. Aragonite resulting from these production are often mixed with calcite (Guo et al., 2007) or calcite and vaterite (Chen and Xiang, 2009). Therefore, these methods may

not be appropriate for specific biomedical applications. Though carbonation methods are found to be useful in industries and environmentally friendly, they are associated with the need for strict control of temperature, purified raw materials, and strenuous gas (CO<sub>2</sub> or combination of CO<sub>2</sub> and N<sub>2</sub>) bubbling phases which are complicated, expensive and time consuming (Wang et al., 2007). Other impurities such as BS-12 are also added to the final products (Wang et al., 2007). Therefore, the top down approach of ANC synthesis from its natural sources, for example cockle shells or sea shells is greatly promising (Islam et al., 2011).

This study is in line with the global efforts at tackling the menace of osteoporosis and its associated complications. It aims at synthesis, physicochemical characterizations and *in vitro* evaluation of the ANC as a potential therapeutic and nanocarrier for sustained slow release of anabolic antiosteoporotic PTH 1-34. The PTH 1-34 - loaded cockle shell aragonite calcium carbonate nanocrystals (PTH-ANC) will go a long way in reducing dosage, cost, side effect and dosing frequency of PTH1-34, towards an effective management of primary and secondary forms of osteoporosis and better compliance from patients.

## 1.2 Statements of Problems

Although osteoporosis has been studied for many years, efforts towards its prevention and treatment still face serious setbacks. No effective prevention and treatment methods exist for this disease. Currently, osteoporosis is being treated by medical, surgical and traditional alternative therapies (Balasundaram *et al.*, 2005; Patel and Hussain, 2010). There exist barriers characteristic to the therapies and their routes of administration. These barriers militate against the successful use of any method of stimulating new bone formation administration (Balasundaram *et al.*, 2005; Jackson, 2006; Patel and Hussain, 2010). Due to their characteristic barriers, the conventional Calcium and Vitamin D supplements given for the treatment of osteoporosis show little effects; hormone replacement therapy used for women suffering from osteoporosis can also increase the risk of other conditions including strokes and breast cancer. Recombinant human parathyroid hormone (PTH 1-34) is often reserved for severe cases and short term usage due to its potential carcinogenic effect. Surgery though temporarily relieves pain, does not provide a full long-term recovery method so is not effective for younger sufferers. Generally, most therapies only aim to maintain bone density instead of returning it to its former state (Jackson, 2006; Patel and Hussain, 2010). There exist two barriers characteristic to the mode of administration of antiosteoporotic agents thus, militating against the successful stimulation of new bone formation by these agents: the agents are often administered systemically thus, causing non-specific bone formation in undesirable, off target areas; locally administered agents rapidly diffuse to adjacent tissues which limit their potential to promote prolonged bone formation in targeted areas of weak bones, necessitating a prolonged unpleasant administration for at least a year if any change is to be seen, and means hardship to the geriatrics (Balasundaram *et al.*, 2005). Consequently, new technology at the nanoscale needs to be developed which coincides with nature in providing effective generation of bone tissue and enabling the successful treatment of osteoporosis (Patel and Hussain, 2010).

### 1.3 Hypotheses

H<sub>1</sub> - Cockle shell-derived calcium carbonate nano-crystal aragonite polymorph (ANC) has therapeutic effects on primary osteoporosis.

H<sub>2</sub> - Cockle shell-derived calcium carbonate nano-crystal aragonite polymorph (ANC) can be used as a nano-carrier in management of primary osteoporosis.

### 1.4 Research Questions

- i. How can ANC and PTH-ANC, be best prepared and characterized as an effective agent and nano-carrier for the management of osteoporosis?
- ii. What is the *in vitro* drug release profile and biocompatibility of PTH-ANC?
- iii. How safe is PTH-ANC on biological systems *in vivo*?
- iv. How effective are ANC and PTH-ANC in the management of post-menopausal osteoporosis?
- v. How effective are ANC and PTH-ANC in the management of senile osteoporosis?

### 1.5 Objectives of the Study

#### 1.5.1 Main Objective

This study was conducted with the aim of investigating the effectiveness of cockle shell-derived CaCO<sub>3</sub> nano-crystal aragonite polymorph (ANC) as a therapeutic and a nano-carrier in the management of primary osteoporosis.

#### 1.5.2 Specific Objectives

- i. To synthesize and characterize the ANC and PTH-ANC.
- ii. To evaluate the *in vitro* hormonal (PTH 1-34) release profile of PTH-ANC and the cytocompatibility of ANC and PTH-ANC.
- iii. To evaluate the *in vivo* acute and repeat dose 28 day toxicities of ANC in young rats.
- iv. To evaluate the antiosteoporotic and hormonal delivery efficacies of ANC in 9 month old rat models of post-menopausal osteoporosis (Type I primary osteoporosis).
- v. To evaluate the antiosteoporotic and hormonal delivery efficacies of ANC in 9 month old rat models of senile osteoporosis (Type II primary osteoporosis).

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## APPENDICES

### Appendix I

#### Drug Content and Drug Encapsulation Efficiency formulae.

$$\text{Drug Content (\%)} = \frac{\text{tdf-fd}}{\text{mnr}} \times 100 \dots\dots\dots 1$$

$$\text{Drug Encapsulation Efficiency (\%)} = \frac{\text{tdf-fd}}{\text{tdf}} \times 100 \dots\dots\dots 2$$

Where: tdf – refers to the total drug fed to the formulation  
fd - refers to the free drug  
mnr – refers to the weight of nano-crystals recovered from the formulation.

## Appendix 2

### TOOLS USED FOR ANC AND PTH-ANC CHARACTERIZATIONS



**Transmission Electron Microscope**



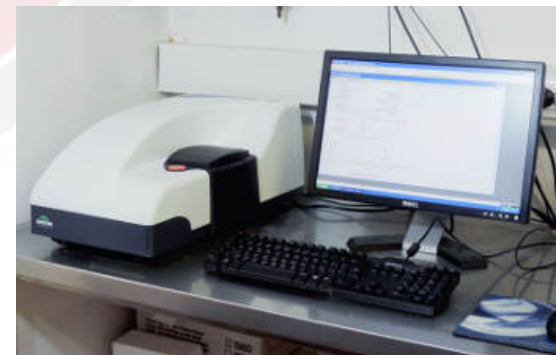
**Field emission scanning electron microscope**



**X-ray powder diffractometer**



**Fourier Transform Infrared Spectrophotometer**



**Malvern Zetasizer**

### Appendix 3

#### YIH DER orbital shaker incubator TU-400



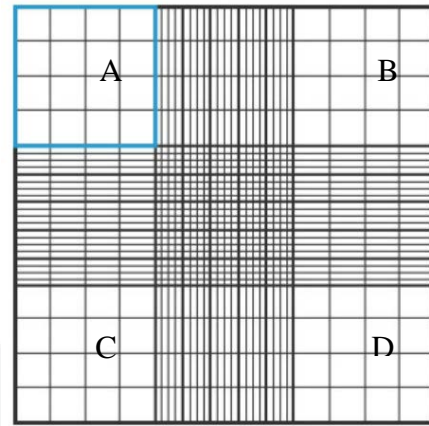
**Appendix 4**  
**Sanyo inCu safe incubator**



