Republic of Iraq

Ministry of Higher Education and Scientific Research
University of Kerbala

College of Veterinary Medicine

Physiology ,Biochemistry and Pharmacology Department

Differentiation and Activation of Osteoblast-Osteoclast Pathway on Bone loss induced by Hypercholestermic Diet in Male Rats

Thesis

Submitted to the Council of the College of Veterinary medicine at University of Kerbala as a Partial fulfillment of the Requirement for the Degree of Master in the Sciences of Veterinary Medicine /physiology

Written by

Duaa Raad Abd Al Ameer

Supervised by

Prof.Dr. Wefak Jbori AI-Bazi

Asst. Prof.Dr. Hayder A. Muhammed

1446A.H **2024A.D**

بسم الله الرحمن الرحيم

(قَالَ رَبِّ إِنِّي وَهَنَ الْعَظْمُ مِنِّي وَاشْتَعَلَ الرَّأْسُ شَيْبًا وَلَمْ أَكُن بِدُعَائِكَ رَبِّ شَقِيًّا)

صدق الله العلي العظيم

(الآية ٤سورة مريم)

Supervisor Certification

We certify that thesis entitled « **Differentiation and activation of osteoblast-osteoclast pathway on bone loss induced by hypercholestermic diet in male rats»** as been prepared by **Duaa Raad Abd Al ameer** under my supervision at the college of Veterinary Medicine, University of Kerbala in partial fulfillment of the requirements for the Master of Degree in the Sciences of Veterinary Medicine in Veterinary Physiology, Biochemistry and Pharmacology.

Supervisors

Prof. Dr. Wefak Jbori Al-Bazi

Asst. Prof.Dr. Hayder A. MUHAMMED

College of Veterinary Medicine
University of Kerbala

The recommendation of the Department

In the view of the above recommendation, I forward this thesis for scientific discussion by the examining committee

Prof. Dr. Ihab Ghazi Mahdi

Vice Dean for Postgraduate studies and scientific Affairs

college of Veterinary Medicine

university of kerbala

Certification of Linguistic Evaluator

I certify that thesis entitled « **Differentiation and activation of osteoblast-osteoclast pathway on bone loss induced by hypercholestermic diet in male rats»** for the student **(Duaa Raad Abd AL Ameer)** was linguistically reviewed by me and the necessary correction has been made. Thus, it is linguistically ready for examination.

Signature

Linguistic Evaluator

Name: Asst. Prof .Dr. Hamed Qattan Jawad

Committee Certification

This is certify this thesis **(Differentiation and Activation of Osteoblast-Osteoclast Pathway on Bone loss induced by Hypercholestermic Diet in Male Rats)** was prepared by **(Duaa Raad Abd Al Ameer)**, We the members of the examining Committee, certify that after reading and examining the student in its content, it is adequate for the ward for the degree of Master in Veterinary Medicine/ Physiology.

Prof. Dr. Rana Fadhil Mousa

College of Veterinary Medicine/ University of Kerbala

(Chairman)

Asst.Prof.

Dr. Lama Walid Khalid

College of Veterinary Medicine/

University of Baghdad

(Member)

Asst.Prof.

Dr. Mayada Sahib Hassan

College of Veterinary Medicine/

University of Kerbala

(Member)

Prof . Dr. Wefak Jbori AI-Bazi

Asst. Prof. Dr. Hayder A. Muhammed

College of Veterinary Medicine/ University of Kerbala

(Member & Supervisor)

Approved by the concile of the college of Veterinary Medicine /University Kerbala

Prof.

Dr. Rana Fadhil Mousa

Head of Department of Physiology,

Biochemistry and Pharmacology

Prof.

Dr. Wefak Jbori Al-Bazi

The Dean of the College of Veterinary

Medicine

Date: / / 2024

Declaration

I hereby declare that this dissertation is my original work except for equations and citations which have been fully acknowledged. I also declare that it has not been previously, and is not concurrently, submitted for any other degree at University of Kerbala or other institutions.

Duaa Raad Abd Al Ameer

/ / 2024

Dedication

From ALLAH to ALLAH for ALLAH sake*……..*

To my Father , who is always a source of pride for me and encouragement…….

To my Mother, who never left me and who always supported me …….

To my husband who supported me and encouraged me in every step……

To my dears... my brother and sisters…….

To my friends and colleagues in my studies and all my teachers…..

I would like to thank everyone who supported me to successfully complete my research……

Duaa Raad Abd AL Ameer

Acknowledgment

Praise be to ALLAH, the First before creation, and the Last after the annihilation of things. May blessings and peace be upon the chosen one, Muhammad, and his faithful family.

I extend my sincere thanks to the Deanship of the College of Veterinary Medicine, University of Kerbala, for heelp me in postgraduate studies in the branch of physiology, biochemistry, and medicines. I also extend my thanks and appreciation to all the teaching staff in the college, including doctors and professors, through their support for me during my postgraduate studies.

As I conclude the composition of my thesis, I wish to express my sincere appreciation and deep gratitude to my esteemed supervisors: **Prof.Dr. Wefak Jbori Al-Bazi** , and Asst. Prof. Dr. Hayder A. Muhammed in the University of Kerbala/ College of Veterinary Medicine for their consistent scientific advice and dedicated guidance, which were pivotal throughout the research and writing phases. They exemplified excellence in their roles as mentors and overseers. I eagerly pray for their enduring health and continuous progress in their scientific endeavors.

Thank you very much to **(Dr. Iman jabber jawad)** for helping me in histological examination.

Thank you very much to **(Dr. Falah Mahmoud)** for helping me in X-ray examination.

Duaa Raad Abd AL Ameer

Abstract

The aim of this study was to examine the impact of a high cholesterol diet on osteoporosis by exploring how hypercholesterolemia can enhance the differentiation and activity of osteoclasts, leading to increased bone resorption and subsequent net bone loss. The experiment was employed twentye male rats aged (1.5-2) months were divided as follows 2 groups : (10) rats were fed normal diet, (10) rats were fed a high cholesterol diet (2%) for 8 weeks serve as HCD group, physiological and biomarker parameters calculation RANK, RANKL, extracellular signal regulated kinase (ERK), tartrate resistance acid phosphate (TRAP), Lipid profile (TC ,TG, LDL , HDL),internal oxidant (MDA) and antioxidant (GSH), electrolytes(calcium, sodium, phosphor, potassium) ,hormones (Calcitonin, parathyroid hormone ,Vit.D) and femur bones were excised to measure of osterix gene expression, histopathological examination after the end of the experimental (8 weeks), radiological image before experimental and after 4 weeks from experimental and after the end of the experimental (8 weeks).

The results of the study showed a significant increase ($P< 0.05$) in the Total cholesterol (TC), Triglycerides (TG), Low density lipoprotein (LDL) in the HCD group compared to the control group. In contrast, a significant decrease ($P< 0.05$) in the (HDL) in the HCD group compared to the control group.

The results showed a significant increase ($P< 0.05$) in the serum of The receptor activator of nuclear factor Kb(RANK), the receptor activator of nuclear factor κB ligand (RANKL), extra cellular signal regulated kinase (ERK) in the HCD group compared to the control group ,while no significant change ($P> 0.05$) in serum Tartrate Resistance Acid Phosphate (TRAP) levels in the HCD group compared to the control group.

The results showed a significant elevated levels ($P< 0.05$) of parathyroid hormone, Calcitonin, and Vitamin D in cholesterol group compared to the control group, this Study showed a significant increase ($P< 0.05$) in the serum of Calcium in the HCD

group compared to the control group. In contrast, no a significant (P> 0.05) in the serum of Sodium, phosphors and potassium in the HCD group compared to the control group by (Na, P, K) .

Also indicated a significant decrease (P< 0.05) in GSH in HCD group compared with the control group. In contrast, a significant increase (P< 0.05) in Malnodialdehyde (MDA) observed in the HCD group compared to the control group.

On the other hand the Osterix gene showed significant up-regulation in the HCD group compared to the control group.

The histopathological examination of the bone tissue in our study showed loss of osteoblasts on borders of trabeculae, necrosis of osteocytes with multiple multinucleated osteoclasts in the HCD group compared to the normal histological section of the control group, which show normal osteocytes in lacunae , regular bone marrow cavities and regular osteoblasts in line on trabecular border.

At the end of experimental animals the study find in the cholesterol groups the study found a radiolucent area at the pelvic bones, femur bone and vertebral of rats with osteoporosis induced by a high-cholesterol diet.

In conclusion: Osterix gene enhances bone matrix mineralization by modulating the expression of genes involved and this a significant increase was associated with the concentration of calcium in serum of hypercholestermic male rats and Monitoring biomarker ERK can provide valuable information about disease progression, treatment response, and potential therapeutic targets in osteoporosis management.

List of Contents

List of Tables

Table of Figures

List of Abbreviations

Abbreviations	Meaning
Acetyl-CoA	Acetyl coenzyme A
AED	Tetracyclic carbon ring
Ach	Acetylcholine
AD	Alzheimer‘s disease
CVD	Cardiovascular Disease
DMSO	Dimethyl Sulfoxide
ELISA	Enzyme-Linked Immunosorbent Assay
GSH	Glutathione
HCD	Hypercholesteremic diet
HCH	Hypercholesterolemia
HDL-C	High-density lipoprotein cholesterol
HMG-CoA	3-hydroxy-3- methylglutaryl coenzyme A
LDL-C	Low-density lipoprotein cholesterol

MDA	Malondialdehyde
MG	Milligram
TGF-β	Transforming growth factor-beta
KG	Kilogram
ROS	reactive oxygen speices
TC	Total cholesterol
TG	Triglycerides
SOD	superoxide dismutase
RANK	Receptor activator nuclear factor Kb
RANKL	Receptor activator nuclear factor Kb ligand
ERK	Extracellular signal regulated kinase
TRAP	Tartrate resistance acid phosphatase
FA	Fatty acid
CAT	Catalase
OPG	Osteoprotegerin
PTH	Parathyroid hormone
Vit D	Vitamin D
BMD	Bone related disease
OSX	Osterix
Ca	Calcium
Na	Sodium
P	Phosphors
K	Potassium

Chapter One

Introduction

Introduction

Osteoporosis is a condition that causes a substantial reduction in bone protein and mineral content, resulting in bone depletion, which is a common metabolic bone disorder, Osteoporosis becomes more prevalent and deadly as individuals age, resulting in a substantial worldwide medical and financial burden **(Bao *et al*.,2023)**

The chronic metabolic bone disease osteoporosis causes a decrease in bone mass, deterioration of the bone structure, and fractures and heightened vulnerability to fractures **(Arandjelovic *et al*., 2021).**

High cholesterol levels can contribute to various physiological conditions, including fat accumulation, cardiovascular disease, and Alzheimer's disease(Ad), thereby causing damage to the body**(Raheem *et al*., 2023).** Osteoporosis and osteopenia are associated with altered bone microstructure, leading to increased fracture risks in animals for example low in calcium and vitamin D and excessive dieting with cholesterol**(You *et al*., 2011).**

Deregulation of cholesterol metabolism in the bone can disrupt osteoblast differentiation and function, impair osteoclast activity, promote inflammation, and contribute to bone loss. Understanding the complex interplay between cholesterol and bone metabolism is important for the development of therapeutic strategies to prevent and treat bone-related disorders **(Tian & Yu 2015).**

Hypercholesterolemia (HCH) is a multifaceted disorder that arises from various factors, encompassing both lifestyle choices and genetic predisposition, Additionally, it is a contributing factor to cardiovascular diseases (CVDs), which account for 172 million fatalities annually**(Cunha *et al*., 2021).** There is a correlation between a decrease in bone mineral density and a high cholesterol diet. In general, animals who are fed a high cholesterol diet have weight loss, even if there is no substantial variation in energy consumption among the experimental animals**(Santos-López *et al*., 2017).**

Recent research indicates that consuming a diet high in fat, known as HFD, not only causes obesity but also triggers metabolic irregularities and the absorption of bone, This leads to a decrease in bone mass and weakened bone strength, ultimately increasing the likelihood of both spontaneous and traumatic bone injuries**(Lian *et al*.,2021).**). This suggests that excess fat is detrimental to bone health in animals**(López-Gómez *et al*.,2022).**

Elevated levels of cholesterol, specifically low-density lipoprotein (LDL) cholesterol, have the potential to penetrate the walls of arteries and undergo oxidative modification,This modified LDL can trigger an inflammatory response and attract immune cells, leading to the formation of fatty plaques in the arteries**(Qin.,2020)**, Reactive oxygen species (ROS) produced during this process can worsen inflammation and contribute to the advancement of atherosclerosis**(Di Pietro *et al*.,2016).**

