UNIVERSITI PUTRA MALAYSIA

ROLE OF PLASMA MEMBRANE TRANSPORTERS (N+/H+ AND HCO3-) IN MEDIATING MAMMALIAN LONGITUDINAL BONE GROWTH AND FRACTURE HEALING

ABUBAKAR ADAMU ABDUL

FPV 2016 26

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By

ABUBAKAR ADAMU ABDUL

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirements for the Degree of Doctor of Philosophy

November 2016

DEDICATION

This project is dedicated to my family and humanity at large, especially my parents for their unconditional love and infinite supports given to me in my life journey.

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the Degree of Doctor of Philosophy

ROLE OF PLASMA MEMBRANE TRANSPORTERS (N^+/H^+ AND HCO_3–) IN MEDIATING MAMMALIAN LONGITUDINAL BONE GROWTH AND FRACTURE HEALING

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November 2016

Chairman : Loqman Mohamad Yusof, PhD
Faculty : Veterinary Medicine

Mammalian long bone growth and secondary bone healing occur by means of endochondral ossification, which involves tightly controlled cellular differentiation of chondrocytes at the epiphyseal region and callus formation at the fracture site. Although the cellular mechanism of chondrocyte differentiation that regulates long bone growth and fracture healing is still poorly understood, plasma membrane transporters were thought to have the mediating roles. This study aimed to investigate the roles of Na^+/H^+ antiporter (NHE1) and HCO_3^- anion exchanger 2 (AE2) in linear bone growth and secondary fracture healing. The specific objectives were: (1) To study postnatal *ex vivo* rat model for longitudinal bone growth investigations. (2) To investigate the role of Na^+/H^+ (NHE1) and HCO_3^- (AE2) exchange across membrane of chondrocytes in mammalian longitudinal bone growth. (3) To investigate the effect of recombinant human growth hormone (rhGH) on the localisation of NHE1 and AE2 membrane transporters on long bone growth in rat. (4) To establish metatarsal fracture model in rats for *in vivo* investigation of secondary bone healing. (5) To investigate the role of NHE1 (Na^+/H^+) and AE2 (HCO_3^-) membrane proteins during secondary bone healing in rat.

Firstly, an experiment was undertaken to determine the suitable age to study bone growth using rat pups *ex vivo* model. The result showed direct bone sectioning for histology was possible across all age groups in metatarsal bone rudiments and in 7-13 day-old pups tibia. However, tibial sectioning was relatively difficult in 14 and 15 day-old rats. Significant differences in tibia and metatarsal growth plate (GP) length was observed among different age groups at different incubation periods (P<0.05). Significant differences of chondrocyte densities in the GP of tibia and metatarsal were recorded before and after 72 hrs incubation. *Ex vivo* longitudinal growth of tibia and metatarsal bone of rats at age of 7-15 day-old was possible under conducive

physiological condition and maximum growth rate was observed in tibia of 10 day-old rats (P10).

Postnatal (P10) metatarsal and tibial bones were cultured for 48 hrs *ex vivo* in the presence of plasma membrane inhibitors of 5-ethylisopropyl amiloride (EIPA) and 4,4'-diisothiocyano-2,2"-stilbenedisulfonic acid (DIDS) for NHE1 and AE2 respectively. The study revealed bone growth suppression by approximately 11% at concentration of 444 µM and 250 µM of EIPA and DIDS respectively. The two inhibitors had no significant effect on the total GP length but significantly affect the total GP and HCZ chondrocytes densities. There was no significant difference between NHE1 and AE2 localisation and fluorescence signaling across GP length. The remarkable suppression of bone growth along with the inhibition of chondrocytes proliferation at the entire GP and HCZ by EIPA and DIDS was an indication that the plasma membrane proteins (NHE1 and AE2) have potential role in bone growth through regulation of chondrocytes density.

In order to determine whether there is effect of bone growth inhibition by EIPA and DIDS on bone growth stimulation under the influence of growth hormone (GH), P10 rat metatarsal and tibia were cultured for 48 hrs in the presence of GH in combination with EIPA, DIDS or the vehicle, DMSO (control). Results showed bone cultured in DMSO recorded steady growth similar as treatment with additional GH. In the presence of GH fluorescence labeling of NHE1 and AE2 membrane proteins along GP was enhanced along with increased in the longitudinal bone growth. However, the combination of GH with EIPA or DIDS suppressed longitudinal bone growth, total GP length, GP chondrocytes density and localisation of NHE1 and AE2 along the GP. The fluorescence labeling of NHE1 and AE2 were also significantly inhibited in EIPA+GH or DIDS+GH treatments.

