UNIVERSITI PUTRA MALAYSIA

THE ROLE OF MEMBRANE TRANSPORTERS (NHE-1 AND AE-2) IN SECONDARY BONE HEALING OF TIBIA-FRACTURED SPRAGUEDAWLEY RATS

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FPV 2018 6

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By

KAREEM OBAYES HANDOOL

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

April 2017

DEDICATION

Consequences of years of study, research and day and night investigation is the present thesis that I would like to dedicate to my beloved My Father and Mother, My brother Ali Obayes Handool, My Wife Halima Hander Ali, My sons Taha (God bless his soul), Tariq and Idris, My daughters Zahra, Athra and Rose and My sisters for their prayer and moral support during my PhD study at Universiti Putra Malaysia.

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

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Chairman : Loqman Haji Mohamad Yusof, PhD
Faculty : Veterinary Medicine

Injuries emerging from orthopedic cases are increasingly becoming one of the major areas of attention in medicine. The development of bone has two major pathways, namely intramembranous bone formation and endochondral bone formation. Chondrocyte swelling describes the process emerging from the net movement of water into the cell which relies primarily on an osmotic gradient. It is likely that there is an important role of transporters which regulate the movement of Na^+ and anions (e.g., $HCO3^-$) across the cell membrane as these are known to be essential for the control of cell volume and pH in a wide range of cell types. This study hypothesizes that plasma membrane transporters have a role in cellular differentiation and regulation of endochondral ossification for secondary fracture healing. The objectives of this study were to evaluate the modified device to induce fracture for secondary fracture healing in a rat model, to study the different cellular stages of endochondral ossification, to evaluate the role of specific plasma membrane transporters (Na^+/H^+ and $HCO3^-$) in secondary fracture healing and to evaluate the effect of EIPA (5-(N-ethyl-N-isopropyl) amiloride and DIDS (4,4'-diisothiocyano-2,2'-stilbenedisulfonic acid) in secondary bone healing by using a rat tibial fracture model. A total of 55 female Sprague-Dawley rats of 8 weeks old were divided into three experiments: normal fracture healing (n=25, control), EIPA (n=15) and DIDS (n=15). Rats were sacrificed at 1, 2, 3, 4 and 6 weeks post-operative and assessed by clinical observation, radiology, histology, immunohistochemistry examination and statistical analysis. The modified device for producing fractures in the rat model is easy, cheap and reproducible, without complications. The result of gross callus area percentage and gross callus index showed significant difference at week 1 compared to the other weeks ($P<0.05$); only four rats had slight comminution

and 21 rats without comminution. A radiographic examination showed clinical union at week 3 in 60% of the rats, and good clinical union (100%) with less callus formation in week 6. Histomorphometric for woven bone, lamellar bone and bone marrow fibrosis percentage area revealed significant differences (*P<0.05*). Proliferative and hypertrophic chondrocyte zones percentage area showed a significant difference (*P<0.05*). Immunoperoxidase staining for NHE-1 and AE-2 revealed significant differences (*P<0.05*) in all weeks compared to week 6.

Following treatment with EIPA and DIDS, gross observation showed that the fracture line was clearly visible until week 4, manual fragment movements continued until week 2 and the callus area was smaller than in normal fracture healing. The X-ray callus index with DIDS treatment showed a significant difference (*P<0.05*). Histomorphometric with EIPA and DIDS treatment showed that the percentage area for woven bone, lamellar bone, periosteal fibrosis and marrow fibrosis revealed a significant difference (*P<0.05*); besides, the proliferative and hypertrophic chondrocyte zones percentage area showed a significant difference (*P<0.05*). Immunohistochemistry density reaction for NHE-1 and AE-2 in EIPA and DIDS showed a significant difference (*P<0.05*), the density reaction started a weak reaction, then declined directly to be absent in week 4 and week 6, whereas in normal fracture healing a strong reaction for NHE-1 started in the first four weeks then declined in week 6; however AE-2 began at a moderate level then increased strongly in weeks 3 and 4 and declined in week 6. The immunohistochemistry result refers to the direct effect of the inhibitors in the NHE-1 and AE-2 chondrocyte transporter proteins. These results suggest that NHE-1 and AE-2 have a role in the endochondral ossification of secondary bone healing. The inhibition of the hypertrophic chondrocyte zone following treatment with EIPA and DIDS, further strengthened the study hypothesis that NHE-1 and AE-2 inhibit fracture healing.