In order to maintain bone balance, three types of bone cells are involved; Osteoclasts, osteocytes, and osteoblasts are three types of cells involved in bone formation and remodeling**(Kim *et al*., 2020**). There are distinct stem cell lineages associated with this cell type; the mesenchyme lineage corresponds to the mesenchymal stem cell type, and the hematopoietic stem cell type corresponds to the hematopoietic stem cell type**(Han *et al*.,2018).**

Osteoclasts, derived from hematopoietic cells, are large multinucleated cells that play a vital role in bone resorption ,Maintaining a balance between osteoclastic bone resorption and osteoblastic bone formation is crucial for preserving bone homeostasis **(Omi& Mishina *et al*., 2022).** On the other hand Osteoclasts, being the exclusive cells capable of bone resorption, have a pivotal role in maintaining bone homeostasis**(Omi& Mishina *et al*., 2022).**

In some studies, scientists often use animal models such as rats to investigate the role of specific molecules, receptor activator RANKL/RANK/OPG pathway system genetic factor or cell types in various biological processes, including bone development and

diseases **(Amin *et al*., 2020**) .This pathway plays a fundamental role in regulating the balance between bone resorption and bone formation, dysregulation of this pathway can disrupt the balance between bone resorption and formation, leading to bone-related diseases (BRD) **(Jin *et al*.,2023).**

Rats are commonly used because they share many physiological and genetic similarities with humans, one of the most important of molecular factor was transcription factor Osterix and RUNK **(Liu *et al*., 2020).** Osterix (Osx), also referred to as Sp7, is a transcription factor that is specific to osteoblasts and contains zinc finger domains. It belongs to the SP/KLF family of transcription factors **(Nakashima et al., 2002;Suske *et al*.,2005),** Osx is present in osteoblast-lineage cells, chondrocytes, and is also highly expressed in different cancer tissues**(Qu *et al*.,2019).**the gene expression was specifically observed in osteoblasts and osteocytes, and to a lesser extent in prehypertrophic and hypertrophic chondrocytes **(Xing *et al*.,2019).**

The receptor activator of nuclear factor Kb (RANK) and Receptor activator of nuclear factor Kb ligand (RANKL) were a protein that expressed on the surface of osteoblasts, which are bone-forming cells, By binding to its receptor, RANK, located on the surface of osteoclast precursor cells, it facilitates the differentiation of these cells into fully mature osteoclasts **(Hooshiar *et al*.,2022).** Osteoclasts are responsible for bone resorption, the process of breaking down bone tissue**(Carrillo-López *et al*., 2021)**, and differentiation of osteoclasts, and inhibits osteoclast apoptosis**(Yari *et al*., 2020).**

Extracellular Signal-Regulated Kinase (ERK) is a type of protein kinase that plays a crucial role in cell signaling pathways , also plays a crucial role in regulating bone cell function and maintaining bone homeostasis **(Xie *et al*.,2021).** ERK activation in osteoclasts promotes their differentiation and survival, thereby contributing to bone resorption, Imbalances in the ERK signaling pathway can lead to skeletal disorders, including osteoporosis**(Guo *et al*., 2020).**

Tartrate-resistant acid phosphatase (TRAP) is an enzyme primarily expressed in osteoclasts and is involved in the breakdown of the mineralized and organic

components of bone during bone resorption. Its activity is tightly regulated and serves as a valuable marker for assessing osteoclast function and bone metabolism**(Nakamura *et al*.,2021).**

Aim of study

The aim of the current study to investigated the effect of a high cholesterol diet on osteoporosis by some serum biomarkers including:

1.Studing of biochemical parameters including serum lipid profile.

2. Studying RANK-RANKL proteins.

2. Serum cellular signaling which include ERK(extracellular signal kinase),TRAP(tartrate resistance acid phosphate) in treated hypercholestermia and control rats.

3. Regulation markers like oxidant (MDA) and antioxidant(GSH) in treated hypercholestermia and control rats.

4. Study of gene expression of (osterix gene) in treated hypercholestermia and control rats.

5. Histopathological examination of femoral bone section in hypercholestermia and control rats.

6. X-ray examination in treated hypercholestermia and control rats.

`

Chapter Two

Literature Review

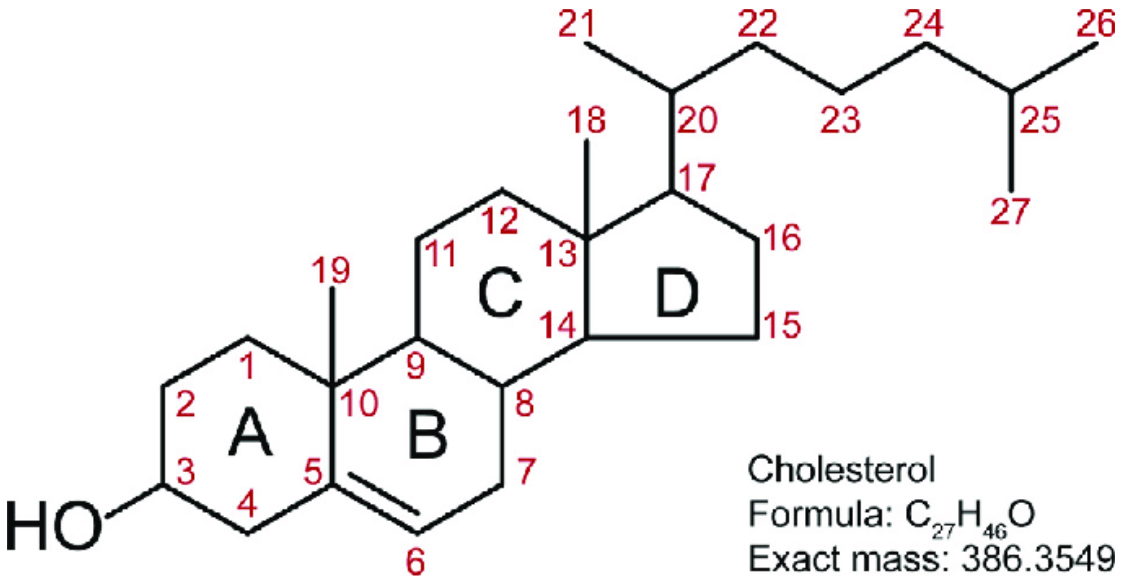
2.1.Cholesterol

Cholesterol is the primary sterol in mammals and plays an essential part in the plasma membrane by regulating fluidity, permeability, and signaling membranes **(Aguilar *et al*., 2021).** It is a lipid molecule with a waxy consistency present in every cell in animal body **(Lakhan *et al*., 2022).** It is produced in the bodies through a complex process that begins with acetyl coenzyme A (acetyl-CoA) and requires the participation of around 30 enzymatic processes **(Duan *et al*., 2022).** The composition of Cholesterol is four connected aromatic hydrophobic rings, a tiny hydrophilic hydroxyl group, and a hydrophobic chain. Mainly, it found inside cells as part of lipid membranes or attached to lipid-binding proteins due to its strong hydrophobicity **(Hofmaenner *et al*., 2022)**

Cholesterol was traveled in the blood within lipoproteins, which are composed of lipids and proteins.Three types of cholesterol: chylomicrons, VLDLs, and HDLs **(Cunha *et al*., 2021).** High levels of cholesterol can lead to osteoporosis and its byproducts influence bone balance by controlling the growth and activation of osteoblasts and osteoclasts **(Bao *et al*., 2023).**

2.1.1. Cholesterol structure:

Generally, cholesterol molecules contain three main parts: 1) a tetracyclic carbon ring (AeD) that is the core of steroids, 2) a polar hydroxyl group attached to ring A, and 3) a non-polar carbon chain attached to ring D. figure (2.1). Cholesterol is a planner molecule because its four rings form a trans conformation. As a result of the double bond between C5 and C6, cholesterol remains rigid **(Li *et al*., 2019)**



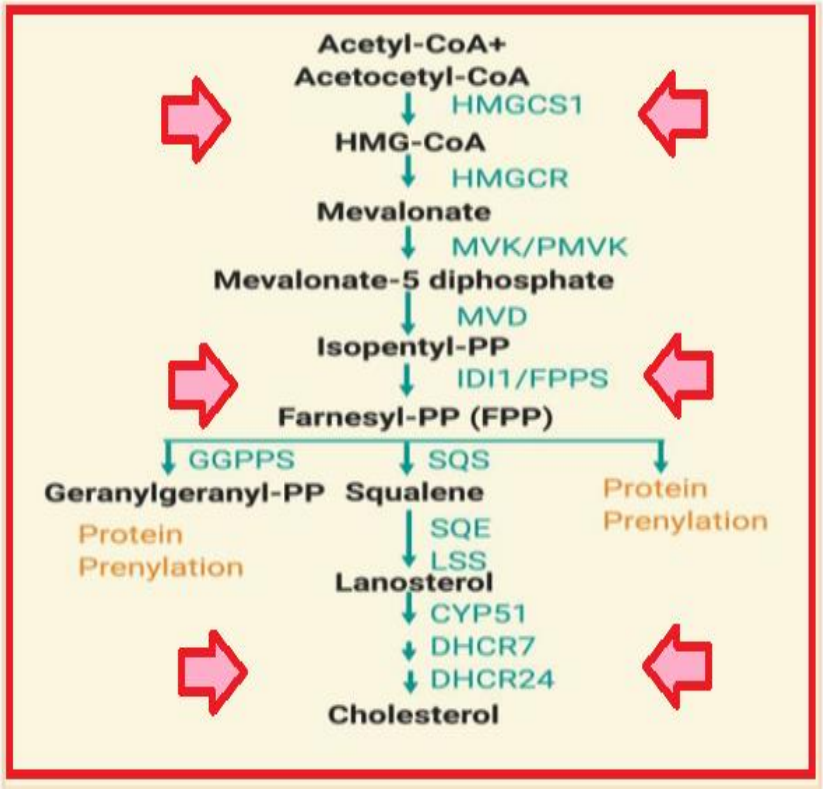
Figure(2.1): Cholesterol structure (Li *et al*., 2019)

2.1.2. Cholesterol Synthesis Pathway:

Cholesterol was synthesized in the cytosol, and the acetyl-CoA needed for cholesterol synthesis can be obtained from several sources, including fatty acid (FA)burning, ketogenic amino acid oxidation, and pyruvate dehydrogenase activation **(Gibbons., 2003).**Acetyl-CoA shuttled out of the mitochondria is in the form of citrate,

which is cleaved into acetyl-CoA and pyruvate by citrate lyase **(Bradshaw., 2021)**.

Although more than 80% of daily cholesterol synthesis occurs in the liver and intestines, there was a bone cells also synthesized cholesterol by mechanism (mevalonate pathway), this synthesis began with acetyl-CoA derived from glucose, glutamine, or acetate metabolism **(Kim *et al*., 2021).** (Figure 2.2)



Figure(2.2): Overview of the cholesterol synthesis pathway (Bradshaw, 2021).

2.1.3. Metabolism of cholesterol:

Cholesterol metabolism involves the breakdown, utilization, and elimination of cholesterol from the body **(Morgan *et al*.,2016).** It is primarily synthesized in the liver,

although other tissues such as the intestines and adrenal glands can also produce it, the synthesis of cholesterol starts with the conversion of acetyl-CoA, a product of glucose metabolism, into a compound called mevalonate **(Hashemi., 2017)**. This process is catalyzed by the enzyme3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMG-CoA), which is the target of cholesterol-lowering drugs called statins, Mevalonate is then converted into cholesterol through a series of enzymatic reactions **(Schade *et al*., 2020)**.

Once synthesized, cholesterol is either used by the cells for various functions or transported through the bloodstream in lipoprotein particles **(Soffientini& Graham.,2016)**. The two main types of lipoproteins involved in cholesterol transport are low-density lipoprotein (LDL) and high-density lipoprotein (HDL), LDL carries cholesterol from the liver to the cells, while HDL helps to remove excess cholesterol from tissues and transport it back to the liver for elimination **(Trajkovska & Topuzovska, 2017).**

Cholesterol can be taken up by cells through receptor-mediated endocytosis, where LDL particles bind to LDL receptors on the cell surface and are internalized into the cell, Inside the cell, it can be used for membrane synthesis, converted into steroid hormones (such as cortisol and testosterone), or stored as cholesterol esters **(Khosravi *et al*., 2018)**.