The present study also established a reproducible transverse mid shaft 3rd metatarsal fracture model for laboratory investigations. The model produced a fracture at the shaft of metatarsal bones that was 100 % transverse, 73% located at mid shaft with minimal fracture angulations based on radiographic evidence (0.48 ± 0.09^{O} at anterior posterior view; 0.78 ± 0.17^{O} at lateral view). There was minimal soft tissue injury, no infection or delayed bone union observed. Varying degree of weight bearing lameness was initially observed but subsequently absent at day six onwards post-surgery. Callus index was observed to peak in week 2 and 3 (2.02 ± 0.1 and 1.99 ± 0.13, respectively) but declined to 1.10 ± 0.04 in week 7 during consolidation period. There was no significant difference between the histological and radiographic healing scores at week 7 post-surgery.

Chondrocytes in fracture callus could be detected as early as first week of bone healing, which peaked after 3 weeks and subsequently declined and ceased at week 6. NHE1 and AE2 localisation was recorded throughout the period of healing but peak signaling was recorded in the first 4 weeks of healing and then significantly declined from week 5 onwards to week 7. The NHE1 localisation was significantly higher than that of AE2 during the healing

period but there was no significant difference of mean localisation signaling score between NHE1 and AE2.

In conclusion, EIPA and DIDS have significantly inhibited longitudinal bone growth, HCZ length and total GP chondrocyte density. Expression of NHE1 and AE2 was affected by the inhibition of EIPA and DIDS in the presence of GH. The transporters were also found to be present in the fracture site at significant high level throughout the first 4 weeks of fracture healing period. This result suggested the possible role of NHE1 and AE2 in longitudinal bone growth as well secondary fracture healing.

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

PERANAN PENGANGKUT MEMBRAN PLASMA (NA^+/H^+ DAN HCO_3^-) DALAM MEMBANTU PERTUMBUHAN LONGITUDINAL DAN PENYEMBUHAN TULANG BAGI MAMALIA

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Pertumbuhan tulang panjang dan penyembuhan tulang sekunder bagi mamalia berlaku melalui proses pembentukan tulang endokondral yang melibatkan pembezaan sel kondrosit yang dikawal rapi di kawasan fiseal dan pembentukan kalus pada tempat kecederaan. Walaupun mekanisma pembezaan sel kondrosit yang mengawalatur pertumbuhan dan penyembuhan kecederaan tulang panjang masih kurang difahami, pengangkut membran plasma dikatakaan dapat memainkan peranan sebagai pengantara. Kajian ini dibuat untuk menyiasat peranan Na^+/H^+ antiporter1 (NHE1) dan HCO_3^- penukar anion 2 (AE2) dalam pertumbuhan linear tulang dan penyembuhan kecederaan sekunder. Objektif khusus adalah: (1) Untuk menjalankan kajian *ex vivo* pada model tikus selepas beranak untuk mengkaji pertumbuhan tulang membujur. (2) Untuk mengkaji peranan pertukaran Na+/H+ ((NHE1) dan HCO_3^- (AE2) merentasi membran kondrosit dalam pertumbuhan tulang membujur mamalia. (3) Untuk mengkaji kesan hormon pertumbuhan manusia rekombinan (rhGH) di lokasi NHE1 dan AE2 membran pengangkutan sepanjang pertumbuhan tulang dalam tikus. (4) Untuk mewujudkan model kecederaan pada metatarsal tikus dalam kajian *in vivo* bagi penyembuhan tulang kedua dan (5) Mengkaji peranan NHE1 (Na+/H+) dan AE2 (HCO_3^-) protein membran semasa penyembuhan tulang kedua tikus.