## Appendix 5



**Haemocytometer**



**Haemocytometer Counting Chambers**

## Appendix 6

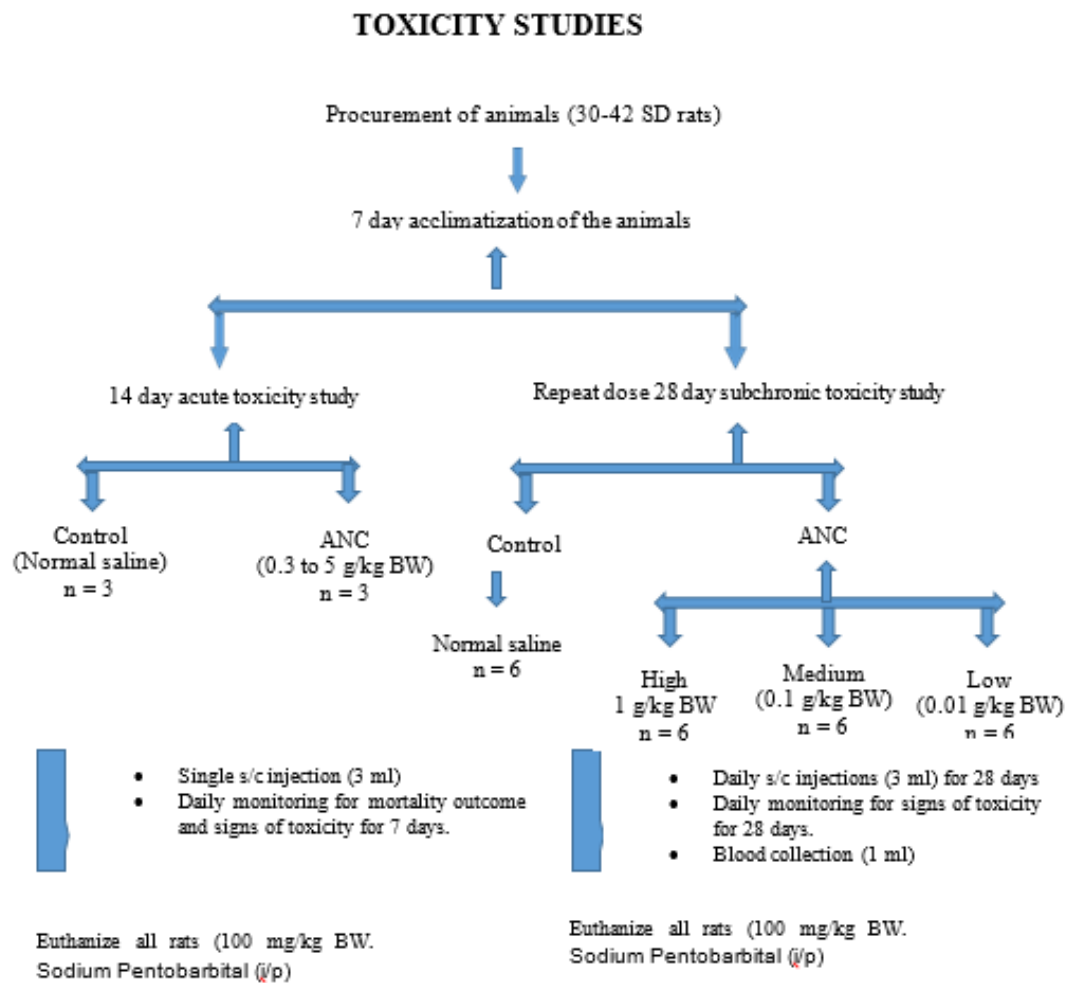
### CALCULATIONS OF TOTAL VIABLE CELLS POPULATION

$$\begin{aligned}
 \text{Average live cells} &= \frac{\text{Sum of live cells counted from chambers}}{\text{Number of chambers counted}} \\
 &= \frac{38 + 36 + 40 + 42}{4} = \frac{156}{4} \\
 &= 39 \\
 \text{Average dead cells} &= \frac{\text{Sum of dead cells counted from chambers}}{\text{Number of chambers counted}} \\
 &= \frac{5 + 4 + 4 + 3}{4} = \frac{16}{4} \\
 &= 4 \\
 \text{Viability (\%)} &= \frac{\text{Number of live cells}}{\text{Number of live + dead cells}} \times 100 \\
 &= \frac{39}{43} \times 100 \\
 &= 91\% \\
 \text{Cell density (cells/mL)} &= \frac{\text{Average live cells} \times \text{dilution factor}}{\text{Volume of a square (mL)}} \\
 &= \frac{\text{Average live cells} \times \text{dilution factor}}{0.0001 \text{ mL}} \\
 &= \frac{39 \times 2}{0.0001} \\
 &= 780000 \text{ cells/mL} \\
 \text{Total viable cells} &= \text{cell density} \times \text{volume of cell suspension.} \\
 &= 580000 \times 4 \\
 &= 3900000 \text{ cells} \\
 &= 3.9 \times 10^6 \text{ cells}
 \end{aligned}$$



## Appendix 7

### STUDY FLOW CHART FOR *IN VIVO* TOXICITY STUDIES



## Appendix 8

### Gold coin mouse pellet: nutrient composition

Ingredient	Percentage
Crude protein (Maximum)	21
Crude fiber (Maximum)	5
Crude fat (Minimum)	3
Moisture	13
Ash (Maximum)	8
Calcium (Minimum)	0.8
Phosphorus (Minimum)	0.4

Gold Coin Specialities Sdn. Bhd., 19, Jalan Perigi Nanas 7/2, KS11Kawasan Perindustrian Pulau Indah 42920 Pulau Indah Selangor Darul Ehsan, West Malaysia.

## Appendix 9

### The Motic Compound Microscope, BA410



## Appendix 10

### REFERENCE BLOOD SERUM BIOCHEMICAL VALUES OF MALE AND FEMALE SPRAGUE DAWLEY RATS

#### Female

Parameters	Units	> 4 weeks		> 13 weeks	
		Minimum value	Maximum value	Minimum value	Maximum value
Albumin/globulin ratio		1	1.2	0.7	0.9
Albumin	g/L	32	47	30	44
Alanine amino-transferase	U/L	13.5	52.5	22.9	64
Alkaline phosphatase	U/L	90.5	769.8	77.6	324.3
Aspartate aminotransferase	U/L	29.5	144.7	47	172.2
Total Bilirubin	µmol/L	0	3.4	0	3.4
Calcium	mmol/L	2.3	2.8	2.3	3.3
Total cholesterol	mmol/L	1.3	9.4	1.4	5.8
Chloride	mmol/L	81.5	105.5	79.6	103.7
Creatinine	µmol/L	26.5	70.7	35.4	88.4
Creatine kinase	IU	253.43	380.23	269.45	361.10
Gamma-glutamyl transferase	U/L	0.1	1.2	—	—
Globulin	g/L	30	36	40	43
Glucose	mmol/L	1.9	12	4.6	9.2
Potassium	mmol/L	2.8	4.8	2.2	5.4
Sodium	mmol/L	132.4	168.3	119.4	154.7
Inorganic phosphorus	mmol/L	2.2	2.6	—	—
Total protein	g/L	57	78	61	87
Triglycerides	µmol/L	0.2	0.7	0.2	0.4
Urea	mmol/L	6.9	30.5	11.1	31.7

Petterinoa and Angetino-Storinob (2006).