(Tall & Yvan-Charvet, 2015) was decided that excess cholesterol that is not used by the cells or eliminated is taken up by HDL particles, HDL particles remove cholesterol from tissues and transport it back to the liver in a process called reverse cholesterol transport, In the liver, cholesterol can be converted into bile acids, which are essential for the digestion and absorption of dietary fats. Bile acids are then secreted into the

intestine, where they aid in the digestion and absorption of dietary fats, including cholesterol. Some of the cholesterol is also eliminated in the feces **(Ticho *et al*., 2019).**

The disruptions in cholesterol metabolism can lead to various health conditions, including hypercholesterolemia which mean high blood cholesterol levels and atherosclerosis which mean the buildup of cholesterol plaques in arteries, these conditions are associated with an increased risk of cardiovascular disease, such as heart attacks and strokes **(Tall & Yvan-Charvet, 2015**).

2.2. Hypercholesterolaemia

Hypercholesterolaemia is a complex condition with multiple causes, including both lifestyle and genetic aspects, it is also a risk factor for cardiovascular diseases(CVDs), which are responsible for 172 million death every year **(Cunha *et al* .,2021)**. It can be classified as primary when the lipid disorder has genetic influence, or secondary, caused by inadequate diet and life style, as well as medications **(Cardiologia *et al*., 2017).**

Observed also that animals fed with a high cholesterol diet developed weight loss, even without significant difference in energy consumption when compared to the control group animal **(Santos-López *et al*., 2017).** Hypercholesterolemia induced by diet also affects the animal's liver, increasing its weight and causing damage, these alterations are probably caused by the higher fat content in the liver **(Tuzcu *et al*., 2017; Cunha *et al*., 2021)**.

The induction of hypercholesterolaemia additionally decreases the activity of some superoxide dismutase (SOD) and catalase (CAT) (important antioxidant enzymes) in the liver, which also decreases the antioxidant defenses **(Harrabi *et al*., 2017).**

2.3. Bone homeostasis

Bone is a rigid organ but plays important roles in animal bodies, it provides mechanical support for the soft tissues and enables mobility, it is also a mineral reservoir to endorse the calcium and phosphate homeostasis in body fluids**(Szekanecz *et al* .,2019)**. As an essential component of the skeleton, bone

tissue provides solid support for the body and protects vital organs **(Wawrzyniak& Balawender,2022)**. Bone tissue is a type of connective tissue characterized by strength and structural support, as external forces are constantly changing, bone maintains its supportive properties by continuous turnover, although this rate is influenced by both genetic and environmental factors, In this regard, bone is

inherently dynamic in nature, as it is being degraded and regenerated **(Su et al., 2019)**.This process occurs through primary bone cells represented by bone-forming osteoblasts, bone-resorbing osteoclasts, and terminally differentiated mechano sensing osteocytes **(Akhmetshina *et al*., 2023)**

2.4. Bone tissue cells:

Bone metabolism is controlled by a variety of environmental signals, the bone cellular compartment responds to these signals by modulating the balance between new bone formation and older bone resorption **(Wawrzyniak& Balawender 2022)**.

The three types of bone cells are primarily related to bone homeostasis, these are osteoblasts, osteocytes, and osteoclasts**(Šromová *et al*., 2023).** These cell types are derived from two separate stem cell lineages, the first is the mesenchymal lineage, and the second is the hematopoietic lineage, It emphasizes the interaction between the immune system and bone and the unique regulation of bone homeostasis **(Han *et al*., 2018)**.

2.4.1. Osteoblast cells

Osteoblasts are derived from precursor cells that can also be stimulated to become muscle, fat, or cartilage ,Osteoblasts have round vesicular nuclei and basophilic cytoplasm, rich in rough endoplasmic reticulum. They are responsible for the

production of a unique collagen-rich material called osteoid, the organic part of the bone matrix **(Molagoda *et al*., 2022).**

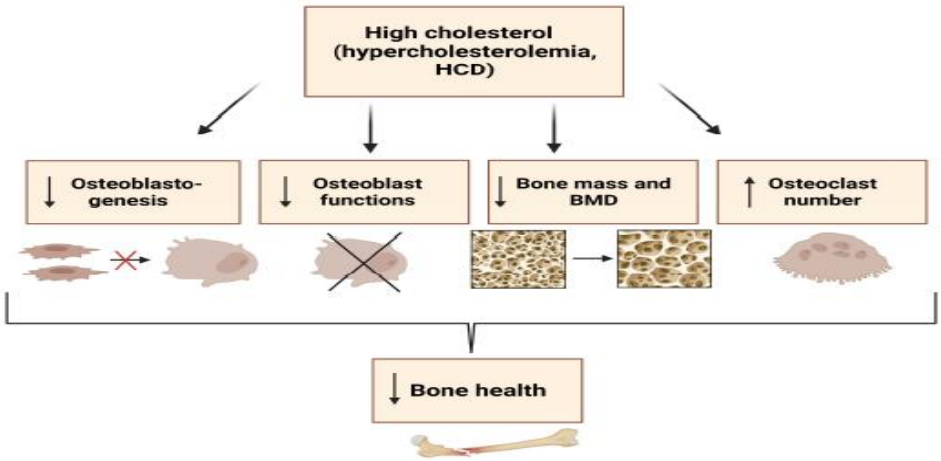
Osteoblasts do not produce an extracellular matrix, they take the form of flattened cells and are spindle-shaped, their nuclei are elongated and adhere closely to the bone surface **(Vancea *et al*., 2021).**only this cells that can give rise to bones in vertebrates. Thus, one of the most important functions of these metabolically active cells is mineralized matrix production **(Mizoguchi *et al*., 2021).**

In normal conditions, these cells can transform or become differentiated to form new bone, After the synthesis of the extracellular matrix is complete, the part of the osteoblasts that has been surrounded by it becomes osteocytes, By providing osteoblasts with the ability to form a calcium- and phosphorus-rich matrix, they guarantee adequate bone hardness and allow them to function properly **(Wawrzyniak & Balawender,2022).**

On the surface of osteoblasts, there is a RANKL glycoprotein that can bind to the RANK glycoprotein on the surface of the osteoclast precursor. This is a way to directly contact these cells and stimulate osteoclast differentiation Osteoblasts secrete proteins that initiate and regulate bone mineralization: osteonectin , osteocalcin , and hydrolases **(Si *et al*., 2020).**They also secrete a protein, osteoprotegerin, which binds to RANKL and prevents osteoblast–osteoclast contact, This inhibits osteoclast precursor differentiation, stabilizes bone, and regulates bone modeling **(Udagawa *et al*., 2021)**. In addition, it has an inhibitory effect on blood vessel calcification, Osteoblast activity is regulated by parathormone, thyroid hormones, growth hormone, vitamin D3, cytokines, growth and differentiation factors, and prostaglandins, adrenal corticosteroids inhibit the activity of these cells **(Si *et al*., 2020).**

2.4.1.1. Role of Cholesterol in Osteoblast

A high cholesterol diet (HCD) and hypercholesterolemia have negative effects on bone health, They manifest as reduced proliferation and differentiation of bone marrow stromal cells, which in turn results in decreased osteoblastogenesis, Such a hyperlipidemic condition affects several aspects of osteoblast function and homeostasis, increases the number and activity of osteoclasts, and decreases bone mass and bone mineral density **(Akhmetshina *et al*., 2023).** Figure(2.3)



Figure(2.3): Summary of the effects of cholesterol on osteoblasts (Akhmetshina *et al*., 2023)

2.4.2. Osteoclast cells

Osteoclasts are large multinucleated cells from hematopoietic origin and are responsible for bone resorption,A balance between osteoclastic bone resorption and osteoblastic bone formation is critical to maintain bone homeostasis **(Omi & Mishina, 2022)**. In the bones the only cells that can resorb bone, play a central role in bone homeostasis as well as bone damage under pathological conditions such as osteoporosis, arthritis, periodontitis, and bone metastasis **(Tsukasaki& Takayanagi,2022)**.

2.4.2.1. Role of Cholesterol in Osteoclasts

Cholesterol is an important component of the cell membrane. It contributes to the structural makeup of the membrane as well as modulates its fluidity **(Huff *et al*., 2017)**. It is constitute a significant portion of lipid rafts, which are membrane signal transducing platforms and play crucial roles in RANK-RANKL signal transduction

during osteoclastogenesis **(Lee *et al*., 2015).** In addition, phosphoinositide, a membrane lipid, regulates calcium signaling and osteoclast differentiation **(Kim *et al*., 2021)**.

However, excess accumulation of cholesterol is highly deleterious to cells and underlies the pathogenesis of a number of metabolic diseases, High cholesterol levels also increase bone turnover, high fat diets in mice promoted osteoclastogenesis, which was followed by a decrease in bone mass **(Kim *et al*., 2021).**

2.5.Osteoporosis

Is a metabolic bone disease that affects hundreds of millions of people worldwide and is characterized by excessive loss of bone protein and mineral content, The incidence and mortality of osteoporosis increase with age, creating a significant medical and economic burden globally **(Bao *et al*., 2023).**

It is characterized by decreased bone mass, microarchitectural deterioration and increased bone fragility as well as the most prevalent chronic metabolic bone disease, subsequent increases in the risk of fracture **(Arandjelovic *et al*., 2021; Yu, *et al* 2021 and Song *et al* 2023)**. Loss results from an imbalance in bone remodeling **(Bashiruddin., 2020; Li., 2020).**

Abnormal bone metabolism and resorption are influenced by many factors, such as genetics, hormones, age, and nutrition, among which dietary factors play an important role **(Feng *et al*., 2021; Song *et al,* 2022).**

A high-fat diet has become a common dietary pattern worldwide **(Chevalier, 2020 and Pan *et al*., 2021)**.

2.5.1.Pathophysiology of Osteoporosis

The traditional pathophysiological models of osteoporosis are based on endocrine mechanisms, for examples are secondary hyperparathyroidism in the elderly due to vitamin D deficiency.In reality, osteoporosis is a multifactorial disease caused by a

complex interplay of genetic, intrinsic, exogenous, and lifestyle factors **(Chin *et al*., 2022).**

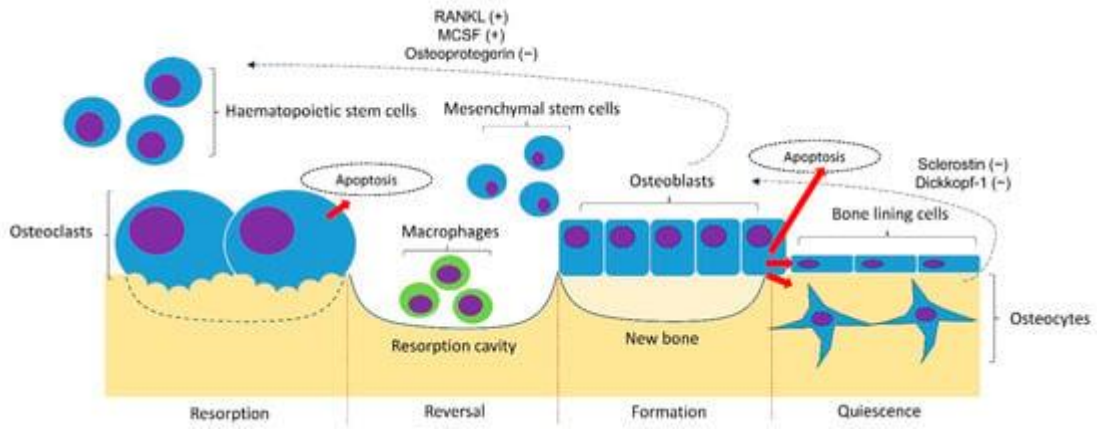
Osteoclasts, osteoblasts, and osteocytes are the three main players in bone remodeling, when bone damage occurs, the macrophage polykaryon-derived

osteoclasts migrate to the damage site and perform bone resorption **(Ma *et al*., 2021).** At the end of bone resorption, osteoclasts undergo apoptosis and produce

apoptotic bodies that may play a role in the subsequent osteogenesis **(Ma *et al*., 2021 and Chin *et al*., 2022).**

After the reversal phase, the mesenchymal stem cell-derived osteoblasts will migrate to the cavity and perform bone formation **(Xiao *et al*., 2016)**

Some osteoblasts will be embedded in the bone matrix they synthesise and differentiate into osteocytes, Osteocytes act as a mechanosensor and play regulatory roles in regulating the bone remodelling process through signalling proteins and via perilacunar remodelling directly **(Prideaux *et al*., 2016).**Figure(**2.4**)



Figure(2.4): Bone remodeling cycle **(Prideaux *et al*., 2016).**

2.6.RANK and RANKL Pathway:

The RANKL/RANK pathway was first known in the mid-1990s as a substantial regulator of bone homeostasis **(Infante., *et al* 2019).**