Eksperimen pertama telah dijalankan untuk menentukan umur yang sesuai bagi mengkaji pertumbuhan tulang menggunakan model *ex vivo* anak tikus. Hasil kajian menunjukkan hirisan tulang untuk histologi boleh dibuat pada semua peringkat umur bagi tulang metatarsus dan pada tulang tibia anak tikus yang berusia 7-13 hari. Namun begitu, hirisan tulang tibia agak sukar diperolehi daripada anak tikus yang berusia 14 dan 15 hari. Perbezaan yang signifikan pada panjang pola pertumbuhan (GP) tibia dan metatarsus diperhatikan secara histologi dalam peringkat umur yang berbeza pada

tempoh inkubasi yang berlainan (P<0.05). Tiada perbezaan signifikan bagi ketumpatan kondrosit dalam GP pada tulang tibia dan metatarsus direkodkan sebelum dan selepas 72 jam inkubasi. Pertumbuhan longitudinal tulang tibia dan metatarsus tikus yang berumur 7-15 hari secara *ex vivo* boleh terjadi di bawah keadaan fisiologi yang sesuai dan kadar tumbesaran yang maksimum didapati pada tibia tikus yang berusia 10 hari (P10).

Dalam kajian yang berikutnya, tulang metatarsus dan tibia P10 telah dikulturkan selama 48 jam secara *ex vivo* dengan penambahan *5-ethylisopropyl amoliride (EIPA)* dan *4,4'-diisothiocyano-2,2"-stilbenedisulfonic acid (DIDS)* masing-masing sebagai perencat membran plasma bagi NHE1 dan AE2. Keputusan menunjukkan perencatan pertumbuhan tulang dalam anggaran 11% masing-masing pada kepekatan 444 µM EIPA dan 250 µM DIDS. Kedua-dua perencat tidak mempunyai kesan yang signifikan pada panjang keseluruhan GP tetapi secara signifikan dapat mengurangkan panjang saiz zon kondrosit hipertrofi (HCZ) dan kepadatan keseluruhan kondrosit GP (P<0.05). Walau bagaimanapun, kepadatan HCZ secara relatifnya kekal malar. Tindakbalas setempat NHE1 dan pengisyaratan berpendarfluor seluruh panjang GP adalah lebih tinggi daripada AE2. Perencatan luar biasa pertumbuhan tulang longitudinal yang setara dengan proliferasi panjang zon HCZ oleh EIPA dan DIDS bersama dengan kepadatan kondrosit yang secara relatifnya malar dalam zon adalah petunjuk yang baik bahawa fenomena pengawal atur isipadu mungkin terlibat dalam proses pemanjangan pertumbuhan tulang di mana NHE1 dan AE2 mungkin mempunyai peranan mengawalselia proses di peringkat sel.

Dalam usaha untuk menentukan sekiranya EIPA dan DIDS mempunyai sebarang kesan perencatan pada pertumbuhan tulang melalui proliferasi rangsangan pertumbuhan tulang oleh hormon pertumbuhan (GH), metatarsus dan tibia tikus P10 telah dikulturkan selama 48 jam dengan kehadiran GH bersama kombinasi EIPA, DIDS atau sarana dan DMSO (kawalan). Keputusan menunjukkan tulang yang dikulturkan dalam DMSO merekodkan pertumbuhan yang stabil manakala penambahan GH dalam media kultur menyebabkan rangsangan langsung pada pertumbuhan tulang longitudinal, panjang keseluruhan GP dan kepadatan keseluruhan kondrosit GP. Dengan kehadiran GH, pelabelan pendarfluor membran protein bagi NHE1 dan AE2 sepanjang GP telah dipertingkatkan selari dengan pertambahan pertumbuhan tulang secara longitudinal. Walau bagaimanapun, kombinasi GH dengan EIPA atau DIDS telah merencatkan pertumbuhan tulang secara longitudinal, panjang keseluruhan GP, kepadatan kondrosit GP serta tindak balas setempat NHE1 dan AE2 sepanjang GP. Pelabelan pendarfluor bagi NHE1 dan penukar anion AE2 didapati dihalang secara signifikan dalam kumpulan yang diberi EIPA+GH atau DIDS+GH.