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

PERANAN PENGANGKUT MEMBRAN (NHE-1 DAN AE-2) DALAM PENYEMBUHAN SEKUNDER TULANG TIKUS SPRAGUE-DAWLEY TIBIA-TERPATAH

Oleh

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April 2017

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Kecederaan yang muncul dari kes-kes ortopedik semakin menjadi salah satu bidang utama perhatian di dalam bidang perubatan. Pembangunan tulang mempunyai dua laluan utama, iaitu pembentukan tulang intramembran dan pembentukan tulang endokondral. Pembengkakan kondrosit menghuraikan proses yang muncul dari pergerakan bersih air ke dalam sel yang bergantung terutamanya kepada kecerunan osmosis. Kemungkinan terdapat peranan penting pengangkut-pengangkut yang mengawal pergerakan Na^+ dan ion negatif (contohnya, HCO_3^-) di seluruh membran sel kerana semua ini dikenali sebagai penting untuk kawalan isipadu sel dan pH bagi pelbagai jenis sel. Kajian ini menghipotesiskan bahawa pengangkut membran plasma mempunyai peranan di dalam pembezaan sel dan pengaturan osifikasi endokondral untuk penyembuhan patah sekunder. Objektif kajian ini adalah untuk menilai peranti yang diubah suai untuk mendorong fraktur untuk penyembuhan fraktur sekunder di dalam model tikus, untuk mengkaji ossifikasi endokondral di peringkat-peringkat sel yang berbeza, untuk menilai peranan pengangkut membran plasma tertentu (Na^+/H^+ dan HCO_3^-) dalam penyembuhan fraktur sekunder dan untuk menilai kesan EIPA (5- (N-etil-N-isopropyl) amiloride dan DIDS (asid 4,4'-diisothiocyano-2,2'-stilbenedisulfonic) dalam penyembuhan tulang sekunder dengan menggunakan model fraktur tibia tikus. Sebanyak 55 tikus betina Sprague-Dawley berumur 8 minggu dibahagikan kepada tiga eksperimen: penyembuhan fraktur normal (n=25, kawalan), EIPA (n=15) dan DIDS (n=15). Tikus-tikus tersebut dikorbankan pada 1, 2, 3, 4 dan 6 minggu selepas pembedahan dan dinilai melalui pemerhatian klinikal, radiologi, histologi, pemeriksaan imunohistokimia dan analisis statistik. Peranti yang diubahsuai untuk menghasilkan fraktur di dalam model tikus adalah mudah, murah dan boleh diulang, tanpa komplikasi. Hasil peratusan kawasan kalus kasar dan indeks kalus kasar menunjukkan

perbezaan yang signifikan pada minggu 1 berbanding minggu-minggu lain *(P<0.05);* hanya empat tikus mempunyai pengecilan sedikit dan 21 tikus tanpa pengecilan. Suatu pemeriksaan radiografi menunjukkan pencantuman klinikal pada minggu 3 dalam 60% daripada tikus tersebut, dengan pencantuman klinikal yang baik (100%) dan pembentukan kalus yang kurang pada minggu 6. Histomorphometri untuk tulang tenunan, tulang lamela dan peratusan kawasan fibrosis sumsum tulang menunjukkan perbezaan yang signifikan *(P<0.05).* Peratusan kawasan zon-zon pembiakan dan hipertrofi kondrosit menunjukkan perbezaan yang signifikan *(P<0.05).* Mewarnakan dengan Immunoperoxidase untuk NHE-1 dan AE-2 menunjukkan perbezaan yang signifikan *(P<0.05)* di semua minggu berbanding minggu 6.