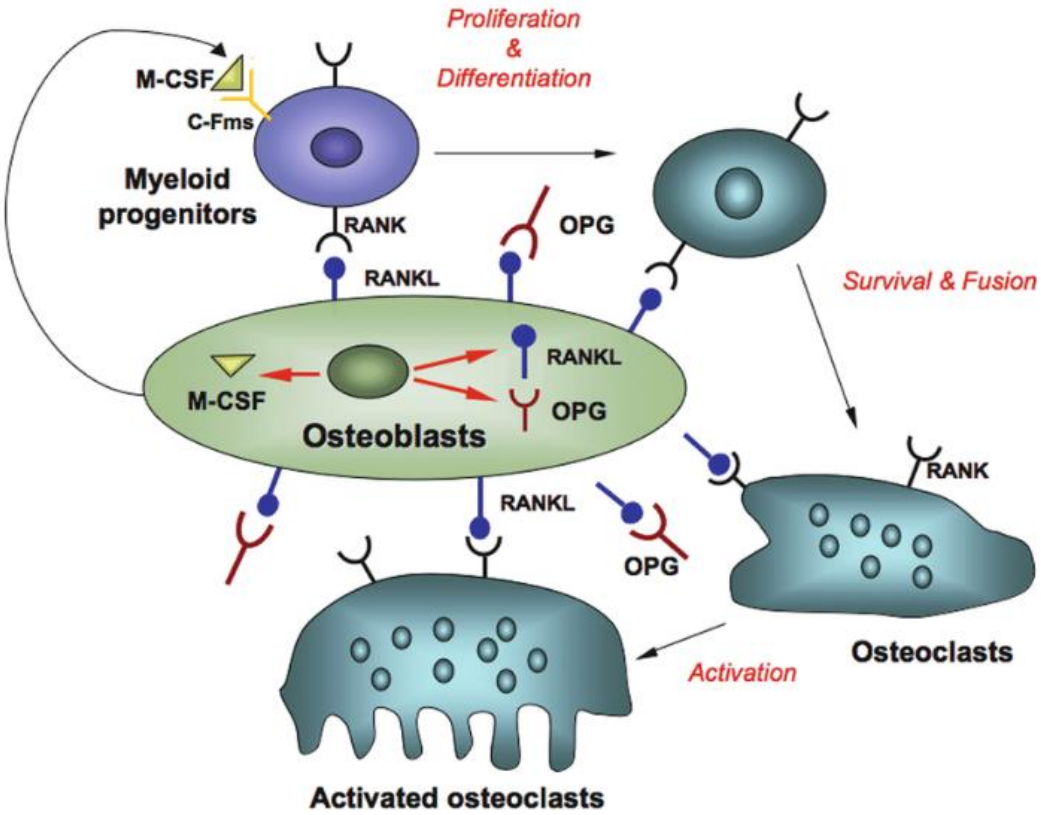
RANK means the receptor activator of nuclear factor *κ*B, and RANKL means receptor activator of nuclear factor *κ*B ligand **(Hooshiar *et al*., 2022),** RANKL, as a pivotal regulator of osteoclastogenesis, is expressed in various cells, such as osteoblasts, osteocytes, preosteoblasts, periosteal cells, dendritic cells, and vascular

cells **(Carrillo-López *et al*., 2021).** It is a ligand for RANK on the surface of osteoclasts **(Yari *et al*., 2020)**

RANKL binds to its receptor RANK, allowing the activation, survival **(Carrillo-López *et al* .,2021)** and differentiation of osteoclasts, and inhibits osteoclast apoptosis **(Yari *et al*., 2020).**

Osteoclast maturation occurs when RANK is activated by RANKL in the osteoclast**,** the osteoclast differentiation, formation, and activation are suppressed by the inhibition of the RANKL/RANK pathway, as well as the bone loss and osteoclastogenesis are promoted and increased by RANKL **(Hooshiar *et al*., 2022).** It is a key modulator of osteoclastogenesis, and thus, mice lacking RANKL exhibited osteoporosis because of osteoclast deficiency, Maturation of osteoclasts happens when RANK is activated in the osteoclasts by RANKL that was produced by osteoblast **(Herwana *et al* , 2020).**

Mature osteoclast adheres to the bone surface and promotes bone resorption by secretion of acid and lytic enzymes (e.g., cathepsin K and tartrate-resistant acid phosphatase) **(Liu and Zhang., 2015)**



Figure(2.5):RANK and RANKEL cascade pathway(Infante *et al*., 2019)

2.7.Osteoblast-Osteoclast signaling pathways

2.7.1. Role of osteoblasts and osteoclasts in bone remodeling:

Osteoclasts and osteoblasts are interconnected through a process known as coupling. Osteoclasts create resorption cavities, and osteoblasts subsequently fill these cavities with new bone tissue, ensuring that bone formation follows resorption, Osteoclasts and osteoblasts are interconnected through a process known as coupling, which ensures the coordination between bone resorption by osteoclasts and bone formation by

osteoblasts. This coupling mechanism is crucial for maintaining bone homeostasis and proper skeletal remodeling **(Sims& Martin, 2020).**

2.7.1.1. Activation of Osteoclasts

Osteoclasts are activated in response to various signals, including the binding of receptor activator of nuclear factor kappa-B ligand (RANKL) to its receptor on osteoclast precursor cells. RANKL is produced by osteoblasts and other cells in the bone microenvironment. When RANKL binds to its receptor, it stimulates the differentiation and activation of osteoclast precursors, leading to the formation of mature osteoclasts**(Yasuda ., 2021).**

2.7.1.2. Bone Restoration and Signaling to Osteoblasts

Once activated, osteoclasts attach to the bone surface and initiate the resorption process, they create a sealed microenvironment called the resorption lacuna, which is acidic and conducive to bone degradation. Osteoclasts secrete acid and enzymes, such as cathepsin K, that dissolve the mineralized bone matrix, while proteolytic enzymes degrade the organic components**(Fu & Shi., 2020).**

During bone resorption, osteoclasts release various factors stored in the bone matrix, including growth factors and cytokines. One of the important factors released is transforming growth factor-beta (TGF-β). TGF-β is involved in signaling to nearby osteoblasts, triggering a response that leads to bone formation**(Sims & Martin., 2020).**

2.7.1.3. Recruitment of Osteoblasts and Bone formation:

The factors released by resorbing osteoclasts, particularly TGF-β, stimulate nearby osteoblasts. Osteoblasts are attracted to the resorption sites, where they receive signals from osteoclasts and the degraded bone matrix. This recruitment of osteoblasts to the resorption lacuna is an essential step in the coupling process**(Jann *et al*., 2020).**

Once recruited, osteoblasts begin synthesizing and depositing new bone matrix,they secrete collagen fibers and other proteins, which form the organic framework for bone mineralization. Osteoblasts also regulate the mineralization process, ensuring the proper deposition of calcium and phosphate ions to form hydroxyapatite crystals**(Lin *et al*., 2020).**

2.7.1.4. Completion of Coupling Cycle:

The process of coupling is cyclic and continuous. As osteoblasts deposit new bone matrix, they gradually fill the resorption lacuna created by osteoclasts,t his leads to the completion of the coupling cycle, where bone resorption is followed by bone formation in the same localized area. This coupling mechanism helps maintain the structural integrity and strength of the bone tissue **(McDonald *et al*., 2021).**

2.8. Hormonal Regulation of Osteoblast and Osteoclast Function:

2.8.1. Endocrine Control of Bone Metabolism:

Hormonal regulation plays a crucial role in controlling the function of osteoblasts and osteoclasts, thereby influencing bone metabolism. Several hormones, produced by various glands and tissues, interact with these bone cells to maintain bone

homeostasis **(Zhou *et al*., 2021).** the insights into the endocrine control of bone metabolism which include the following:

2.8.2. Parathyroid Hormone (PTH)

PTH is a key regulator of calcium and phosphate homeostasis. When blood calcium levels decrease, the parathyroid glands secrete PTH, which exerts effects on both

osteoblasts and osteoclasts through Osteoblasts activation by PTH stimulates osteoblasts to produce and release factors such as receptor activator of nuclear factor kappa-B ligand (RANKL), which promotes osteoclast formation and activation and Osteoclasts activation by PTH enhances osteoclast activity, leading to increased bone resorption and the release of calcium and phosphate into the bloodstream **(Shaker & Deftos.,2023)**

2.8.3. Calcitonin

Calcitonin, produced by the thyroid gland, acts as a counterbalance to PTH by inhibiting osteoclast activity and promoting bone formation. It helps regulate calcium levels by Osteoclasts inhibition through directly inhibits osteoclast function and reducing bone resorption as well as Osteoblasts deposition by stimulates osteoblasts to deposit new bone matrix and decrease calcium release from bone **(Xie *et al.*,2020).**

2.8.4.Interplay of Osteoblasts, Osteoclasts, and Vitamin D: Insights into Skeletal Development and Bone Metabolism

Vitamin D, a crucial hormone, plays a regulatory role in bone metabolism and influences the activities of both cell types by during embryonic and postnatal skeletal development, osteoblasts and osteoclasts work together to shape and remodel bone tissue, Osteoblasts secrete collagen and other proteins, forming the organic matrix that provides the structure of bone. This matrix is then mineralized through the deposition of calcium and phosphate ions, facilitated by osteoblasts. Osteoclasts, on the other hand, remove excess or improperly formed bone through bone resorption.

This process allows for the precise modeling and shaping of bones, ensuring proper skeletal development **(Weivoda *et al*.,2020)**

2.8.4.1. Role of vitamin D with osteoblast:

Osteoblasts are responsible for synthesizing and depositing new bone tissue. They express receptors for vitamin D, enabling them to respond to its active form, calcitriol.Calcitriol stimulates osteoblast differentiation and activity, leading to increased bone formation. Osteoblasts also produce osteocalcin, a protein involved in bone mineralization. Furthermore, vitamin D regulates the expression of genes associated with osteoblast function, ensuring optimal bone formation **(Katica& Tepekoy, 2020).**

2.8.4.2. Role of vitamin D with osteoclast:

Vitamin D indirectly influences osteoclast activity by regulating the production of two important factors: receptor activator of nuclear factor kappa-B ligand (RANKL) and osteoprotegerin (OPG). RANKL promotes osteoclast formation and activation, while OPG acts as a decoy receptor for RANKL, inhibiting osteoclast function. Vitamin D enhances the production of OPG, helping to control osteoclast activity and prevent excessive bone resorption**(Khalaf & Almudhi,2022).**

2.9. TRAP and ERK Signaling in Skeletal Development and Bone Metabolism

2.9.1. Tartrate-Resistant Acid Phosphatase (TRAP):

Is an enzyme that plays a role in bone metabolism. It is predominantly produced by osteoclasts, the cells responsible for bone resorption, TRAP is involved in the breakdown of bone tissue by promoting the acidification of the resorption lacunae, allowing for the dissolution of hydroxyapatite, the mineral component of bone **(Mira-Pascual, 2019).**

In the context of osteoporosis, TRAP is often used as a marker of osteoclast activity, Increased levels of TRAP in the blood or urine can indicate higher osteoclast activity and increased bone resorption, which are characteristic features of osteoporosis,

Measuring TRAP levels can be helpful in diagnosing and monitoring the progression of the disease **(Kuo& Chen,2017)**.