Kajian ini juga telah menunjukkan keratan rentas model retakan bahagian pertengahan metatarsus ketiga menghasilkan keputusan yang boleh diulang semula bagi mengkaji peranan pengangkut membran dalam penyembuhan tulang sekunder. Model kecederaan menghasilkan retakan pada bahagian tengah tulang metatarsus yang melintang pada kadar 100% di mana 73%

didapati pada pertengahan tulang dengan penyudutan yang minimal apabila dianalisa menggunakan radiografi (0.48 ± 0.09° pada pandangan anterior posterior; 0.78 ± 0.17° pada pandangan lateral). Kecederaan tisu lembut yang minimal, ketiadaan jangkitan atau penyambungan tulang yang tertunda telah direkodkan. Namun begitu, pada permulaannya, pelbagai tahap ketempangan galas berat diperhatikan yang kemudiannya beransur hilang mulai hari ke-6 selepas pembedahan. Indeks kalus dilihat mencapai tahap paling tinggi pada minggu ke-2 and ke-3 (masing-masing pada 2.02 ± 0.1 dan 1.99 ± 0.13) tetapi berkurangan menjadi 1.10 ± 0.04 pada minggu ke-7 semasa peringkat pengukuhan. Skor penyembuhan yang diperhatikan secara histologi dan radiografi masing-masing adalah 3.5 ± 0.13 dan 3.75 ± 0.25 (berbanding dengan skor maksimum penyembuhan iaitu 4) pada minggu ke-7 selepas pembedahan. Kajian ke atas proliferasi kondrosit (CP) menggunakan antibodi monoclonal mencit terhadap antigen proliferasi sel nuklear (PCNA) menunjukkan kondrosit dalam kalus retakan boleh dikesan seawal minggu pertama penyembuhan tulang, mencapai tahap tertinggi selepas minggu ke-3 dan kemudiannya berkurangan dan berhenti pada minggu ke-6. Terdapat perbezaan yang signifikan (P<0.05) pada CP pada selang masa penyembuhan yang berlainan. Tindakbalas setempat NHE1 didapati lebih tinggi secara signifikan dari tindak balas setempat AE2 semasa tempoh penyembuhan tetapi tiada perbezaan purata skor pengisyaratan setempat yang signifikan antara NHE1 dan AE2. Kesimpulannya, EIPA dan DIDS secara signifikannya boleh menghalang pertumbuhan tulang longitudinal, panjang HCZ dan kepadatan keseluruhan kondrosit GP walaupun dengan kehadiran GH. Ekspresi NHE1 dan AE2 juga dipengaruhi oleh perencatan disebabkan oleh EIPA dan DIDS walaupun dengan kehadiran GH. Kedua-dua pengangkut juga ditemui hadir dalam tempat kecederaan pada ahap yang tinggi secara signifikan sepanjang empat minggu tempoh penyembuhan kecederaan. Keputusan ini menunjukkan potensi peranan NHE1 dan AE2 dalam pertumbuhan tulang longitudinal dan juga penyembuhan tulang sekunder.

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I certify that a Thesis Examination Committee has met on 25 November 2016 to conduct the final examination of Abubakar Adamu Abdul on his thesis entitled "Role of Plasma Membrane Transporters (N^+/H^+ and HCO_{3-}) in Mediating Mammalian Longitudinal Bone Growth and Fracture Healing" 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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Date: 28 February 2017

This thesis was submitted to the Senate of the Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

µg	Micro gram
µL,	Micro liter
µM	Micro molar
ABC	Avidin biotin complex
AE2	Anion exchanger 2
ANOVA	Analysis of variance
AP	Anterior posterior view
AQP	Aquaporin membrane protein
ARF	Animal resource facility
BMP	Bone morphogenic protein
BSA	Bovine serum albumin
Caγ	Calcium gamma channel protein
CC	Capacitative coupling
cm	Centimeter
CMF	Combine magnetic field
CO_2	Carbon dioxide
DAB	3,3'-Diaminobenzidine
DBM	Demineralized bone matrix
DC	Direct current stimulation
DIDS	DIDS (4,4-diiodothiocyano-2,2-stilbenedisulphonate)
DMSO	Dimethyl sulphate
ECM	Extra cellular matrix
EGP	Epiphyseal growth plate
EHCZ	Early hypertrophic chondrocytes zone