Selepas rawatan dengan EIPA dan DIDS, pemerhatian kasar menunjukkan bahawa garis fraktur itu jelas kelihatan sehingga minggu 4, pergerakan serpihan manual berterusan sehingga minggu 2 dan kawasan kalus adalah lebih kecil daripada dalam penyembuhan fraktur normal. Indeks kalus X-ray dengan rawatan DIDS menunjukkan perbezaan yang signifikan *(P<0.05).* Histomorphometri dengan EIPA dan rawatan DIDS menunjukkan bahawa peratusan kawasan untuk tulang tenunan, tulang lamela, fibrosis periosteum dan fibrosis sumsum menunjukkan perbezaan yang signifikan *(P<0.05);* selain itu, peratusan kawasan zon-zon pembiakan dan hipertrofi kondrosit menunjukkan perbezaan yang signifikan *(P<0.05).* Reaksi ketumpatan immunohistokimia untuk NHE-1 dan AE-2 dalam EIPA dan DIDS menunjukkan perbezaan yang signifikan *(P<0.05)*, tindak balas ketumpatan memulakan tindak balas yang lemah, kemudian menurun secara langsung dan tidak ada di minggu 4 dan minggu 6, sedangkan pada penyembuhan fraktur normal reaksi yang kuat untuk NHE-1 bermula pada empat minggu pertama kemudian menurun pada minggu 6; bagaimanapun AE-2 bermula pada tahap sederhana kemudian meningkat dengan kukuh pada minggu 3 dan 4 dan menurun pada minggu 6. Hasil immunohistokimia merujuk kepada kesan langsung daripada perencat dalam protein pengangkut kondrosit NHE-1 dan AE-2. Keputusan-keputusan ini menunjukkan bahawa NHE-1 dan AE-2 mempunyai peranan dalam ossifikasi endochondral penyembuhan tulang sekunder. Perencatan zon hipertrofi kondrosit selepas rawatan dengan EIPA dan DIDS, mengukuhkan lagi hipotesis kajian bahawa NHE-1 dan AE-2 merencatkan penyembuhan fraktur.

ACKNOWLEDGEMENTS

First of all, I am immensely grateful to Almighty “Allah” for His blessings in giving me the strength and encouragement to conduct this research and everything I have.

Second, I would like to express my special and heartiest appreciation to my main supervisor Dr. Loqman Haji Mohammad Yusof, Head Department of Companion Animal Medicine and Surgery, Faculty of Veterinary Medicine, University Putra Malaysia, for his kindness, who dedicated his time to teaching me. His patients, encouragements and efforts in conducting and accomplish this research is that much which without his helps carrying this study was impossible.

I’d mainly like to thank and appreciate Assoc. Prof. Dr. Md. Sabri Mohd Yusof. Head Department of Veterinary Pathology & Microbiology, Faculty of Veterinary Medicine, University Putra Malaysia, member of supervisory committee, who actively helped to evaluate the histological slides, for his excellent feedback and suggestions.

I would especially thank to my supervisory committee member Assoc. Prof. Dr. Jalila Abu, Head Department of Veterinary Clinical Studies, Faculty of Veterinary Medicine, University Putra Malaysia, other member of supervisory committee, for her helpful guidelines and discussion through the years of study and research. My sincere gratitude goes to Prof. Dr. Mohamed Ariff Omar and Dr Mahdi Ibrahimi Department of Preclinical Veterinary Sciences, Faculty of Veterinary Medicine, University Putra Malaysia, who taught and directed me statistics.

I’d mainly like to thank and appreciate Assoc. Prof. Dr. Faez Jesse Firdaus, Dr. Lau Seng Fong and Dr. Mazlina Mazlan Department of Veterinary Pathology & Microbiology, Faculty of Veterinary Medicine, University Putra Malaysia, for her helpful guidelines and discussion through the years of study and research.

Many thanks goes to my friends who helped me in the surgeries: Dr. Abubakar Adamu Abdul, Dr. Sahar Mohammed Ibrahim, Dr. Kah ying, and Dr. Ubid Kaka, Small animal surgery staff, histopathology laboratory staff and clinical laboratory staff.