## Male

Parameters	Units	> 4 weeks		> 13 weeks	
		Minimum value	Maximum value	Minimum value	Maximum value
Albumin/globulin ratio		0.5	1.1	0.6	0.8
Albumin	g/L	24	44	29	41
Alanine aminotransferase	U/L	19.1	78	34.9	218.1
Alkaline phosphatase	U/L	162.3	769.7	131.6	459
Aspartate aminotransferase	U/L	38.4	215.3	56.1	201.8
Total bilirubin	µmol/L	0	5.1	0	5.1
Calcium	mmol/L	2.3	3	2.1	2.9
Total cholesterol	mmol/L	1.7	14.6	1.9	4.6
Chloride	mmol/L	87.7	106.6	81.5	104
Creatinine	µmol/L	26.5	70.7	35.4	79.6
Creatine kinase	IU	317.03	392.07	300.84	421.94
Gamma-glutamyl transferase	U/L	0	3.2	—	—
Globulin	g/L	29	47	40	49
Glucose	mmol/L	3.3	9.1	5	11.2
Potassium	mmol/L	2.8	5.8	2.9	5.3
Sodium	mmol/L	132.1	170.4	121.9	162.6
Inorganic phosphorus	mmol/L	2	2.8	—	—
Total protein	g/L	58	75	65	81
Triglycerides	µmol/L	0.3	0.6	0.2	0.4
Urea	mmol/L	6.6	31.4	10.8	34.4

Petterinoa and Angetino-Storinob (2006).

## Appendix 11

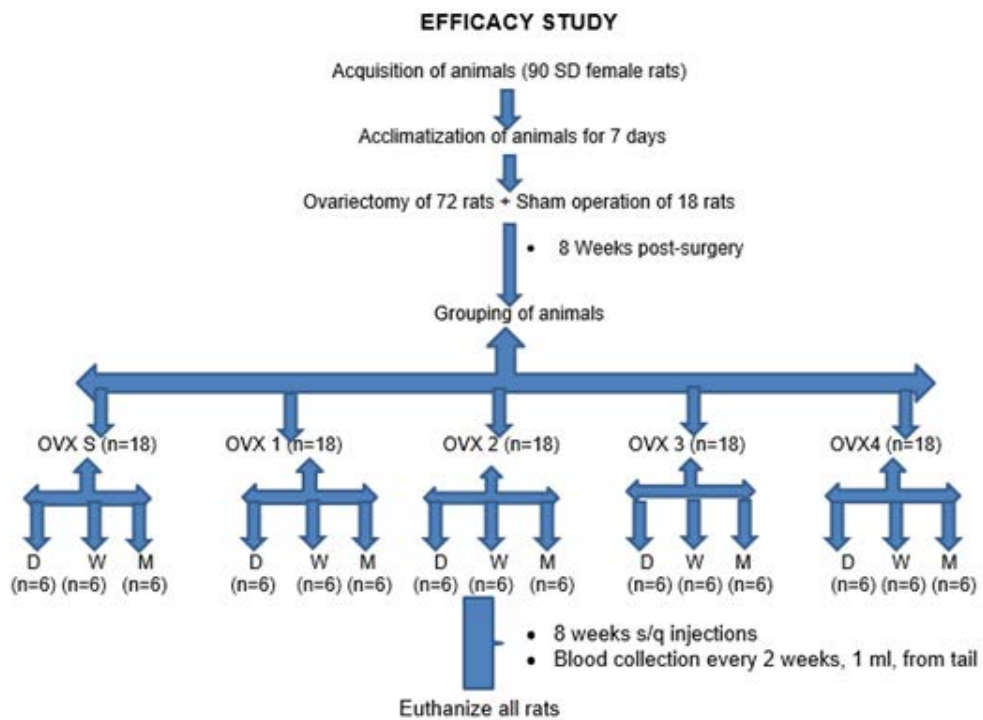
### REFERENCE HAEMOGRAM MALE AND FEMALE SPRAGUE DAWLEY RATS

Parameter	Unit	Male range (2.5 – 97.5%)	Mean (males)	Mean (females)
Red blood cells (RBCs)	$\times 10^6/\mu\text{L}$	7.34 – 8.85	8.14	8.19
Haemoglobin (Hgb)	g/dL	14.7 – 17.3	15.9	15.9
Haematocrit (Hct)	%	44.9 – 51.7	48.5	46.5
Mean cell volume (MCV)	fL	55.1 – 64.2	59.7	56.9
Mean cell haemoglobin (MCH)	pg	18.6 – 20.7	19.6	19.5
Mean cell haemoglobin concentration (MCHC)	g/dL	31.3 – 34.4	32.8	34.3
Red cell distribution width (RDW)	%	11.3 – 14.2	12.4	11.5
Absolute reticulocytes	$\times 10^6/\mu\text{L}$	0.114 – 0.399	0.236	0.195
Reticulocytes		1.3 – 4.94	2.81	2.28
Platelets	$\times 10^3/\mu\text{L}$	903 – 1594	1159	1146
White blood cells (WBCs)	$\times 10^3/\mu\text{L}$	6.63 – 20.35	12.43	12.02
Neutrophils	$\times 10^3/\mu\text{L}$	0.37 – 2.63	0.95	0.72
Lymphocytes	$\times 10^3/\mu\text{L}$	6.10 – 18.45	10.85	10.79
Monocytes	$\times 10^3/\mu\text{L}$	0.04 – 0.50	0.20	0.16
Eosinophils	$\times 10^3/\mu\text{L}$	0.02 – 0.27	0.11	0.15
Basophils	$\times 10^3/\mu\text{L}$	0.01 – 0.12	0.05	0.05
Large unstained cells	$\times 10^3/\mu\text{L}$	0.04 – 0.35	0.14	0.12

Weiss and Wardrop (2010).

## Appendix 12

### STUDY FLOW CHART FOR ANC AND PTH-ANC EFFICACY STUDY



**KEY:** SD rat = Sprague-Dawley rat, ANP = Cockle shell derived Calcium carbonate nanoparticle Aragonite polymorph, PTH = Synthetic parathyroid hormone (PTH 1-34), C = Control, OVX<sub>s</sub> = Sham ovariectomized rat to be administered normal saline(placebo). OVX<sub>1</sub> = ovariectomized rat to be administered normal saline. OVX<sub>2</sub> = ovariectomized rat to be administered normal saline + PTH. OVX<sub>3</sub> = ovariectomized rat to be administered normal saline + PTH + ANP. OVX<sub>4</sub> = ovariectomized rat to be administered normal saline + ANP.