EIPA	(5-(N-ethyl-N-isoprophyl) amiloride)
FCS	Fetal calf serum
FGF	Fibrolast growth factor
g	Gram
GA	Glutaraldehyde
GH	Growth hormone
GP	Growth plate
GPC	Growth plate chondrocytes
H&E	Hematoxyline and eosin staining
H_2O_2	Hydrogen peroxide
HCO_3^-	Hydrogen bicarbonate ion
HCZ	Hypertrophic chondrocytes zone
HMA	Hexamethylene amiloride
hr	Hour
IACUC	Institutional animal care and used committee
IF	Immuno-fluorescence
IGF	Insulin-like growth factor
IgG	Immunoglobuline G
IHC	Immunohistochemestry
IHH	Indian hedgehog
IMM	Induced micro motion
IP	Immuno Peroxidase
IU	International unit
K_{ATP}	Potassium ATP channel protein
kg	Kilogram

$KHCO_3$	Potassium bicarbonate
LHCZ	Late hypertrophic chondrocytes zone
LIUS	Low intensity ultrsound
M	Molar
MIBA	5-(N-methyl-N-isobutyl) amiloride
mL	Milliliter
mL^{-1}	Per milliliter
mM	Milimolar
mm	Millimeter
mV	Milivolt
N^+/H^+	Sodium and Hydrogen ions
Naγ	Sodium gamma channel protein
NBCs	Sodium couple bicarbonate cotransporter
NCX	Sodium calcium exchanger
ng	Nano gram
nm	nano meter
NHE1	Sodium hydrogen exchanger 1
NKCC1	Sodium potassium chloride co transporter 1
NMDA-R	N-methyl D-aspartate receptor
PAP	Peroxidase anti-peroxidase
PBS	Phosphate buffer saline
PEMF	Pulse electromagnetic field
PCZ	Proliferative chondrocytes zone
PDGF	Platelet derived growth factor
pH	Potential for hydrogen ion

PMCA	Plasma membrane Ca^{2+} ATPase
PTH	Parathyroid hormone
rhGH	Recombinant human growth hormone
RHT	Ruthenium hexamine trichloride
RMP	Resting membrane potential
RVD	Regulatory volume decrease
RVI	Regulatory volume increase
SEM	Standard error of means
SLC	Solute carrier protein
SPSS	Statistic for social sciences
TGF	Transforming growth factor
TGP	Total growth plate
TRPV	Transient receptor potential cation channel
UV	Ultraviolet light
v/v	Volume by volume
VEGF	Vesicular endothelial growth factor
w/v	Weight by volume
wtn	Want signaling pathways
α-MEM	Alpha modified essential media

CHAPTER 1

INTRODUCTION

1.1 Background of the Study

Bone is one of the organs of the musculoskeletal system which provide shape, physical support, to the vital organs of the body and facilitate the body movement (Clarke, 2008; Oryan *et al*., 2015). It is rigid, hard, and it can be easily regenerated and repaired (Taichman, 2005). It help store the marrow, serve as a storage facility for calcium, phosphorus, growth factors and cytokines, it is also involves in acid-base balance and endocrine regulation of energy metabolism (Peavey, 2003; Sanchez, 2006, Lakhkar *et al*., 2013).

Bone growth and development occur in two phases during embryonic development when bone tissues starts to develop and the second phase during postnatal life (Summerlee, 2001). The growth and development of the cortical component of the bone is usually taking place either through intramembranous or endochrondral ossification (O'Connor *et al*., 2010). Intramembranous ossification involves formation of flat bones, particularly those of craniofacial origin, which includes bones of the skull, facial bones, and the clavicle (Anderson and Shapiro, 2010; Long and Ornitz, 2013). In this process bone is develop directly from the mesenchymal stem cells precursors (Clendenning and Mortlock, 2012). The mesenchymal cells form an aggregate, which are then invaded by blood vessels and subsequently differente and proliferate into mature osteoblasts (Colnot *et al*., 2004; Yoshida *et al*., 2008; McBratney-Owen *et al*., 2008). The mature osteoblasts will then secrete osteoid, which directly laid down the foundation which will become flat bones (DeLise *et al*., 2000; Dallas and Bonewal, 2011) as shown in Figure 1.1.