Last but not least Abuljawadain and my family. It is difficult to overstate my gratitude to Abuljawadain, my mother, brother and wife. They helped me to overpass my difficult time with their love and supports. Accomplishing this way was impossible without relying on them. My wife had a significant position in these years. Pushing me forward by encouraging me was her most important feature in finishing this difficult task. My brother was one of my embossed features in starting and continuing my study here whom I really would like to thank to.

I certify that a Thesis Examination Committee has met on 28 April 2017 to conduct the final examination of Kareem Obayes Handool on his thesis entitled "The Role of Membrane Transporters (NHE-1 and AE-2) In Secondary Bone Healing of Tibia-Fractured Sprague-Dawley Rats" 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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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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I hereby confirm that:

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Declaration by Members of Supervisory Committee

This is to confirm that:

- the research conducted and the writing of this thesis was under our supervision;
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Signature: _____
Name of Member of Supervisory Committee: Associate Professor Dr. Sabri Mohd Yusof

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Name of Member of Supervisory Committee: Associate Professor Dr. Jalila Abu

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Ab	Antibody
ACUC	Animal care and user committee
AE	Anion exchanger
Akt	Protein kinase B (PKB), also known as Akt
ANOVA	Analysis of Variance
ASCT	Amino acid transporters,
ASICs	Acid sensing ion channels
BAX	A gene located on chromosome 19q13.3-q13.4
BM	Bone marrow fibrosis
BMPs	Bone morphogenic proteins
BO	Bio-Oss
C	Cartilage
ºC	Degree celsius
Ca.	Calcium
CA	Callus area
cAMP	Cyclic adenosine monophosphate
$Ca_5(PO_4)_3(OH)_2$	Hydroxyapatite
CA	Callus area
CB	Cortical bone
CI	Callus index
Cl^-	Chloride ion
cm	Centimeter
CO2	Carbon dioxide
CSD	Critical sized defect

CT	Connective tissue
DBM	Demineralized bone matrix
DEPC	Diethylpyrocarbonate
DIDS	4,4'-diisothiocyano stilbene-2,2'-disulfonic acid
DPX	Distrene, Plasticizer, Xylene
DMSO	Dimethyl Sulfoxide
eAE	Erythroid Anion Exchangers
ECG	Electrocardiography
ECM	Extracellular matrix
EIPA	5-(N-Ethyl-N-isopropyl)-Amiloride
eNOS	Endothelial Nitric Oxide Synthase
ERM	Ezrin-Radixin-Moesin.
ESF	External skeletal fixation
FGF	Fibroblast growth factor
Fig.	Figure
g	Gram
G.	Groups
GAGs	Glycosaminoglycans
GF	Growth factor
GLUT	Glucose-transporter
H^+	Hydrogen ion
HA	Hydroxyapatite
HB	Host bone
HCL	Hydrochloride
H & E	Hematoxylin and Eosin
HCO_3^-	Bicarbonate

HMIT	Hydrogen myo-inositol transporter
HOCH2CH2NH3^{+}	Ethanolamine
$HOCH_2CH(COO\text{-})NH_3^{+}$	Phosphate serine
HCZ	Hypertrophic chondrocyte zone
IC50	Median inhibitory concentration
i.e.	Id est.
IGF	Insulin growth factor
IHC	Immunohistochemistry
IL	Interleukin
IM	Intramedullary pin
i.m	Intramuscular
IP	Intraperitoneal
IU	International unit
I.V	Intravenous
K^{+}	Potassium ion
Ki	The inhibitory constant of drug
KO	Knockout
KVp	Kilo voltage-peaks
Lat.	Lateral
Lb	Lamellar bone
LM	Light microscope
MAs	Mill-ampere-seconds
MCSF	Macrophage colony stimulating factor
MFS	Major facilitator superfamily
MB	Mature bone
ml	Milliliter