### Appendix 13

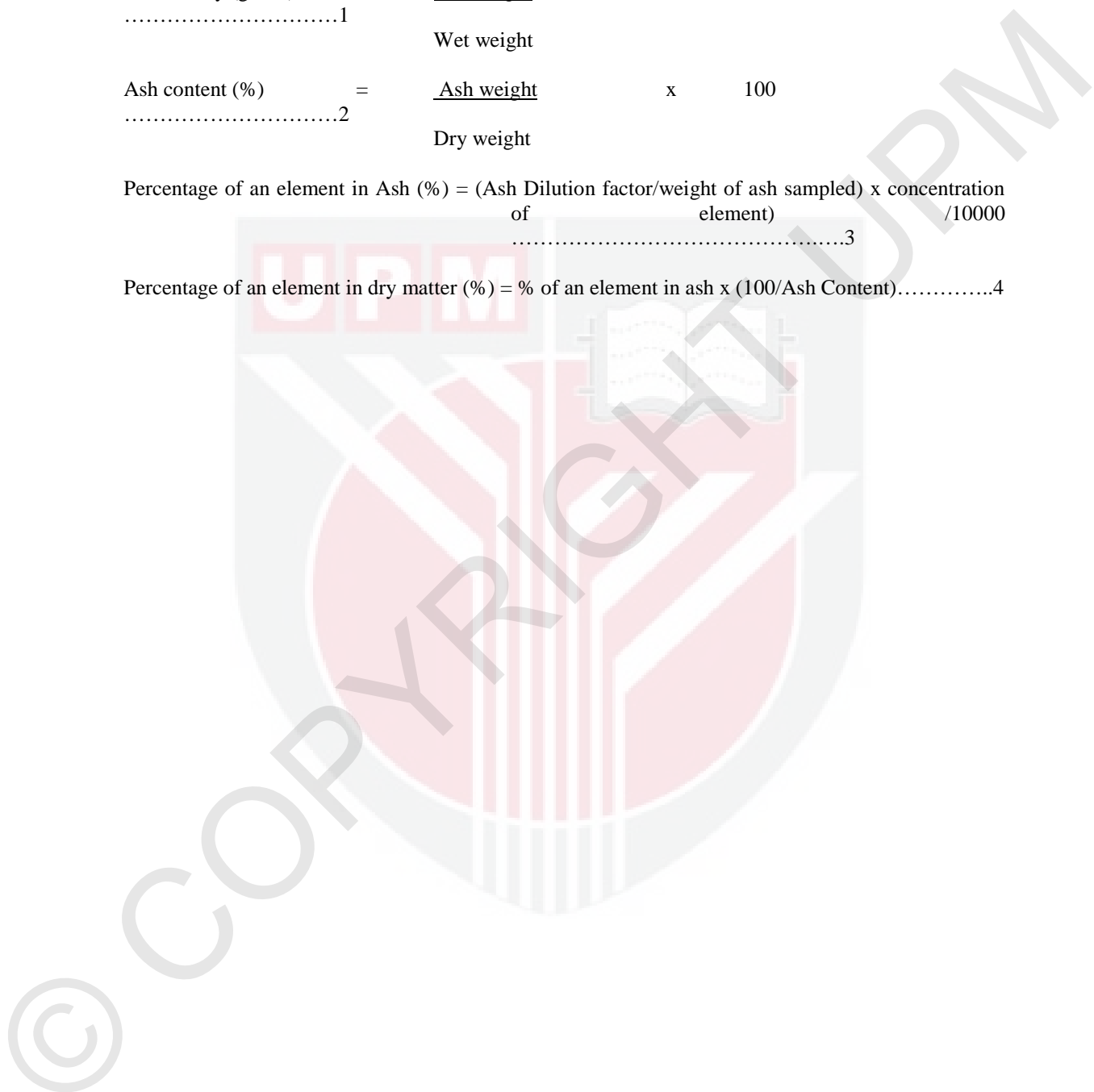
#### ANALYSES OF FEMORAL ASH AND CALCIUM AND PHOSPHORUS CONTENT

$$\text{Ash density (g/cm}^3\text{)} \dots\dots\dots 1 = \frac{\text{Ash weight}}{\text{Wet weight}} \times 100$$

$$\text{Ash content (\%)} \dots\dots\dots 2 = \frac{\text{Ash weight}}{\text{Dry weight}} \times 100$$

$$\text{Percentage of an element in Ash (\%)} = \frac{(\text{Ash Dilution factor/weight of ash sampled}) \times \text{concentration of element}}{10000} \dots\dots\dots 3$$

$$\text{Percentage of an element in dry matter (\%)} = \% \text{ of an element in ash} \times (100/\text{Ash Content}) \dots\dots\dots 4$$





## Appendix 14

Protocol for Immunohistochemical staining of proximal tibial for evaluation of proliferation of osteogenic cells following 8 weeks subcutaneous administration of regimens of PTH1-34, ANC and PTH-ANC.

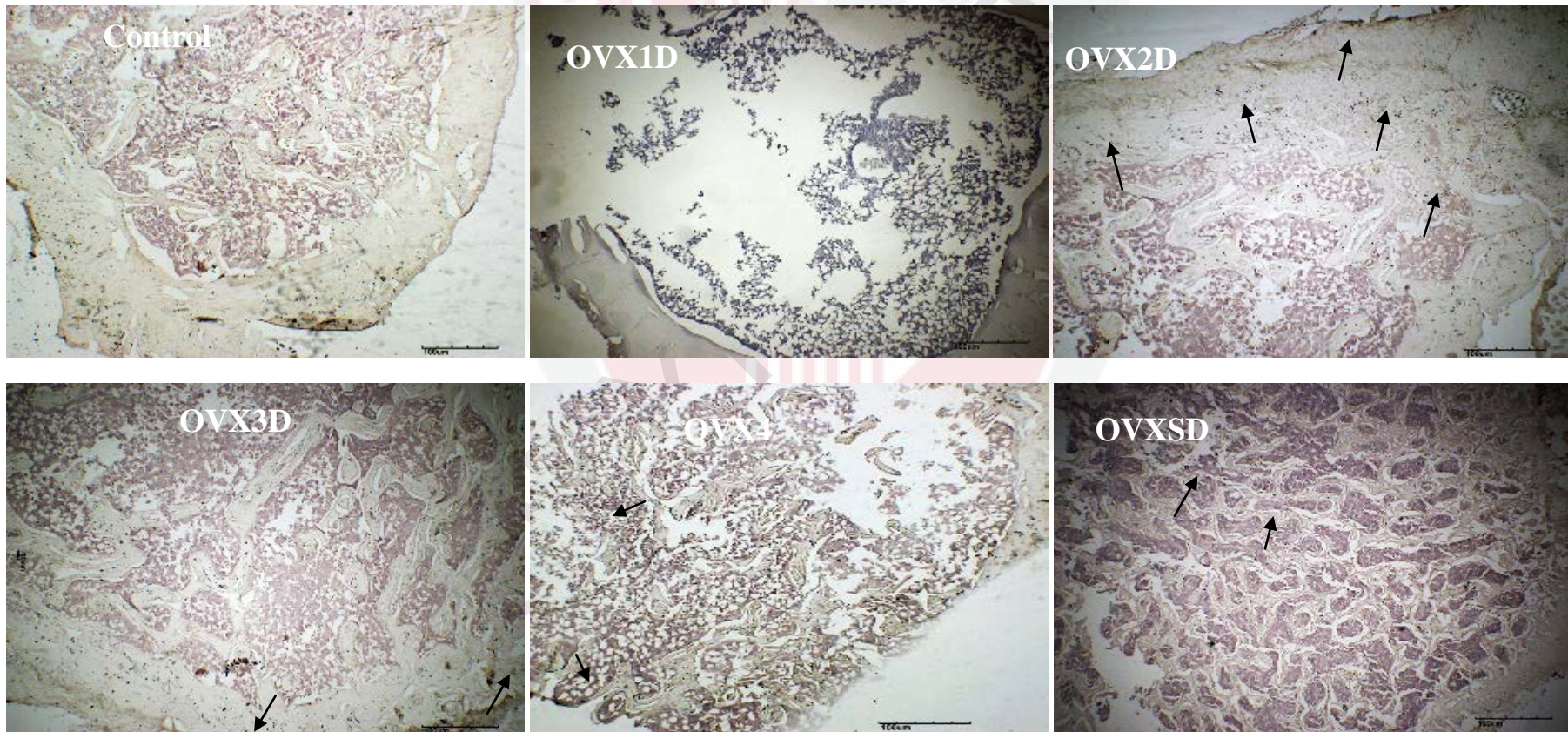
- i. Routine tissue section (4-6 $\mu$ m) were deparaffinised in xylene, 100% ethanol, and 70% ethanol for 5 minutes each.
- ii. The slides were washed in deionized water for 1 minute with stirring, excess liquids were aspirated from the slides afterwards.
- iii. Antigen retrieval: slides were placed in a container and cover with 10 mM sodium citrate buffer, pH 6.0, heat at 95°C for 20 minutes in a microwave.
- iv. The slides were allowed to cool for 20 minutes, washed in deionized water 3 times for 2 minutes each and excess liquids were aspirated from the slides.
- v. The slides were Incubated for 5-10 minutes in 0.5% H<sub>2</sub>O<sub>2</sub> in Phosphate Buffered Saline (PBS), washed in PBS twice for 5 minutes each, and their sections Incubated for 30 minutes in 1.5% blocking serum in PBS.
- vi. Excess blocking serum were bolted from slides and the sections Incubated with primary antibody for 1 hour at 37°C.
- vii. Slides were washed 3 times in PBS for 5 minutes each and sections incubated for 30 minutes with biotinylated secondary antibody.
- viii. Slides were washed with 3 changes of PBS for 5 minutes each and Incubated sections for 30 minutes with AB enzymes reagent.
- ix. Slides were washed with 3 changes of PBS for 5 minutes each and sections Incubated in 1-3 drops peroxidase substrate for 10 minutes.
- x. Slides were washed in deionized water for 5 minutes, counter stained in haematoxylin for 1 minutes and immediately washed with several changes of de ionized water (5 times).
- xi. Slides were then destained with acid alcohol, washed with tap water, dehydrated with 2x70% ethanol for 10 seconds each, 2x100% 10 seconds each, 3x Xylenes for 10 seconds each, and had their excess xylene wiped.
- xii. 1-2 drops of permanent mounting medium were then placed and the slide covered with coverslip.
- xiii. The slides were later observed and captured using the Motic Compound Microscope BA410 (Appendix 7). The Motic Images Plus 2.0 software was used to analyse the images before they were being captured at x 100 magnification.