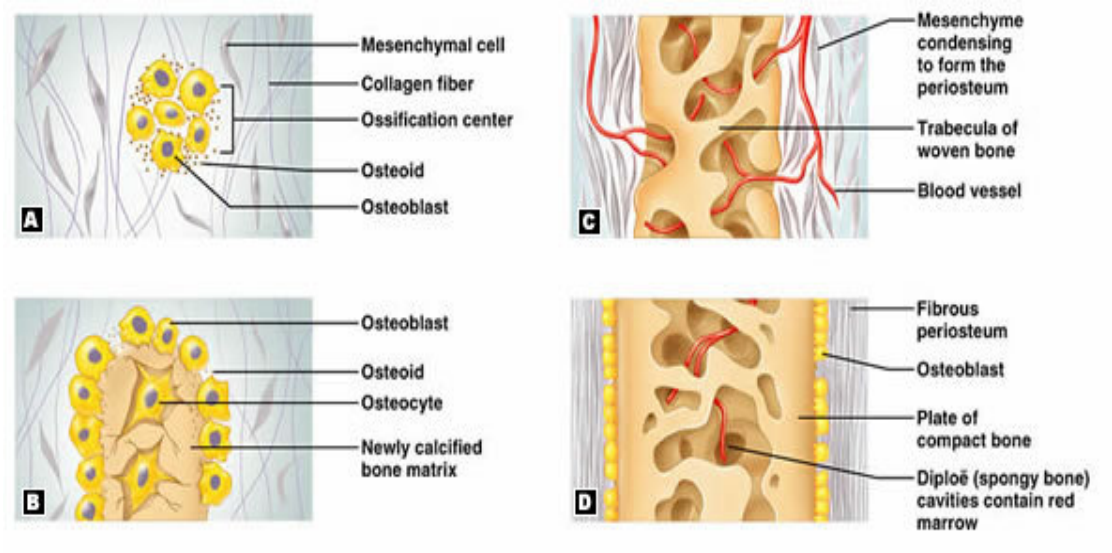

Figure 1.1 : Schematic diagram of sequential stages of intramembraneous ossification. Adapted from Pearson Education Inc., Cambridge, 2006

Endochondral ossification is relatively more complex than intramembraneous process of bone growth and development; it utilizes an intermediary stage of cartilage formation for the development of long bones. Secondary bone healing also occurred through the endochondral process of bone formation (Kronenberg, 2003; Mackie *et al*., 2011). Endochondral ossification is initiated by embryonic mesenchymal cells that aggregate to form a cartilaginous template which resemble the size and shape of the developing bone (DeLise *et al*., 2000; Staines, *et al*., 2013). The chondrocytes within the cartilage template then differentiate to form proliferative chondrocytes which subsequently undergoes morphological change into hypertrophic chondrocytes (Nilsson and Baron, 2004; Dowthwaite *et al*., 2004), the hypertrophic chondrocytes then undergoes programmed cell death before becoming vascularized, mineralized and invaded by osteogenic cells, and other precursor cells as shown in Figure 1.2 (Ornitz and Maries, 2002; Emons *et al*., 2009; Long *et al*., 2014).