mm	Millimeter
mM	Mill mole
MOBL	Mesenchymal osteoblasts
mRNA	Messenger Ribonucleic Acid
MV	Millivolts
MSC	Mesenchymal stem cell
no	Number
Na^+	Sodium ion
NB	New bone
NCSD	Non-critical sized defect
NBCs	Sodium bicarbonate transporters
NHE	Sodium Hydrogen Exchanger
NKCC	Sodium, potassium, and chloride transporter
No	Number
NO	Nitric Oxide
NPPB	5-nitro-2-(3-phenylpropyl-amino) benzoic acid
OB	Original bone
OH	Hydroxyl group
OPGL	Osteoprogerin Ligand
OSTB	Osteoblasts
OSTP	Osteoporosis
p.	Page
p	P-Value
PBS	Phosphate buffer solution
PDGF	Platelet derived growth factor
Pf	Periosteal fibrosis

PGE	Prostaglandin E
PH	Power of hydrogen
pHe	Extracellular pH
pHi	Intracellular pH
PI3K	Phosphatidylinositol-3-OH kinase
pp.	Pages
PCZ	Proliferative chondrocyte zone
RANKL	Receptor Activator for Nuclear Factor κ B ligand
ROI	Region of interest
S	Sample
SAU	Sindh Agriculture University
S/C	Subcutaneous
SLC	Solute carrier family
SOBL	Surface osteoblasts
SPSS	Statistical package for social science
SE	Standard error
SEM	Scanning electron microscope
SITS	Stilbene derivatives
SO4	Sulfate
STD	Standard deviation
TGF	Transforming growth factor
TGF-B	Transforming growth factors-b
TM	Transmembrane
TNFR	Tumor necrosis factor receptor
UDC	Ursodeoxycholate
UPA	Urokinase-type plasminogen activator

UPM	Universiti Putra Malaysia
Vas	Vascularization
Wk	Week
Wb	Woven bone
W/V	Weight/Volume
μm	Micromillileter

CHAPTER 1

INTRODUCTION

Major injuries of bone associated with multiple trauma and traffic accidents, which lead to prolonged periods of treatment with significant socioeconomic impacts, are still considered as basic health issues in advanced countries (Sfeir *et al.,* 2005; Fayaz *et al*., 2011). Bone fracture healing can occur via two techniques endochondral bone formation and intramembranous bone formation. Secondary bone healing includes the classical phases of injury, haemorrhage, inflammation, soft callus formation, mineralization callus, and remodelling callus.

This process of secondary bone healing strictly be similar to endochondral ossification, which includes a cartilage template being substituted by bone (Shapiro, 2008). Endochondral ossification is essential processes during fetal development of the mammalian skeletal system also an essential process during the rudimentary formation of long bones, the growth of the length of long bones, (Brighton *et al*., 1973) and the natural healing of bone fractures (Brighton and Robert, 1986; Fayaz *et al*., 2011). Bone healing utilized the similar formation designs as bone growth by enlargement of chondrocytes, however the specific approach of healing is determined through the biomechanical environment delivered (Kim *et al*., 2013; Sathyendra & Darowish, 2013).

The plasma membrane is the borderline that separates the living cell from its surroundings and exhibits selective permeability, which permits certain material pass more easily than others do. Phospholipid bilayer, cholesterol and protein are the fundamental structure of the plasma membrane. For cellular biological homeostasis to be maintained, molecules must be removed from and transported into organelles and cells. This crucial function is accomplished through way of transport proteins that exist in intracellular membranes and in the cytosol. These proteins control the inflow of vital ions, nutrients, environmental toxins, cellular waste, and other xenobiotics, which play vital roles in cellular homeostasis and enable the movement of vital biological molecules such as sugars, amino acids, nucleotides, and vitamins through cellular membranes against or along their electrochemical gradients (Kim, 2002; Eraly, *et al*., 2004).

In the human genome about two thousand transporter-associated genes, underlining their biological importance and role in cellular homeostasis was discovered. The solute carrier families carry out more than three hundred transporters from these transporter genes.