2.9.2. Extracellular signal-regulated kinase (ERK):

is a member of the mitogen-activated protein kinase (MAPK) family, and it plays a significant role in various cellular processes, including cell proliferation, differentiation, and survival**(Park.,2023).**ERK phosphorylates a wide range of substrates, including transcription factors, kinases, cytoskeletal proteins, and other signaling molecules, By phosphorylating these targets, ERK modulates their activity and influences various cellular processes **(Guo *et al*., 2020).** ERFK plays a role in Cell Proliferation and Differentiation; signaling is critically involved in cell proliferation and differentiation processes, Activation of ERK promotes cell cycle progression by inducing the expression of genes involved in cell cycle regulation and DNA synthesis, ERK also plays a role in promoting cell differentiation by activating specific transcription factors that drive cellular differentiation programs **(Martin-Vega& Cobb,2023).**

2.10.Monitoring of gene expression on for determine bone osteoporosis

2.10.1.Osterix gene expression

Osterix (Osx) was first discovered by**(Nakashima *et al*., 2002)**, Osx, also known as Sp7, is a zinc finger-containing osteoblast-specific transcription factor belonging to the SP/KLF family (**Suske *et al*., 2005**)

It is play role in embryonic skeletal development, osterix continues to be expressed in mature osteoblasts and is required for their function in bone remodeling and repair. It regulates the expression of various genes involved in osteoblast maturation and mineralization, thus contributing to the maintenance of bone homeostasis**(Liu *et al*.,2020).** Osx is expressed in osteoblast-lineage cells, chondrocytes and also over

expressed in various cancer tissues **(Qu *et al*., 2019).** It is specifically expressed in osteoblasts

and osteocytes and, albeit at lower levels, in prehypertrophic and hypertrophic chondrocytes, while not expressed in osteoclasts **(Xing *et al*., 2019)**

Osterix has long been known to be an essential transcription factor for the osteoblast differentiation and bone mineralization. Emerging evidence suggests that Osx not only plays an important role in intramembranous bone formation, but also affects endochondral ossification by participating in the terminal cartilage differentiation, its essentiality in skeletal development and bone formation **(Liu *et al*., 2020),** Osx has become a new research hotspot in recent years 8 **(Liu *et al*., 2020).**

2.10.1.1.Interplay of Osterix with TRAP and ERK Signaling

Osterix, a key transcription factor involved in osteoblast differentiation and maturation, interacts with various signaling pathways, including TRAP (Tartrate-Resistant Acid Phosphatase) and ERK (Extracellular Signal-Regulated Kinase) signaling. These interactions play important roles in osteoblast function and bone remodeling:

2.10.1.1.1.TRAP Signaling:

TRAP is an enzyme produced by osteoclasts, which are responsible for bone resorption. While TRAP is primarily associated with osteoclasts, recent research has revealed its involvement in osteoblast function as well **(Hendrijantini *et al*.,2021)**.

2.10.1.1.2.Osteoclast-Osteoblast AND TRAP Crosstalk:

Osteoclasts and osteoblasts communicate through secreted factors and signaling molecules, establishing a dynamic crosstalk between bone resorption and formation. TRAP, produced by osteoclasts, can influence osteoblast differentiation and activity.

Regulation of Osterix Expression: Studies have shown that TRAP can modulate the expression of Osterix in osteoblasts. Increased levels of TRAP have been associated

with decreased Osterix expression. This suggests that TRAP signaling may play a role in regulating osteoblast differentiation and maturation by influencing Osterix levels**(Kim *et al*.,2020)**

2.10.1.1.3. ERK Signaling:

ERK is a member of the mitogen-activated protein kinase (MAPK) signaling pathway, which regulates various cellular processes, including cell proliferation, differentiation, and survival, ERK signaling has been implicated in osteoblast differentiation and function, and it can also influence Osterix activity**(Sun *et al*.,2021)**.Regulation of Osterix Expression: ERK signaling can modulate the expression of Osterix during osteoblast differentiation. Activation of the ERK pathway has been shown to enhance Osterix expression, promoting osteoblast differentiation and maturation, and Osterix Phosphorylation: ERK signaling can also phosphorylate Osterix, which can impact its activity and function. Phosphorylation can affect Osterix's ability to bind to target DNA sequences and regulate gene expression. The precise effects of Osterix phosphorylation on osteoblast function are still being investigated**(Wu *et al*., 2023)**.

Chapter Three

Methodology

3. Methodology

3.1. Materials

3.1.1. Instruments and Equipment:

All the devices utilized as a part of this study are summarized in table (3.1)

Table 3.1. Apparatus and equipment with their manufactures.

No.	Apparatus & Equipment	Company	Manufactures
1.	Anatomicalset(Scissors, Forceps, Scalpel)	Chemo lab	China
2.	Balance	Denver	Germany
3.	Beakers (100, 250, 500, 1000)	Chemo lab	India
4.	Centrifuge	Hettich	Germany
5.	Colony flask	Chemo lab	India
6.	Cotton	India	Entrepreneur
7.	Digital balance	Denver	Germany
8.	Digital camera	Canon	China
9.	ELIZA printer	Epson	Japan
10.	ELIZA reader	Biotek	USA
11.	Eppendorf's tubes	Chemo lab	India
12.	Filter paper	Chemo lab	India
13.	Gel tube	Chemo lab	India
14.	Incubator	Lab tech	Korea
15.	Insulin syringe	eldawlia	Egypt
16.	Light Microscope	Olympus	Japan
17.	Micropipettes(different volumes)	dragonmed	China
18.	Microscope with camera	Olympus	Japan
19.	Microtome	Leica RM	USA
20.	Pipette tips (10 – 1000) µl volume	Chemo lab	China

21.	Refrigerator	Denka	Japan
22.	Sensitive balance	Sartorius	Germany
23.	Slide & cover slip	Chemo lab	China
24.	Spectrophotometer	EMCLAB	Germany
25.	Surgical gloves	Chemo lab	China
26.	DXA machine	Hologic	USA
27.	Syringe (1 ml, 5 ml)	Chemo lab	China
28.	Test tubes	Chemo lab	China
29.	Vortex	Sturat	United
30.	Water bath	labtech	Korea

3.1.2.Chemicals and Kits

All the chemicals and the standard kits used in this study are shown in **table (3.2)**

Table 3.2: Chemicals and Kits with their suppliers.

No.	Chemicals & Kits	Company	Suppliers
1.	Cholesterol powder	Avonchen	UK
2.	Normal saline		
3.	Eosin Stain	Himedia Lab	India
4.	Formalin 37 %	Chemanol	SA
5.	Hematoxylin Stain	Himedia Lab	India
6.	Xylene	Alph chemika	India
7.	Glutathione(GSH) kit	laboratorio ct	Italy
8.	Malondialdehyde (MDA) kit	Elabscience	USA
9.	Lipid profile kit	Biolabo	Italy
10.	Rat RANk (Receptor Activator Of Nuclear Factor Kappa B)	ELK bioticnology	China

11.	Rat sRANKL(SolubleReceptor Activator of Nuclear factor-kB Ligand)	ELK bioticnology	China
12.	ERK(extracellular regulated kinase)	ELK bioticnology	China
13.	TRACP(tartarate resistance acid phosphate)	ELK bioticnology	China
14.	Dimthylsulphoxid DMSO	LOBA	China

3.2. Examination methods

3.2.1. Experimental protocol

Twenty white male rats weight (100g±75g) were used in this study and came from the College of Pharmacy at the University of Kerbala in Iraq. They ranged in age from (4–6) weeks; and the animals were housed in clean, specialized plastic enclosures, to start experimental design the rats were placed in clean box compartment. We utilized a 12-hour light cycle and a relative humidity of 50±5%. The subjects were retained for two weeks so that they could adjust to the usual testing conditions. The experiment began on 25th September and ended on 23rd November. In this experiment, the temperature of the room was maintained at (23-26) C° by using a room thermostat. Continuous ventilation vacuum was used to keep the air in the room changing continuously, and the animal was fed on pellets of fresh ration prepared from soybeans and corn.

3.2.2. Experimental Design

Two groups of twenty white male rats were randomly split and given the following treatments (8 weeks). One half of this group of rats was fed a regular meal orally as the control group, and the other half was fed a cholesterol-rich meal for eight weeks, comprising 2% cholesterol (w/w)**(Cunha *et al*.,2021 & Sawale *et al* .,2016)**.

3.3.Ethical approve

The study was conducted at the anatomical facility of the College of Veterinary Medicine at Kerbala University in Iraq, under the reference number (UOK.VET.PH.2023.076).

3.4. Feed intake & dose:

The body Feed intake of all rats was recorded in order to determine the correct dose to be given to each animal**.**

3.5. Collect of the blood samples:

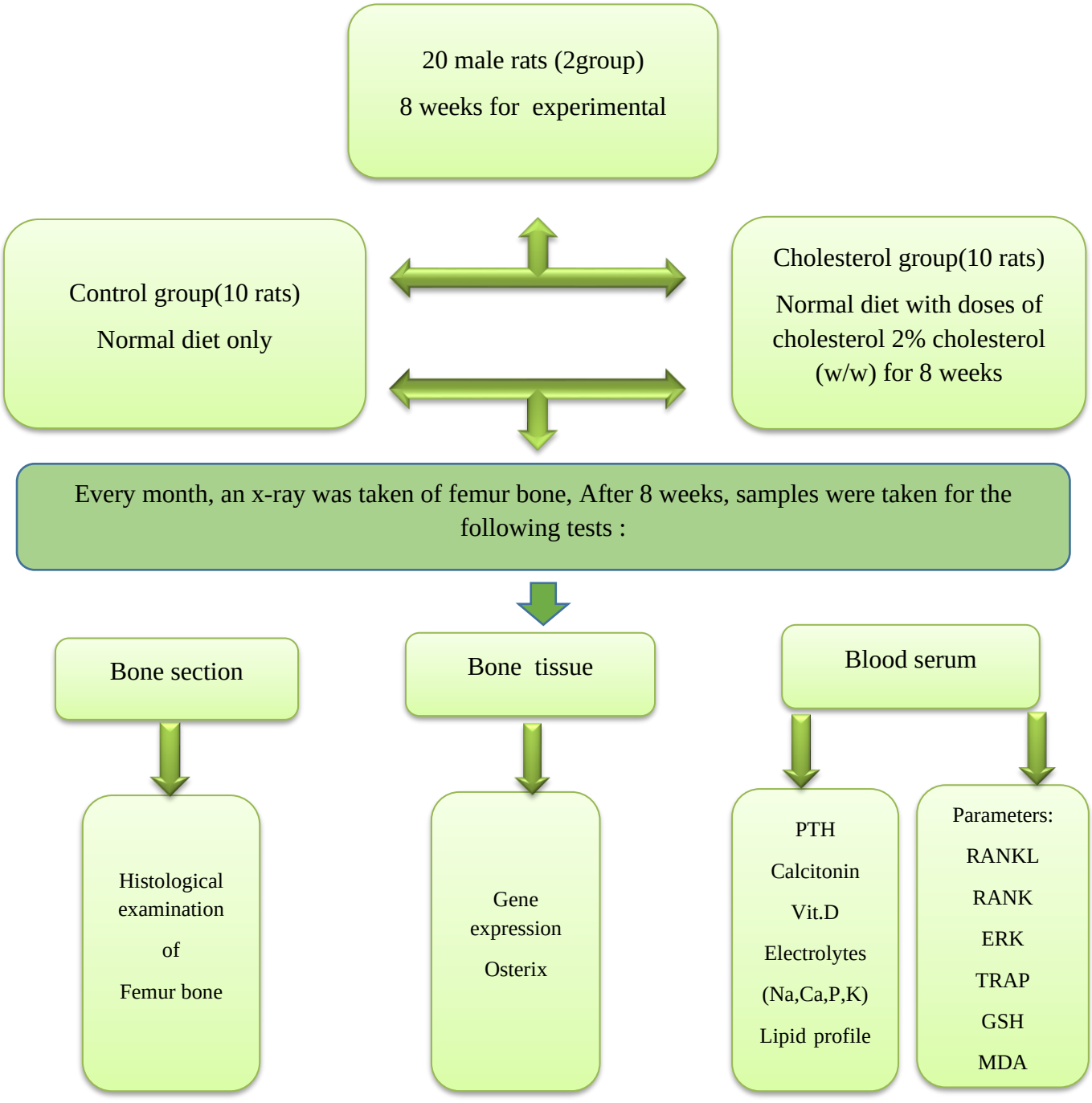
During the trial, blood samples were obtained after 8 weeks of the trial, with the animals being controlled and comfortable with ketamine and xylazine before the blood was taken. The serum was centrifuged for 5 minutes at 4000 revolutions per minute in a special gel tube after being extracted from the heart by the heart puncture method. As soon as the serum had been separated, it was placed in Eppendrof tubes for storage and kept in the fridge (-30 °C).

3.6. Bone tissues Collection:

Rats were sacrificed by chloroform anesthesia after completion of the experiment, and the animals were dissected for brain sampling. After taking bone samples, we conducted two tests on them, first examination that takes 50mg from bone tissue for gene expression and histopathological examination as shown below:

3.6.1.Osterix gene expression (Osx): TRIzolTM Reagent was used to preserve the bone tissue until it could be extracted and studied in a lab.

3.6.2.Histopathological examination: The bone tissue they preserved in 10% formalin in sterile plastic containers for the histological examination.



Figure(3.1) Experimental Design

3.7. Determination of serum Lipid profile:

3.7.1. Determination of serum total cholesterol (TC):

Total Cholesterol (TC) was detected by the use of Enzymatic method described by **(Allain *et al*., 1974)**, as illustrated in **appendixes I.**

3.7.2. Determination of serum Triglycerides (TG):

Total Serum TG was detected by the use of Fossati and Prencipe method associated with Trinder reaction. Reaction scheme **(Fossati & Prencipe, 1982),** as illustrated in **appendixes II**.