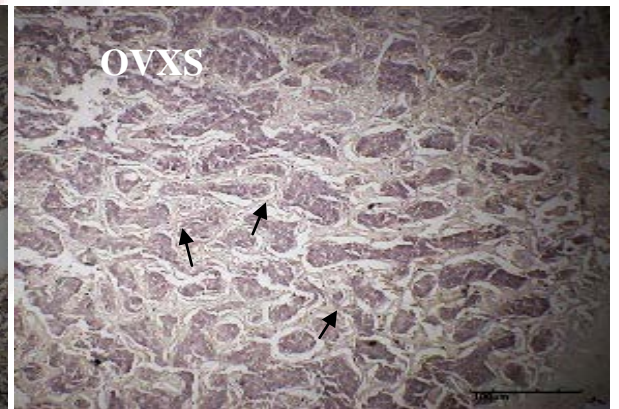
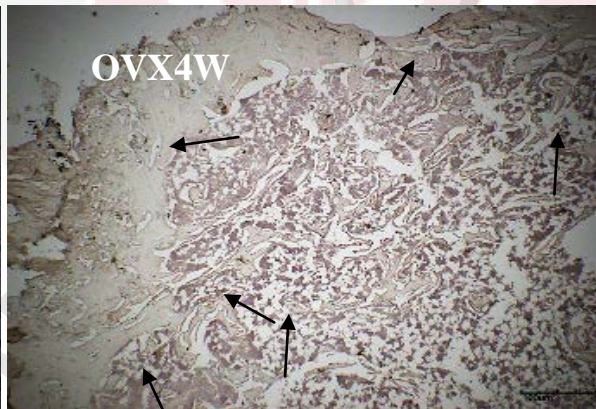
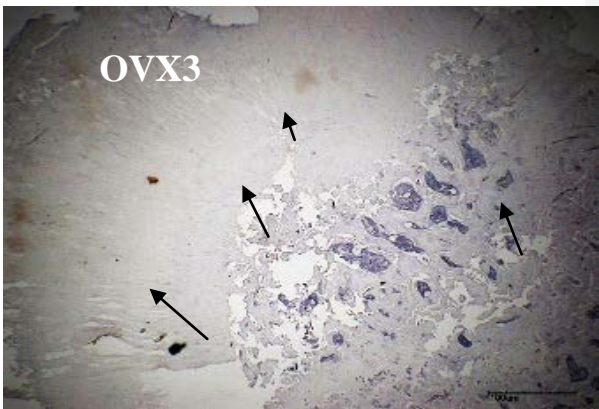
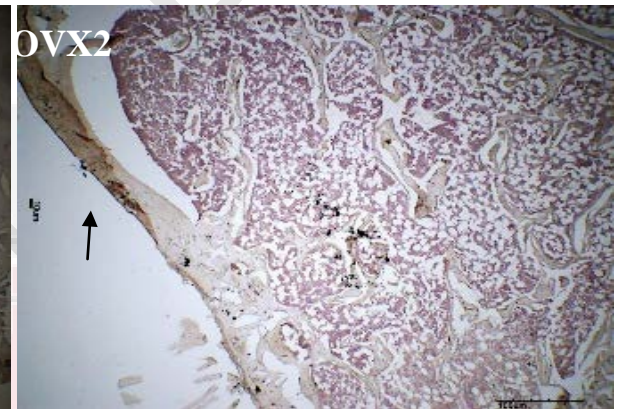
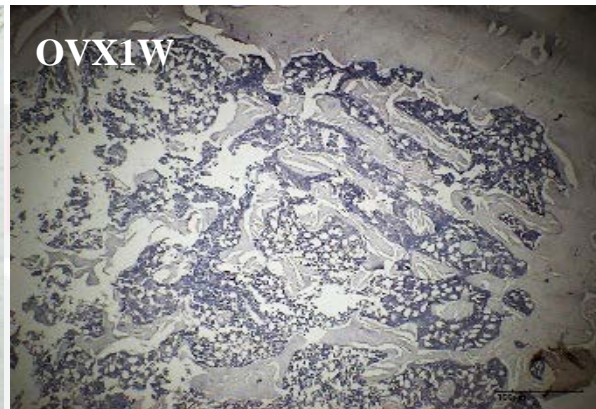
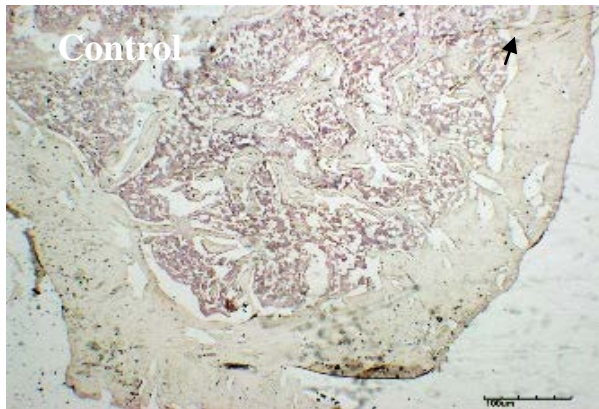
### Precautions:

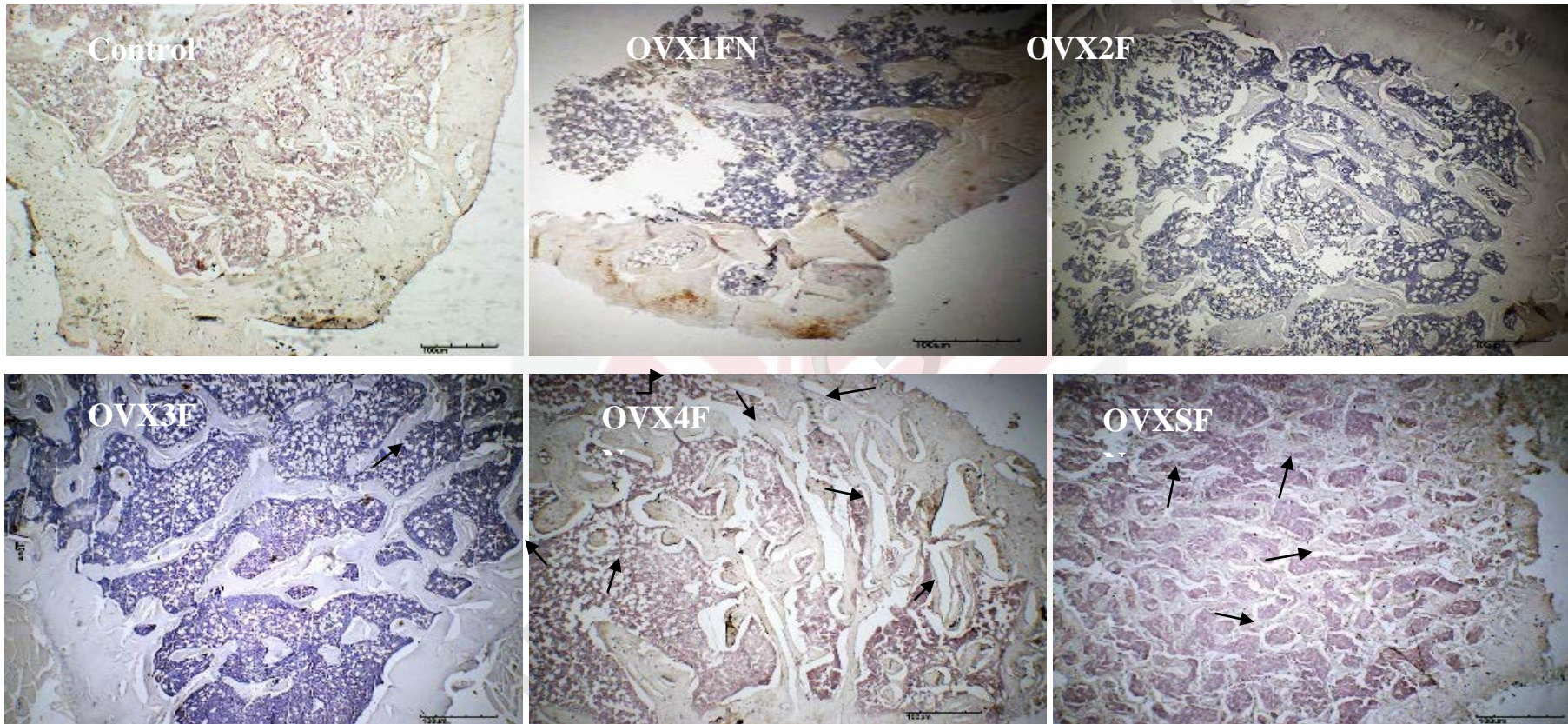
- Staining system reagents were allowed to reach room temperature prior to use
- Tissue sections were not be allowed to dry out at any time during procedure
- Sufficient reagents were used to cover the specimens, approximately 100-500 $\mu$ L were used per slide.  
(please place in appendix)

## Appendix 15

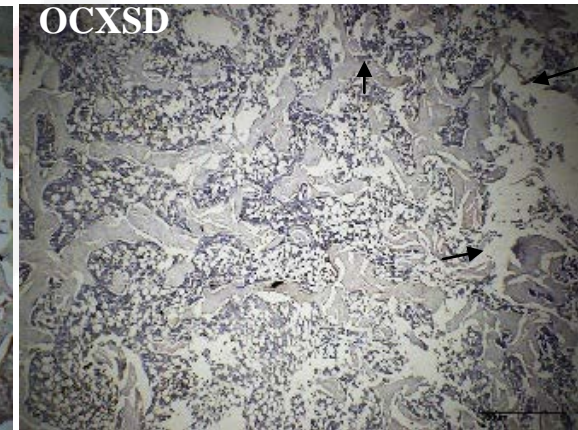
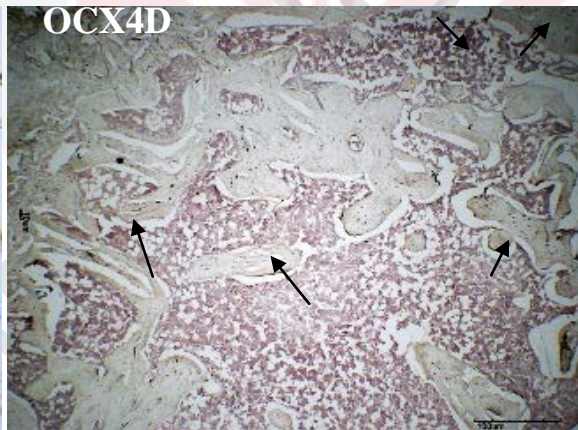
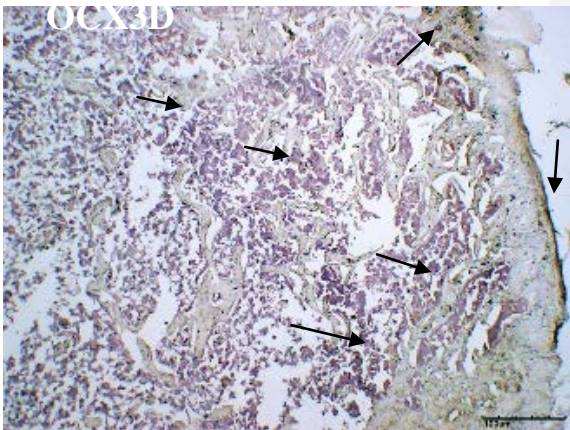
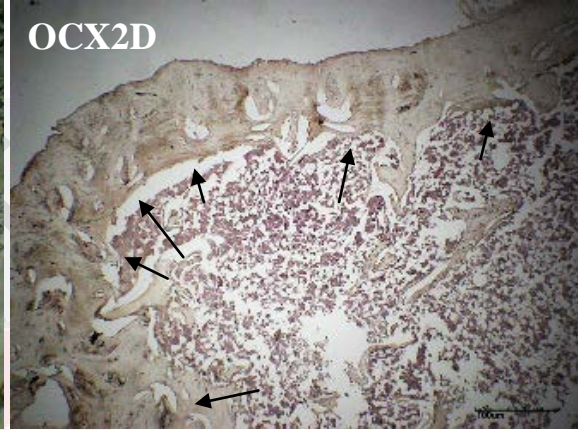
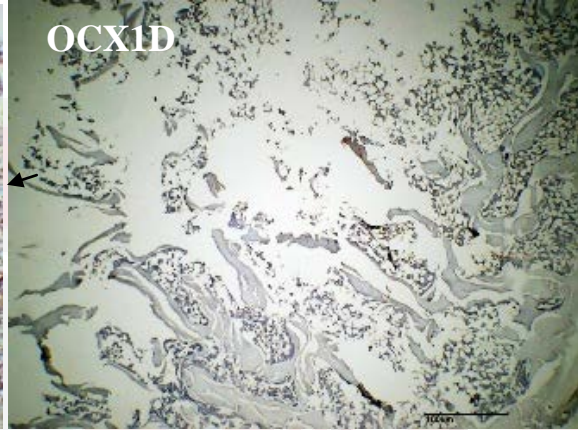
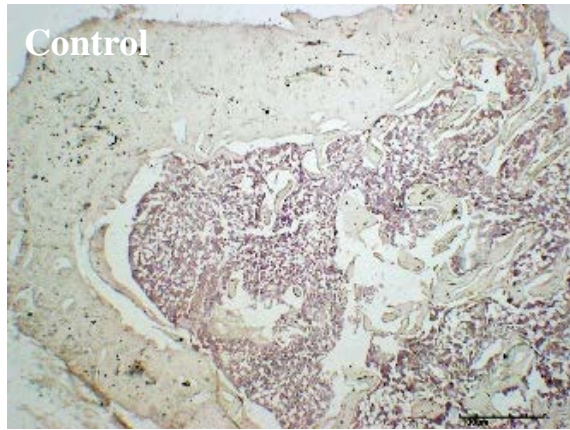
- a) Immunohistochemical scoring of proliferation of ovariectomized SD rats' tibiae after 8 weeks of subcutaneous administration of regimens of PTH 1-34, ANC and PTH-ANC.