The sodium/hydrogen ion exchangers (NHEs) are fundamental transporter proteins in the solute carrier family 9 (SLC-9) and perform important functions in transepithelial salt, acid and base transport, and regulation of extracellular and intracellular pH, cell volume regulation, growth, proliferation, differentiation and apoptosis (Landowski *et al*., 2008).

The second major group of anion exchangers-bicarbonate cotransporter family is the sodium bicarbonate transporters (NBCs), which perform a crucial role in acid-based movement in most tissues and cell types including kidney, heart, liver, blood cells, intestine, stomach, pancreas, central nervous system and reproductive systems (Abduladze *et al*., 1998).

Bush *et al*. (2010) in previous study provided an indication of the role of the Na-K-2Cl cotransporter (NKCC1) in volume increased of growth plate hypertrophic chondrocyte zone. However, another study on the role of anion exchanger (AE-2) and Na^+/H^+ antiporter (NHE-1) in bone growth plate chondrocytes has been reported (Loqman *et al*., 2013). This significance is due to the fact that indirect bone healing can occur in the similar formation design of enlargement of chondrocytes in endochondral ossification of secondary bone healing. This indicates the role of the transporters that control the movement of anions (e.g., HCO_3^-) and Na^+ through chondrocyte cell membranes.

Many diseases for instance cystic fibrosis, non–insulin-dependent diabetes, type I cystinuria, mellitus haemolytic anaemia, epilepsy and schizophrenia acquired or inherited are produced via defective or dysregulation expression of transporter proteins (Landowski *et al*., 2008). For example, cystic fibrosis is a common life-limiting autosomal recessive genetic disorder, with highest prevalence in Europe, North America, and Australia. The disease is caused by mutation of a gene that encodes a chloride-conducting transmembrane channel called the cystic fibrosis transmembrane conductance regulator (CFTR), which regulates the anion transport and mucociliary clearance in the airways. Functional failure of CFTR results in mucus retention and chronic infection and subsequently in local airway inflammation that is harmful to the lungs (Elborn, 2016).

Currently, studies investigating animal models of secondary fracture bone healing are still few and data on the different cellular stages of endochondral ossification in secondary bone fracture healing are limited. Insufficiency of information also exists on the role of specific plasma membrane transporters (Na^+/H^+ and HCO_3^-) in chondrocytes for secondary bone fracture healing.

Orthopedic surgery and orthopedics, similar to other specialties, have been developed through the requirement and responsibility. Orthopedic surgeons have developed the capability to avoid major losses of a bone's function and actually, they can prevent expectable death. They try to find excellence in their art, by making sure that the patient achieves optimal condition in the shortest period throughout the safest procedures and possible methods with lost economic losses.

Delayed healing results in an increased duration of immobilization in non-union or mal-union of the bone ends, increases the risk of joint stiffness, and is associated with Long-term morbidity, hospitalization, care patients and death, also increases the risk of inadequate fracture alignment. Long fracture healing period, suffering to patients and large costs for society, a pharmacological therapy, the high socioeconomic costs and the complications impaired in bone healing, bone regeneration and the targeted therapies were the major problem in bone fracture healing.

This study was intended to evaluate the role of specific cell plasma membrane transporters and cellular differentiation of endochondral ossification process in secondary fracture healing using laboratory animal models.

Hypothesis:

Specific plasma membrane transporters (Na^+/H^+ and HCO_3^-) play a significant role in cellular differentiation and regulation of endochondral ossification in secondary bone healing.

Objectives:

This study was conducted in recognition of the fact that plasma membrane transporters are important in cellular differentiation and regulation of endochondral ossification for secondary fracture healing. The specific objectives of this study were:

1- To evaluate the modified three-point bending pliers to induce fracture using *in vivo* animal model for secondary bone fracture healing.
2- To study the different cellular stages of endochondral ossification in secondary bone fracture healing.
3- To determine the role of specific plasma membrane transporters (Na^+/H^+ and HCO_3^-) in secondary bone healing.
4- To evaluate the effect of EIPA in secondary bone healing in the rat tibial fracture model.
5- To evaluate the effect of DIDS in secondary bone healing in the rat tibial fracture model.

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