3.7.3. Determination of serum low-density lipoprotein (LDL):

Serum low density lipoprotein-cholesterol concentration was measured depending on equation **(Friedewald *et al*., 1972)**, as illustrated in **appendixes III.**

3.7.4. Determination of serum high-density lipoprotein (HDL):

Serum HDL-C concentration was measured enzymatically by utilizing HDL-C kit , according to **(Naito and Kaplan, 1984)**, as illustrated in **appendixes IV.**

3.8. Determination of serum Biomarker of Bone:

3.8.1. Determination of serum RANK:

Serum RANK was detected by the use of a special Elisa kit depending on the source**(Valverde *et al*., 2020)** as as illustrated in **appendix V.**

3.8.2. Determination of serum RANKL:

Serum RANKL was detected by the use of a special Elisa kit depending on the source **(Findlay & Atkins.,2011)**as illustrated in **appendix VI.**

3.8.3. Determination of serum extra cellular regulates kinas (ERK):

Serum extra cellular regulate was detected by the use of a special Elisa kit depending on the source**(Leevers & Marshall.,1992)** as illustrated in **appendix VII.**

3.8.4. Determination of serum tartrate-resistant acid phosphatase (TRAP):

Serum tartrate-resistant acid phosphatase (TRAP) was detected by the use of a special Elisa depending on the source**(Miyazaki *et al.*, 2003)** kit as illustrated in **appendixVIII.**

3.9. Determination of serum Hormones & vitamin D that effect on bone:

3.9.1. Determination of serum Calcitonin:

Serum Calcitonin was detected by the use of a special Elisa kit depending on the source**(Weissel *et al*.,1991)** as illustrated in **appendix IX.**

3.9.2. Determination of serum parathyroid hormone (PTH):

Serum parathyroid was detected by the use of a special Elisa kit depending on the source**(Aloia *et al*., 2006)** as illustrated in **appendix X**.

3.9.3. Determination of serum Vit D:

Serum Vit D was detected by the use of a special Elisa kit depending on the source **(Zerwekh., 2008)** as illustrated in **appendix XI**.

3.10. Determination of serum Electrolyte:

3.10.1. Determination of serum Calcium (Ca):

Serum Calcium was detected by the use of a special kit depending on the source**(Robertson *et al.*, 1979)** as illustrated in **appendix(XII)**

3.10.2. Determination of serum phosphorus (P):

Serum phosphorus was detected by the use of a special kit depending on the source **(Drewes ., 1972)** as illustrated in **appendix(XIII)**

3.10.3. Determination of serum sodium (Na):

Serum sodium was detected by the use of a special kit depending on the source**(Berry *et al.*,1988)** as illustrated in **appendix(XVI)**

3.10.4. Determination of serum potassium (K):

Serum sodium was detected by the use of a special kit depending on the source**(Wong *et al*., 1985)** as i illustrated in **appendix(XVII)**

3.11. Evaluation of Biochemical Parameters:

3.11.1. Determination of Serum Malondialdehyde (MDA) concentration (µ mol /L):

Malondialdehyde was estimated by Thiobarbituric acid (TBA) assay method of **(Buege & Aust, 1978)** on spectrophotometer, as illustrated in **appendixes(XVIII)**

3.11.2. Determination of serum Reduced Glutathione (GSH) concentration:

Reduced glutathione was measured following the method **of (Nabi & Nabi .,2014).** as illustrated in **appendixes(XIX)**

3.12. Determination of Gene expression:

Animals were anesthetized and dissected so samples could be obtained (the head of the femur bone). TRIzolTM Reagent/thermofisher scientific was used to preserve the organs in clean dark containers. The first strand of cDNA is synthesized using a Roche or Promega first strand cDNA synthesis kit. The primer mixture consists of an anchored oligo(dT)18 primer and a random hexamer primer. Delivery the extracted total RNA were treated with DNase enzyme to remove the trace amounts of genomic DNA from the eluted total RNA by using samples (DNase enzyme) and done according to the method described by promega company, USA instructions as follow according to **(You et al.,2011),** Following synthesis of cDNA, gene specific primers can be used to determine the level of gene (Bioneer /Korea), 20µl reactions Prepare GoTaqR 1-Step RT-qPCR Reaction Mix as a single batch that includes the common components, such as Go TaqR 1-Step RT-qPCR Master Mix, CXR dye, nuclease-free water and Go Script RT Mix. Divide the batch into individual volumes then add the remaining components; quantify the mRNA expression level of Osterix bone resorption cytokines, the relative amounts of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA were analyzed. RNA extraction, cDNA synthesis, and real-time PCR were performed as described by **(Yang *et al.*, 2012).** This study used primer sequences as shown in Table(3.3).

Table (3.3): Nucleotides sequences for OSX gene and House keeping gene

Gene of interest		Primers	PCR
OSX	F	5-GGAAAGGAGGCACAAAGAAGC-3	100 bp
	R	5-CCCCTTAGGCACTAGGAGC	
Housekeeping gene		**Primers**	
GADPH	F	5-AGCCCAAGATGCCCTTCAGT-3	88 bp
	R	5-CCGTGTTCCTACCCCCAATG-3	

3.13.Histopathological study

After sterilizing the rats with anesthesia, samples (femur bone) were removed during dissection. The organs were stored in 10% formalin within marked plastic containers. formalin ware replaced after 24 hours and left until histological sectioning could be performed**(Kadhem *et al*.,2023)**

3.14. X-ray examination:

X-rays of the experimental animals were examined before the start of the experiment, and bone examination continued every 4 weeks until osteoporosis It happened after (8 weeks) of treatment.

3.15.Statistical analysis :

Graph Pad Prism 8.0 was used to conduct the statistical analysis. The standard of significance for the analysis was $P < 0.05$, and the data points were reported as mean Standard Error.

Chapter four

Results and Discussion

4. Results & Discussion:

4.1. Effect of Hypercholesterolemia diet on serum lipid profile:

The current study shows that there was a significant (P< 0.05) increase in the (TC, TAG,LDL-C)in the treated group as compared with the control group while there was a significant decrease in the HDL-C in the treated group as compaare with the control group as shown in figure (4-1).And this result is in agremant with **(Gao *et al*., 2023; Jayalekshmi *et al*., 2023 and Mustofa *et al*., 2024).**

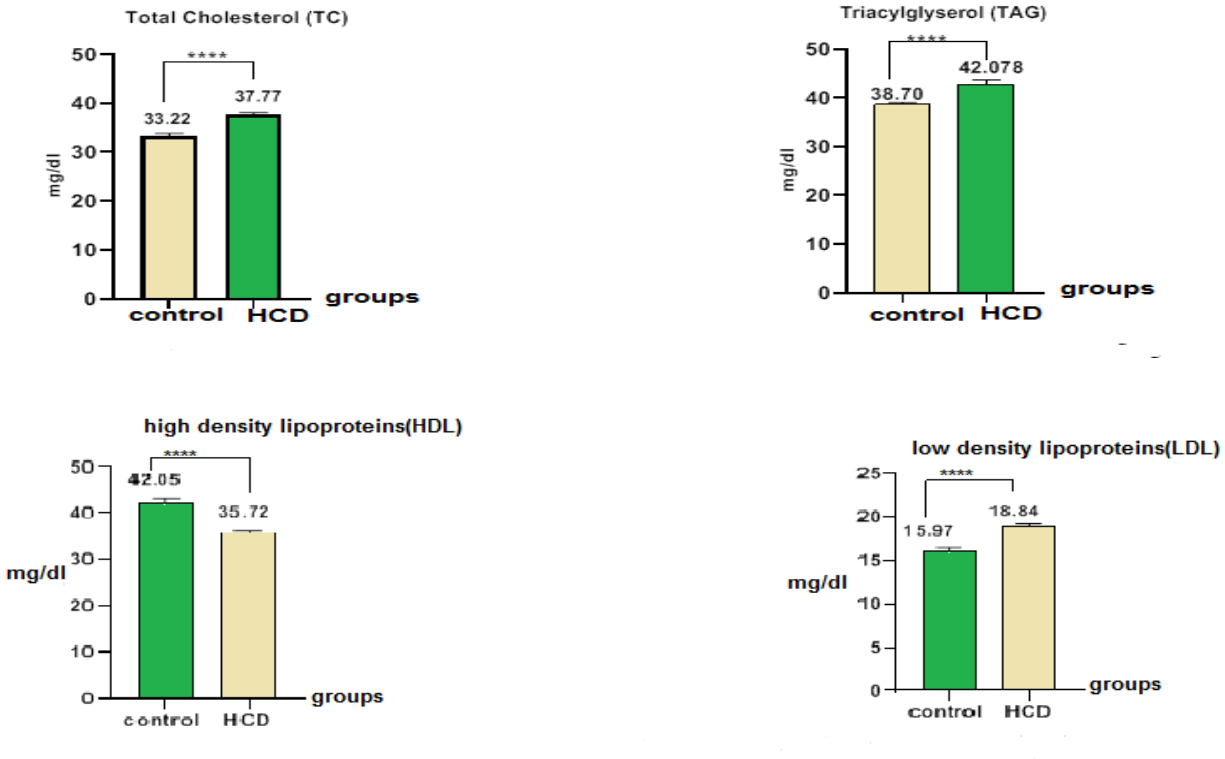


Figure (4-1): Effect of Hypercholesterolemia diet on serum lipid profile

It showed a significant difference in serum TG,TC.HDL,LDL levels between the cholesterol and control groups. (P< 0.05) :Control group rats revived normal diet daily for 8 weeks , hypercholesteremic diet (HCD) group (rats received 0.2mg/kg BW cholesterol) daily for8 weeks

In the current research, a diet high in cholesterol raised TG compared to the control group this is inagreement with **(Mahdi *et al*., 2021).** Hyperlipidemia most often occurs as a consequence of some disorder, hyperlipidemia even can also occur spontaneously after a meal of high-fat diet **(Id *et al*., 2018).**

The study showed a substantial rise in blood LDL levels in those on a high cholesterol diet, in comparison to the control group. , as well as there was an important reduction in the levels of HDL in the bloodstream of individuals following a cholesterol-rich meal. compared to the control group . this result may occur due to the absorbed cholesterol gets into the circulation and may be a factor in the rise in LDL cholesterol **(Guo.,** ***et al*** **2024)** . Some of studies reverse to dietary cholesterol is transported in the bloodstream within lipoprotein particles called chylomicrons. As these particles are broken down, chylomicron remnants are formed, releasing cholesterol into the bloodstream. This cholesterol can contribute to an increase in LDL cholesterol **(Gugliucci** ***et al*****., 2023).** Also the studies have shown that chronic conception of high cholesterol diet increase serum LDL. Also cholesterol has an impact on high-density lipoprotein cholesterol (HDL-C or "good" cholesterol) levels **(Chiesa** ***et al*** **., 2019)**, HDL cholesterol is known for its protective role in cardiovascular health, as it helps transport cholesterol away from the arteries to the liver for excretion **(Nessler** ***et al*** **.,2019),** Diets high in cholesterol may contribute to inflammation and oxidative stress in the body. These factors can impact the functionality of HDL cholesterol and may reduce its effectiveness in protecting against atherosclerosis **(März** ***et al*** **., 2017;& Jasim** ***et al*** **.,2021).**

Low-density lipoprotein is a chief carrier of cholesterol to the cells. High dietary fat adds to its abundance causing pathological complications above a threshold level **(Goldstein & Brown, 2015; and Khatana** ***et al*****., 2020).** While cholesterol is central to many healthy cell functions, it also can harm the body if it is allowed to reach abnormal blood concentrations **(Khatana** ***et al*****., 2020).**

High cholesterol diets also decrease bone marrow stromal cell proliferation and differentiation, which results in decreased osteoblastogenesis Under these circumstances, the functionality and equilibrium of osteoblasts are disrupted, leading to an increase in osteoclast activity and quantity, ultimately resulting in a decrease in bone mass **(Akhmetshina** ***et al*****., 2023).** Concurrently, dyslipidemia, characterized by Low-density lipoproteins (LDL) and total cholesterol (TC) are elevated. coupled with

reduced levels of high-density lipoproteins (HDL), heightens the susceptibility to atherosclerosis and associated cardiovascular diseases **(Purva *et al*., 2020),** the common occurrence of this condition and its detrimental effects on the cardiovascular system make it a significant clinical concern **(Hussein *et al*., 2024).** And in the current study, a diet high in cholesterol raised TC, TG compared to the control group. This result agrees with **(Jasim *et al*., 2024).**

4.2 Effect of Hypercholesterolemia diet on bone biomarkers:

4.2.1 Effect of Hypercholesterolemia diet on serum Receptor Activator of Nuclear Factor Kappa-B (RANK) marker:

The study shows a significant increase in serum RANK marker in the Hypercholesterolemia diet group (0.2300 ±0.0059) ng/ml as compared with the control group (0.1729±0.0078) ng/ml as shown in figure (4-2), this study was in agreement with **(Pacifico *et al*., 2018 and Mangu *et al*., 2022)** who found positive associations between OPG and IR as well as between OPG and nonalcoholic fatty liver disease (NAFLD) and RANKEL expression was significantly higher in the HCD group, respectively.