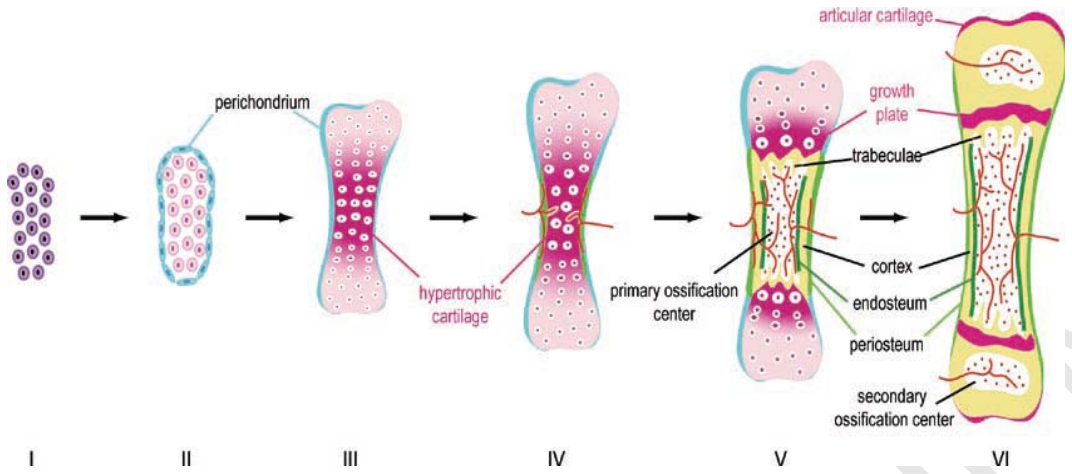

Figure 1.2 : Stages of endochondral ossification
(i) mesenchymal condensations, (ii) cartilage template, (iii) chondrocyte hypertrophy, (iv) vascular invasion, (v) formation of the primary ossification center, and (vi) formation of the secondary ossification center, and (vi) formation of the secondary ossification center and longitudinal growth. Adapted from Pearson Education Inc. Cambridge, 2006

There are numerous signaling factors that influenced the longitudinal bone growth these includes endocrine, paracrine and transcription factors. The signaling pathways play key roles in proliferation and differentiation of numerous cells that that take part during bone development (Yang and Karsenty, 2002; O'Connor *et al*., 2010; Staines *et al*., 2013). The endocrine factors are hormones that are involves in the regulation of bone growth development which includes; growth hormone, thyroid hormone, parathyroid hormone, calcitoriol, parathyroid hormone, estrogen, testosterone and calcitonin (Woods *et al*., 2007; Bobick and Kulyk, 2008; Beier and Loeser, 2010; Wit and Camacho-Habner, 2011). There are several paracrine signaling pathways that were reported to have played critical roles during bone growth. These includes; wtn signaling, Indian hedge hog [IHH], transforming growth factor [TGF-β], bone morpho genic protein [BMP], vesicular endothelial growth factor [VEGF], fibroblast growth factor [FGF] and platelet derived growth factor [PDGF] (Cooper *et al*., 2013; Abad *et al*., 2002; Joeng and long, 2014; Cho *et al*., 2012; Zhang *et al*., 2012; James, 2013; Maxhimer *et al*., 2015; Rahma *et al*., 2015). Bone growth is also regulated by many genes, majority of which are transcription factors which play important roles in the expression of genes that contribute significantly to bone growth, the most important transcription factors includes Sox9, Runx2, Dlx5, Twist1, c-Fos/AP-1, NF-κB, MITF, and NFATc1 (Nakashima and Crombrugghe, 2003; Guenou *et al*., 2005; Komori, 2006; Zhou *et al*, 2006; Yavropoulou and Yovos, 2008).

There are numerous ion channels and transporter proteins that were hypothesized to have potential role in in the course of bone growth and development according to Barret-Jolley *et al*., (2010); Lewis *et al*., (2011a). The functional activities of these ion channels and transporters were reported to have been taking place in the articular chondrocytes of the bone, which subsequently affect longitudinal bone growth (Lewis *et al*., 2013). The ion channels and transporter proteins so far reported to have potential role in long bone growth includes; NKCC (Na^+, K^+ and Cl^-), NMDA-R (Ca^+ and Na^+), AQP ($3H_2O$), ENaC (Na^+), Naγ (Na^+), Caγ (Ca^{2+}), K_{ATP} (K^+), TRPV4 (Ca^+ and Gd^+), TRPV6 (Gd^{2+}), TRPV5 (econazole Gd^{3+}), KZP (K^+), CIC (Cl^-), PMCA (Ca^{+2}), NCX ($3Na^+$), NHE (Na^+ and H^+), AE (HCO_3^- and Cl^-), VGSC (Na^+) and VGCC (Ca^+) (Bush *et al*., 2010; Butterworth, 2010; Lewis *et al*., 2011b; Yool and Campbell, 2012; Loqman *et al*., 2013; Karakas and Furakanu, 2014).

The ion channels and membrane proteins directly affect the functional activities of the chondrocytes physiology through resting membrane potential (RMP), which subsequently bring about regulation of chondrocytes volume. The chondrocytes volume regulation occurred when the RMP is at a steady state according to Hoffman *et al*., (2009); Lewis *et al*., (2011a). The physiological functions of the entire ion channels can be pharmacologically antagonised using their specific or non specific chemical antagonist. However, the mechanisms of action of most of the ion channels transporters that bring about modulation of chondrocytes physiology in bone elongation is either unknown or still controversial (Barrett-Jolley *et al*., 2010).