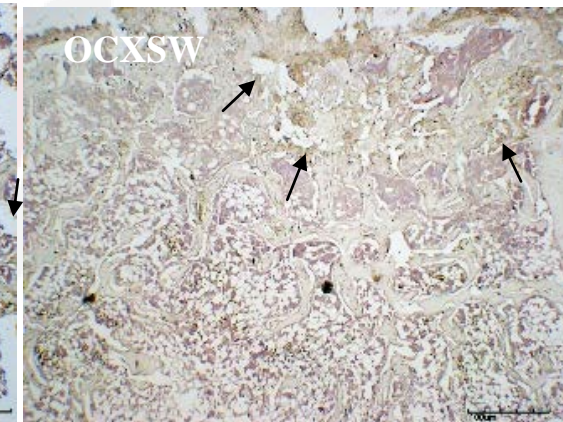
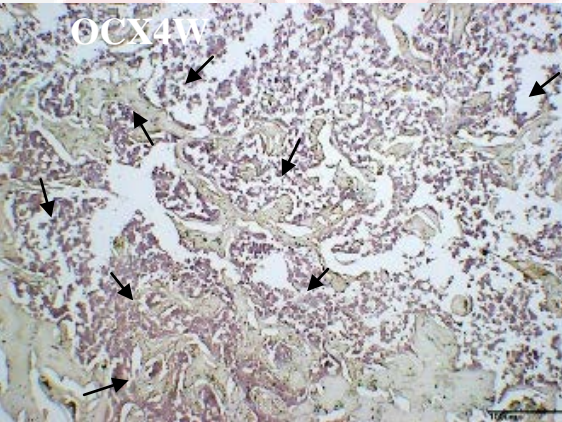
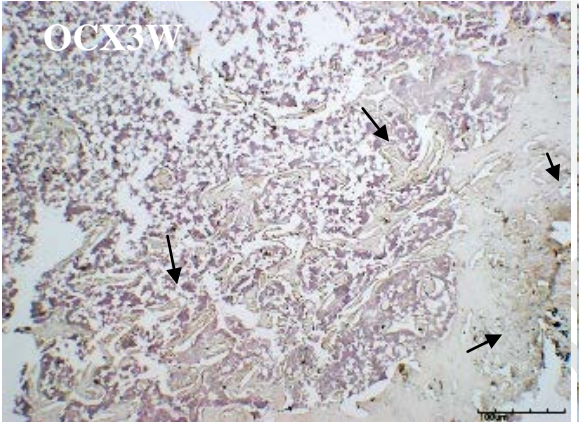
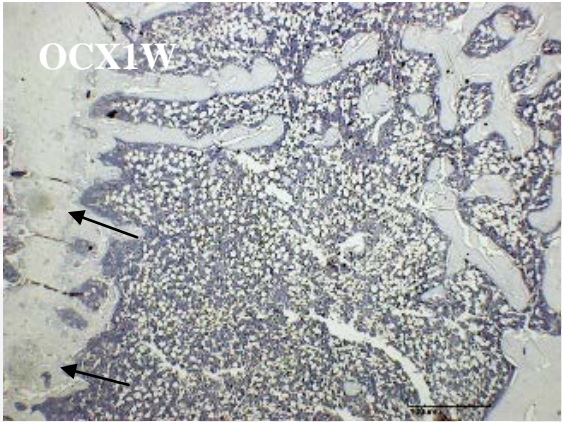
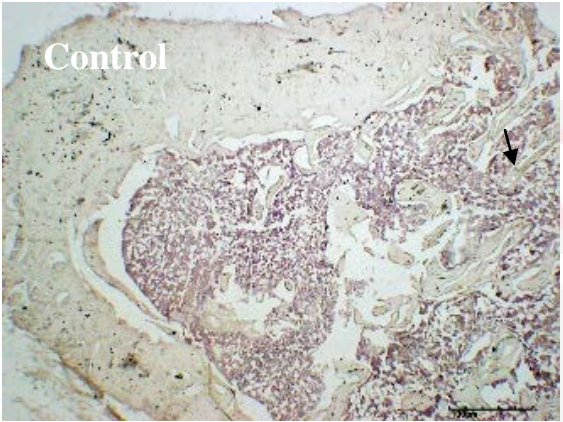