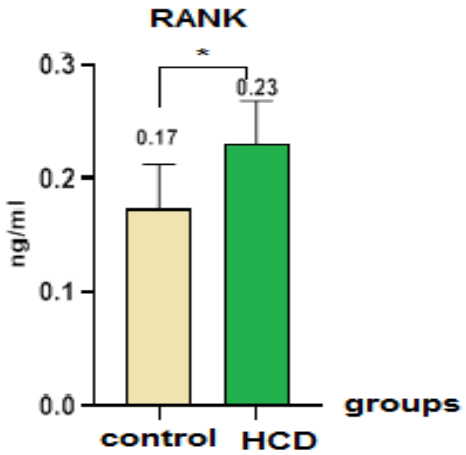


Figure (4-2): Effect of Hypercholesterolemia diet on serum RANK

There was a significant difference in the level of serum RANK in the high cholesterol diet group compare control group (P< 0.05): Control group rats revived normal diet daily for 8 weeks , hypercholesteremic diet (HCD) group (rats received 0.2mg/kg BW cholesterol) daily for 8 weeks

The RANK-mediated stimulation of osteoclasts expedites the resorption process which eliminates and breaks down older or damaged bone structure proteases, increase of serum RANK in the HCD group has significant clinical implications for bone health. Abnormal increase of RANK can lead to excessive bone resorption and bone loss and will lead for conditions such as osteoporosis, rheumatoid arthritis, and bone metastases **(Mathis *et al*., 2018 and Li *et al* ., 2022)**.

High levels of cholesterol in diet can promote lipid peroxidation a process that generates reactive oxygen species (ROS) and oxidative stress which has been linked to increase apoptosis of osteocytes can lead to increased RANK secretion that promotes osteoclastogenesis and bone resorption **(wang *et al*., 2022 and Akhmetshina *et al*., 2023)**.

The increase in RANK secretion observed in response to disrupted osteoblast functionality further amplifies the activation of the RANK signaling pathway and the subsequent osteoclast-mediated bone resorption. This creates a vicious cycle wherein osteoclast activity is heightened, leading to further impairment of bone formation and exacerbation of bone loss**(kim *et al*., 2020; and Zhang *et al* ., 2022)**.

Studies found that Cholesterol can indeed influence the expression and activity of RANK on the surface of osteoclasts and cholesterol-rich environments can upregulate the expression of RANK on the surface of osteoclast precursor cells, This upregulation occurs in response to the presence of cholesterol-derived molecules or alterations in lipid composition within cell membranes. Cholesterol-rich microenvironments may also enhance the binding affinity of RANKL to its receptor, RANK, thereby promoting RANK activation and downstream signaling **(de Munter *et al*., 2015 ;and Ascone *et al*., 2020).**

On the other hand, high cholesterol diets also decreases bone marrow stromal cell proliferation and differentiation which results in decreased osteoblast genesis the functionality and equilibrium of osteoblasts are disrupted leading to an increase in

osteoclast activity and increase RANK secretion **(you *et al*., 2011 ; Cortez *et al*., 2013 ;and Yin *et al*., 2019) .**

4.2.2 Effect of Hypercholesterolemia diet on serum Receptor Activator of Nuclear Factor Kappa-B Ligand (RANKL) marker:

The study shows a significant increase in serum RANKL in the Hypercholesterolemia diet group (44.72±3.89) ng/ml as compared with the control group (16.39± 2.67) ng/ml as shown in figure (4-3) and this result was in agreement with **(Khalaf & Almudhi , 2022)** who were found increased of RANKL concentration in experimental rat which was induced via vitamin D deficiency.

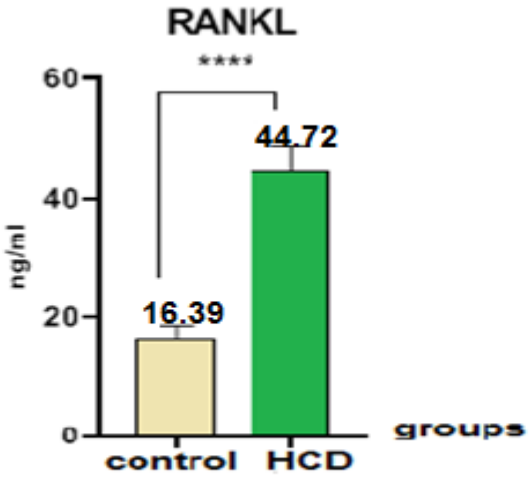


Figure (4-3): Effect of Hypercholesterolemia diet on serum RANKL

A significant difference in the level of serum RANKL in the high cholesterol diet group compare control group (P< 0.05): Control group rats revived normal diet daily for 8 weeks , hypercholesteremic diet (HCD) group (rats received 0.2mg/kg BW cholesterol) daily for 8 weeks.

The common high levels of cholesterol in the bloodstream can lead to chronic low-grade inflammation which affects bone metabolism inflammatory cytokines such as interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-alpha) promote osteoclast activity which increases RANKL production and suppress osteoblast activity leading to loss of bone mass **(Lombardi *et al* ., 2019 ;and Kim *et al* ., 2020).**

May be due to high cholesterol levels which lead to oxidative stress that disrupts the balance between bone resorption and formation. Oxidative stress promotes osteoclast genesis and inhibits osteoblast function further exacerbating bone resorption **(Yaob *et al* .,2021 ;and Che *et al* ., 2022).** Oxidative stress can activate transcription factors such as nuclear factor kappa B (NF-κB) and activator protein 1 (AP-1), which are known to regulate RANKL gene expression. These transcription factors can bind to the promoter region of the RANKL gene and enhance its transcription, leading to increased RANKL production **(Ma *et al*., 2020; and Wang *et al*., 2022)**

On the other hand, **(Reiss *et al* ., 2018)** found that the vascular calcification due to atherosclerosis can also impact bone health which high cholesterol levels contribute to vascular calcification and the same factors that promote calcification in blood vessels can also affect bone mineralization and resorption.

Also high cholesterol levels can affect the transcriptional regulation of the RANKL gene in osteoblasts. Cholesterol-rich environments activate transcription factors liver X receptors (LXRs) which can directly increase RANKL gene expression **(Goel & Vohora,2021).**

4.2.3 Effect of Hypercholesterolemia diet on serum Extracellular Signal-Regulated Kinase (ERK):

The study showed a significant increase in serum ERK in the hypercholesterolemia diet group (2.161 ± 0.057) ng/ml as compared with the control group (1.566± 0.034) as shown in figure (4-3) and this result is inagreement with **(Roberts, 2012)** who illustrated that ERK can be activated through stimulation of G protein-coupled receptors (GPCRs) and release of Gβγ subunits, or through activation of growth factor-stimulated tyrosine kinase receptors, The activation of Gβγ subunits after stimulation of GPCRs, ERK will be activated through transactivation of growth factor tyrosine kinase receptors such as the epidermal growth factor (EGF) receptor, on that basis the epidermal growth factor receptor (sEGFR) levels are elevated in patients with increase blood glucose and cholesterol levels **(Lee *et al*., 2022).**

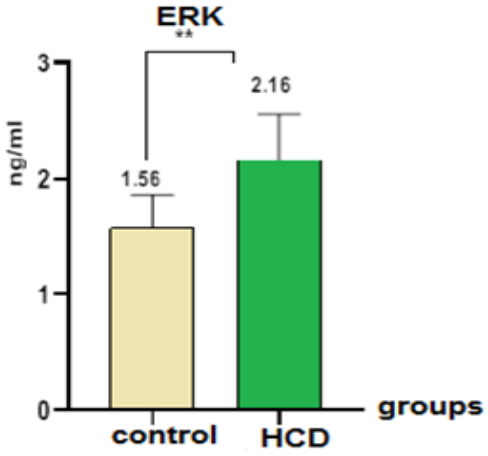


Figure (4-4): Effect of Hypercholesterolemia diet on serum ERK

A significant difference in the level of serum ERK in the high cholesterol diet group compare control group (P< 0.05): Control group rats revived normal diet daily for 8 weeks hypercholesteremic diet (HCD) group (rats received 0.2mg/kg BW cholesterol) daily for 8 weeks.

The studies showed an increase in ERK activity in hypercholesterolemia diet group in comparison to control group the ERK signaling pathway has been studied in relation to osteoporosis, and its activation or inhibition may have implications for bone health, the signaling of ERK has been associated with increased osteoblast (bone-forming cells) activity and bone formation, whereas the inhibition of it signaling may result in decreased osteoblast activity and bone loss **(Mandal, 2015 and Li *et al*., 2016).**

Hypercholesterolemia can lead to the activation of receptor tyrosine kinases (RTKs), such as the Epidermal Growth Factor Receptor (EGFR)**(Wang, 2016)**, and, Platelet-Derived Growth Factor Receptor (PDGFR) **(Charbonneau *et al* ., 2016),** as well as vascular endothelial Growth Factor Receptor (VEGFR)**(Shaik *et al* ., 2020)** . those signaling receptors linked and activation of RTKs stimulates lead to downstream signaling cascades, including the ERK pathway **(Jain *et al*., 2018).**

Hypercholesterolemia can lead to alterations in lipid raft composition affecting the localization and activation of signaling molecules including those involved in the ERK pathway. The arrangement of lipid rafts may enhance the clustering and activation of RTKs and downstream effectors, leading to sustained ERK activation **(Degirmenci *et al* .,2020)** . Also High cholesterol levels can induce oxidative stress which can activate

ERK signaling pathways. Oxidative stress is known to activate various intracellular signaling cascades including ERK in a variety of cell types. Therefore oxidative stress induced by a hypercholesterolemic diet may contribute to increased ERK activation in bone cells **(Rezatabar *et al* .,2019).**

Hypercholesterolemia is often associated with low-grade systemic inflammatory cytokines which can activate ERK signaling pathways in various cell types including those involved in bone metabolism osteoblasts and osteoclasts. Thus, a hypercholesterolemic diet may indirectly influence ERK activation through its pro-inflammatory effects like IL-6 and TNF-α **(Srivastava *et al*., 2022).**

Cholesterol metabolites such as oxysterols have been shown to influence ERK activation in osteoblasts. Some of study using oxysterols are oxidized derivatives of cholesterol and act as ligands for certain nuclear receptors which activation of LXRs by oxysterols that can lead to the stimulation of ERK signaling pathways in osteoblasts, and will be promoted their differentiation and function **(Morello *et al* ., 2009 and Mutemberezi *et al*., 2016).** The ERK signaling pathway has been extensively investigated in the context of osteoporosis, and its activation or inhibition can potentially impact bone health, activation of the ERK signaling pathway has been linked to enhanced osteoblast (cells responsible for bone formation) activity and increased bone formation and conversely, inhibiting the ERK signaling pathway may lead to reduced osteoblast activity and a consequent loss of bone mass **(Matsushita *et al*., 2009 ; and Miranda *et al*., 2024).**

4.2.4 Effect of Hypercholesterolemia diet on serum Tartrate-Resistant Acid Phosphatase (TRAP):

The study shows a no significant increase in serum TRAP in the Hypercholesterolemia diet group as compared with the control group as shown in figure (4-5) and this result is on the contrary with **(Tamiya *et al*., 2021).**

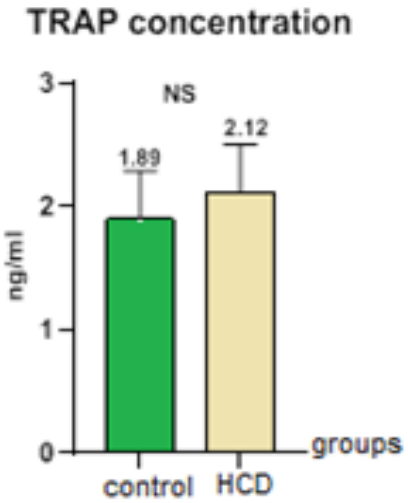


Figure (4-5): Effect of Hypercholesterolemia diet on serum TRAP

There is no significant increase in serum TRAP in the hypercholesterolemia diet group as compare with the control group (P > 0.05): Control group rats revived normal diet daily for 8 weeks , hypercholesteremic diet (HCD) group (rats received 0.2mg/kg BW cholesterol) daily for 8 weeks.