Currently there is no known report of their potential roles in fracture healing, however it can be hypothesized that, they may have similar potential role to play in the course of fracture healing, because longitudinal bone growth and fracture healing both occure through the process of endochondoral ossification.

Currently, studies were conducted on three ion channels (NKCC1, NHE1 and AE2) on their roles in chondrocytes hypertrophy with possible effect on longitudinal bone growth (Bush *et al*., 2010; loqman *et al*., 2013). The results of these studies have revealed that NKCC1, NHE1 and AE2, membrane transporter proteins act on hypertrophic chondrocytes via regulatory volume mechanism which bring about bone growth. The three membrane proteins transporters belong to solute carrier (SLC) classes, with different families and functional classes (Landowski *et al*., 2012; Saier Jr *et al*., 2013). Generally, the SLC transporter protein comprises genes that are responsible for passive transport, coupled ion transport and ion exchange (Hoffman *et al*., 2009).

Previous studies that were conducted to investigate role of plasma membrane transporters in long bone growth utilized metatarsal bone model for *ex vivo* bone growth. Although metatarsal bone is a typical long bone and has advantage of having multiple number bone rudiments that can be use to

have adequate sample size for statistical analysis but most at time is difficult to carefully harvest them with intact articular cartilage becaue of the smaller size of the bone. Therefore, there is need to explore other long bones like tibia that could be use as an *ex vivo* bone growth model in order to overcome the challage associated to the use of metatarsal bone.

Previous *ex vivo* bone growth model also used embryonic and post natal rat pup at day 7 (P7) bones. The choice of the embryonic and post natal P7 bone was that, both were proven not to be mineralized; hence there is no need to decalcify them in order to maintain their *in situ* morphological structure when histologically sectioned as reported by Loqman *et al*., (2010). However, there is no known documented age limit at which post natal bone is completely mineralized.

Previous investigators have hypothesized that, the cyclical process of endochondral bone formation occurred within a period of 24 hrs cycle, however, Chagin *et al*., (2010) and Okubo *et al*., (2013) reported that, the complete cyclical period of endochondral ossification can take place in more than 24 hrs period. This also warrant further investigation, hence our bone culture growth period was extended to 72 hrs with every 24 hrs change of media and taking bone length parameters.

1.2 Problems Statement

1. Bone growth disorders and complications of fracture healing lead to great economic and social impact to the public health, individual life quality and losses in livestock industry. Therefore, experimental investigations involving role of plasma membrane transport in bone growth and fracture healing may help explore the mechanism and pathogenesis of certain musculosketetal conditions.
2. Growth plate chondrocytes (GPC) hypertrophy has been implicated to be main determinants of bone growth rate through endochondral ossification but the mechanism is not fully understood.

1.3 Justification of the Study

The outcome of the study is expected to confer better understanding of the fundamental cellular mechanism of cell differentiation and tissue growth under normal and pathologic condition with emphasis on the role of plasma cell membrane transporters proteins. The plasma membrane protein in future could be of clinical relevance in mediating the cellular healing process of secondary fracture bone healing. Previous *ex vivo* studies of long bone growth have established potential roles of NKCC1, NHE1 and AE2 plasma membrane proteins during long bone growth.

1.4 Research Hypothesis

Plasma membrane transporters (Na^+/H^+) and (HCO_3^-) may play role in chondrocytes differentiation process that can contribute positively to the rate of mammalian long bone growth and secondary fracture healing

1.5 Objectives

The main objective of the work is to investigate the role of specific plasma membrane transporters (Na^+/H^+) and (HCO_3^-) during long bone growth and secondary fracture healing

Specific objectives:

1. To study postnatal *ex vivo* rat model for longitudinal bone growth investigations
2. To investigate the role of Na^+/H^+ (NHE1) and HCO_3^- (AE2) exchange across membrane of chondrocytes in mammalian longitudinal bone growth
3. To investigate the effect of recombinant human growth hormone (rhGH) on the localisation of NHE1 and AE2 membrane transporters on long bone growth in rat
4. To establish metatarsal fracture model in rats for *in vivo* investigation of secondary bone healing
5. To investigate the role of NHE1 (Na^+/H^+) and AE2 (HCO_3^-) membrane proteins during secondary bone healing in rat

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