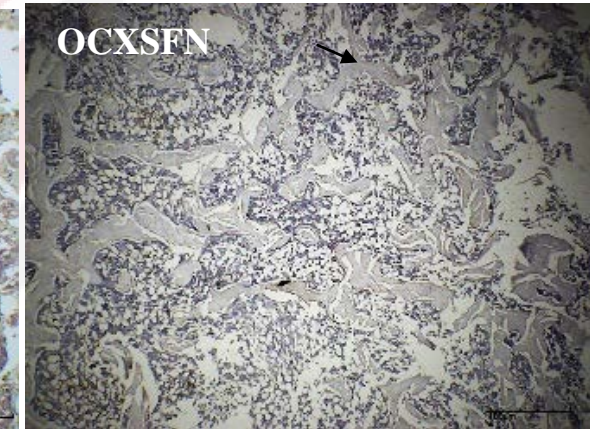
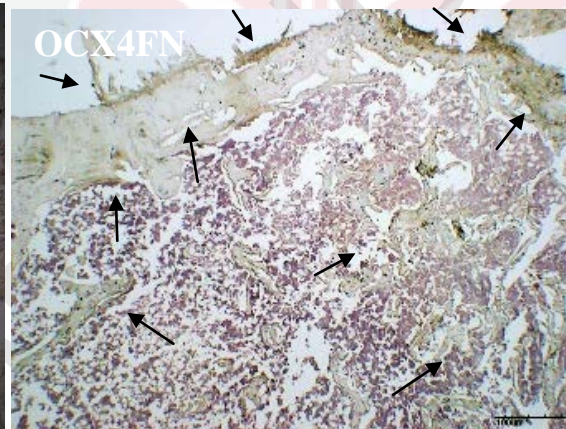
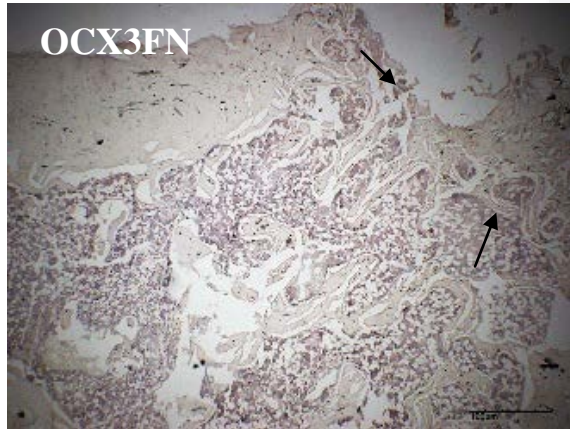
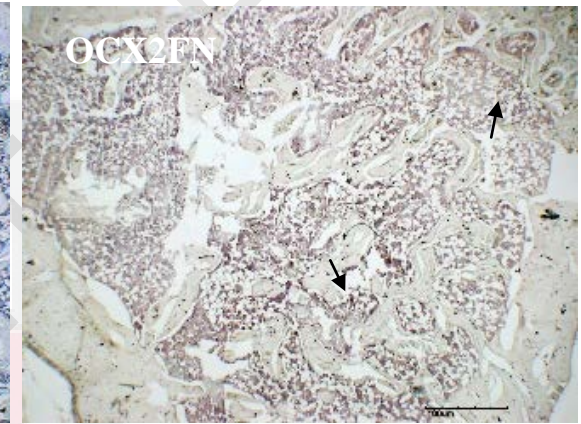
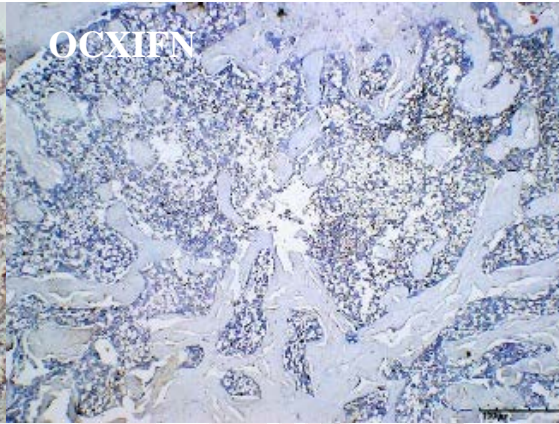
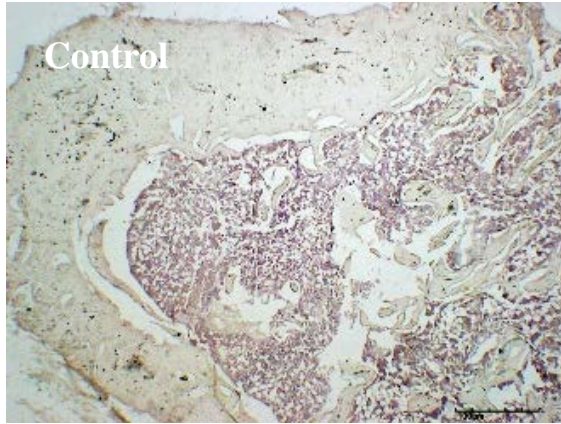




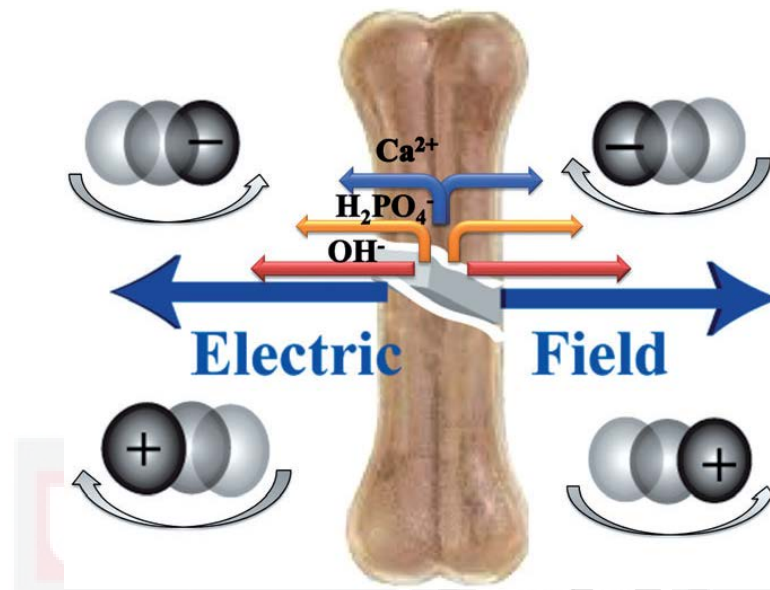
b) Immunohistochemical scoring of proliferation of orchietomized SD rats' tibiae after 8 weeks of subcutaneous administration of regimens of PTH 1-34, ANC and PTH-ANC.







## Appendix 16



Depiction of the electric field induced by the ion gradient and the resultant particle migration. The lengths of the arrows next to the ions represent their relative mobility. The generated electric field points outwards away from the crack. Accordingly, the negatively charged particles move towards and positively charged particles move away from the crack. Source: Yadav et al. (2013).



## BIODATA OF STUDENT

The student, Alhaji Zubair Jaji was born on the 17<sup>th</sup> October, 1971 in Maiduguri, Borno State, Nigeria. After completing primary and secondary education, he graduated as a doctor of veterinary medicine and later as a master of veterinary science (anatomy) holder from the Faculty of Veterinary Medicine, University of Maiduguri, Nigeria in the years 2000 and 2009. He was employed by the University of Maiduguri as an Assistant Lecturer in the year 2007. He transferred his services to the Faculty of Veterinary Medicine, University of Ilorin, Nigeria as a Lecturer I in the year 2011. He got a PhD (Anatomy) admission with the Universiti Putra Malaysia in the year 2012 under the supervision of Prof MD Zuki Abu Bakar @ Zakaria. The aim of his research was to study the blood parameter and tissue changes in osteoporotic rat model treated with cockle shell - derived calcium carbonate nanocrystals



## LIST OF PUBLICATIONS

### Manuscripts accepted for publication

Alhaji Zubair Jaji, Md Zuki Abu Bakar @ Zakaria, Rozi Mahmud, Loqman Mohamad Yusof , Mohamad Hezmee Mohamad Noor, Tijani Isa, Fu Wenliang and Nahidah Ibrahim Hammadi. (in print). Synthesis, Characterization and Cytocompatibility of Potential Cockle-Shell Aragonite Nanocrystals for Osteoporosis Therapy and Hormonal Delivery. Nanotechnology, Science and Applications.

Alhaji Zubair Jaji, Md Zuki Abu Bakar @ Zakaria, Rozi Mahmud, Loqman Mohamad Yusof , Mohamad Hezmee Mohamad Noor, Yusuf Abba, Tijani Isa, Saffanah Khuder Mahmood. (in print). Safety Assessments of Subcutaneous Doses of Aragonite Calcium Carbonate Nano-Crystals in Rats. Journal of Nanoparticle Research.

### Conference abstract

**Zubair Jaji**, Md Zuki Abu Bakar Zakaria, Rozi Mahmud, Loqman Mohamad Yusof and Mohamad Hezmee Mohamad Noor (2016). PTH-ANC for osteoporosis management. 9th World Drug Delivery Summit. Zubair Jaji et al., J Pharm Drug Deliv Res 2016, 5:3(Suppl). June 30-July 02, 2016 New Orleans, USA. <http://dx.doi.org/10.4172/2325-9604.C1.009>



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