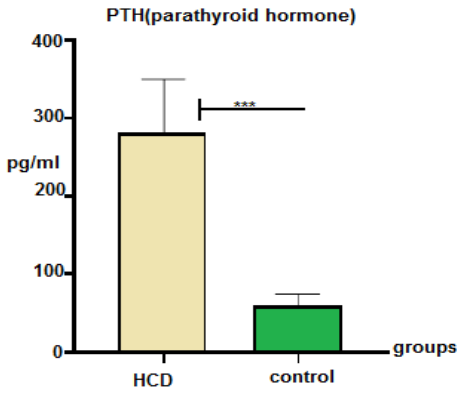
The current investigation revealed no significant disparity in serum tartrate-resistant acid phosphatase (TRAP) levels between the control group and the treated group, as depicted in figure (4-4). These causes may be attributed to rats can have individual variability in their TRAP response to osteoporosis induction. Some rats may see a notable rise in serum TRAP levels, while others may not. These variable changes may be attributed to hereditary causes, hormone changes, or other underlying variables that impact bone metabolism **(Garnero,2008**).When bone resorption increases leading to higher levels of TRAP in the bloodstream. Therefore, the measuring serum TRAP levels can provide valuable information about the rate of bone turnover and the extent of bone resorption occurring in the body. The elevated serum TRAP levels may indicate increased bone resorption and could be used as a diagnostic marker for osteoporosis **(Mederle *et al* ., 2018).**

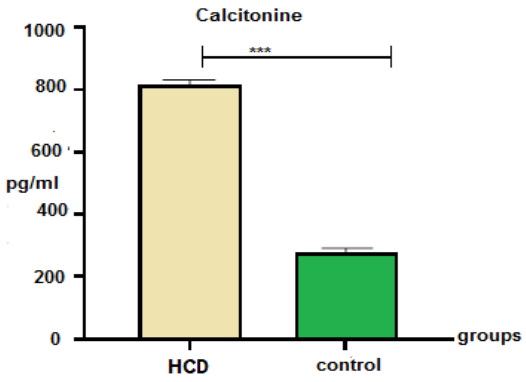
This study was in disagreement with some of studies have shown that TRAP levels and activity are elevated in individuals with osteoporosis compared to healthy controls. This indicates increased osteoclast activity and bone resorption, which are

characteristic features of osteoporosis. TRAP levels have been correlated with bone mineral density (BMD) and can be used as an indicator of bone turnover and disease severity **(Chung *et al*., 2005).**

4.3. Effect of Hypercholesterolemia diet on serum parathyroid hormones and calcitonin and vitamin D concentration:

The current study shows a significant ($p \leq 0.05$) increase in serum PTH, calcitonin and Vit.D in Hypercholesterolemia diet group as compared with the control group as shown in figure (4-6) and this result is in agreement with **(Qin *et al*.,2015).**





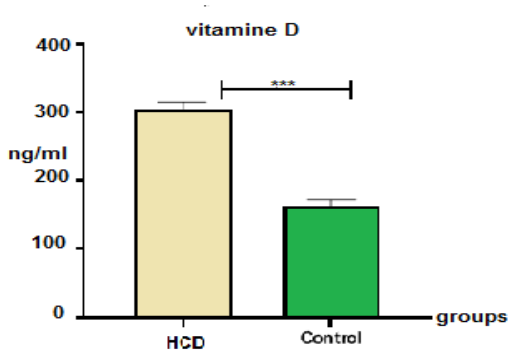


Figure (4-6): Effect of Hypercholesterolemia diet on serum PTH, Calcitonin and Vit.D

There was a big difference in the level of serum PTH, Calcitonin and Vit.D in the high cholesterol diet group compare control group (P< 0.05): Control group rats revived normal diet daily for 8 weeks ,hypercholesteremic diet (HCD) group (rats received 0.2mg/kg BW cholesterol) daily for 8 weeks

The production of PTH by the parathyroid glands in response to low blood calcium levels in experemntal animals. Its primary function is to increase blood calcium levels by stimulating bone resorption (the breakdown of bone tissue), increasing the release of calcium from bone into the bloodstream **(Hachani *et al*., 2010)** PTH also enhances the reabsorption of calcium in the kidneys and promotes the activation of vitamin D, which further aids calcium absorption from the intestine **(Pirih *et al*., 2012).**

Calcium-sensing receptors (CaSR) play a crucial role in regulating PTH secretion by sensing changes in extracellular calcium levels and the intracellular, Oxidative stress induced by highly diet cholesterol has been associated with changes in the function of the calcium-sensing receptor (CaSR) **(Acharya *et al*.,2024).** The CaSR is a G protein-coupled receptor that plays a crucial role in maintaining calcium homeostasis in the body by sensing changes in extracellular calcium levels. It is primarily expressed in parathyroid glands **(tu *et al*., 2024)**. Oxidative stress induced by highly diet cholesterol can impact the expression levels of the complex intracellular pathways regulate CaSR signaling, and oxidative stress syndrome may impair the receptor's signal-transmitting capacity in response to changes in calcium levels **(Minaychev *et al*., 2024).**

Prolonged exposure to oxidative stress may contribute to parathyroid hyperplasia, a condition characterized by the hyper activates of the parathyroid glands, and this acetate could be a compensatory response to oxidative damage, aiming to maintain adequate PTH production. However, the hypertrophic changes may lead to dysregulation of PTH secretion, leading to the connection between oxidative stress and the endocrine function of the parathyroid glands, This can interrupt these signaling cascades, which can lead to imbalance in the level of the (PTH) in the cholesterol grouping as compare with control group.

The study was in agreement with **(Stein *et al*., 2013)** who found the skeletal effects of primary hyperparathyroidism (a condition characterized by excessive PTH production) on bone microstructure and stiffness in postmenopausal women, The author utilized advanced imaging techniques to assess cortical and trabecular bone microarchitecture and mechanical properties. The study highlights the adverse impact of elevated PTH levels on bone quality and stiffness, which can contribute to the development of osteoporosis.

Also hypercholesterolemia can disrupt calcium homeostasis, which may indirectly affect PTH secretion. High cholesterol levels may impair calcium absorption in the intestines or inhibit calcium reabsorption in the kidneys, leading to hypocalcemia. In response to hypocalcemia, the parathyroid glands increase PTH secretion to stimulate calcium release from bone and enhance renal calcium reabsorption, thereby restoring calcium balance **(Wongdee *et al*., 2019).**

The study shows that hypercholesterolemia has been linked to alterations in vitamin D metabolism which can impact PTH levels **(Chen *et al*., 2014).** Vitamin D is essential for calcium absorption in the intestines and regulates PTH secretion through negative feedback mechanisms. Some of the study illustrated deficiency vitamin D metabolism in case of hypercholesterolemia PTH secretion may increase to compensate for inadequate calcium absorption and maintain calcium homeostasis**(Papadopoulou *et al*., 2021).**

4.4. Effect of Hypercholesterolemia diet on serum electrolyte:

The results of the current study showed no significant differences in the concentration of electrolytes, including potassium, phosphate and sodium, in the blood of the rats under the experiment except for calcium where a significant (P< 0.05) increase was recorded in hyper cholesterol group (22.90± 1.402) mg/dl from control groups (8.200± 0.57) mg/dl as shown in figure (4-7),(4-8)

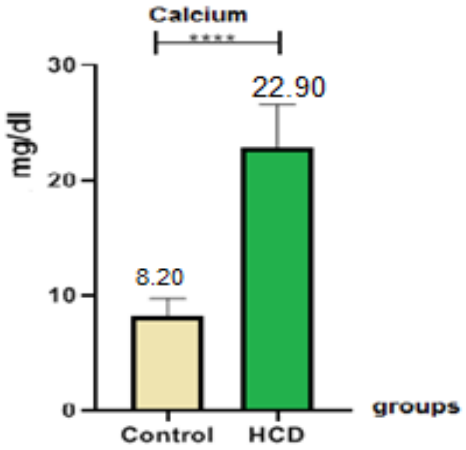


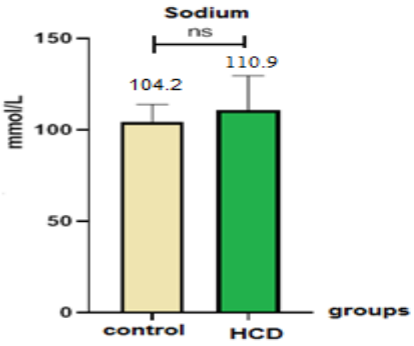
Figure (4-7): Effect of Hypercholesterolemia diet on serum calcium

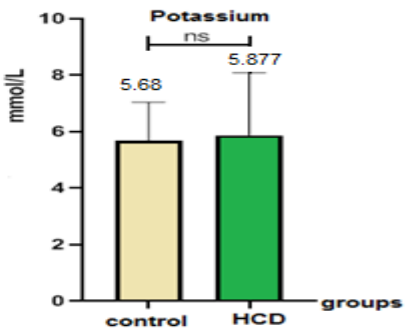
There was a significant difference in the level of serum Calcium in the high cholesterol diet group compare control group (P< 0.05): Control group rats revived normal diet daily for 8 weeks hypercholesteremic diet (HCD) group (rats received 0.2mg/kg BW cholesterol) daily for 8 weeks

From the current study the result shows that there is an increase in the level of calcium in the blood serum of the rats of the experiment when compared to the control group, perhaps the reason is that the calcium element was affected by the change it occurred from the hormones PTH, calcitonin and vitamin D and this leads to the withdrawal of calcium from the bone and an increase in its concentration in the blood serum of hypercholesterima diet groups **(Bhattarai *et al* .,2020).** Vitamin D directly raises the serum calcium by protein synthesis in the intestinal wall which promotes intestinal calcium transport and absorptions. Vitamin D increases the reabsorptions of calcium and phosphorus from renal tubular, Parathyroid hormone is very sensitive and

response to any change in extracellular calcium which acts in conjunction with active vitamin D to regulate calcium **(Riggs & Parfitt, 2015)** . This study was inagreement with **(Peng *et al*., 1997)**, who found an acute marked increase in total serum calcium was observed after a single iv injection of lead. It was also inagreement with **(Sawy *et al*., 2022)** who found treat female rats' osteoporosis using natural sources rich in calcium (permeate with kiwi or fig). and there are no significant differences in the amount of sodium, potassium, and phosphorus when comparing the experimental group to the control group. On the other hand withdrawal of calcium from osteocyte as a result of increase of RANK and RANKL led to a case of bone rickets and increasing calcium concentration in the blood serum **(Kitaura *et al*., 2020).**

Some research believes that an increase antioxidant factors may lead to the occurrence of high cholesterol which may lead to a state of apoptosis of bone cells and thus the bone cells die and their components are excreted outside the cells, which lead to an increase in the amount of calcium in the blood **(Bellido *et al*., 2019).**





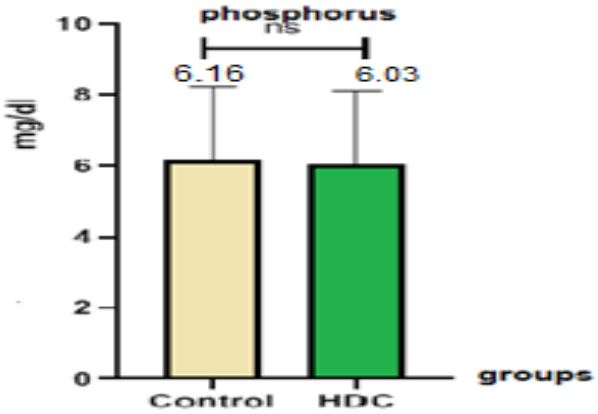


Figure (4-8): Effect of Hypercholesterolemia diet on serum sodium,potassium and phosphorus

There was no a significant difference in the level of serum sodium ,potassium ,phosphorus in the high cholesterol diet group compare control group (P< 0.05): Control group rats revived normal diet daily for 8 weeks, hypercholesteremic diet (HCD) group (rats received 0.2mg/kg BW cholesterol) daily for 8 weeks.

The study doesn't find any significant differences in Phosphorus levels in both groups, however previous studies have reported that high phosphorus diets directly stimulate PTH secretion **(Hernández *et al*., 1996).** Other electrolytes, such as sodium and potassium, are also important for overall health but have a lesser direct impact on bone metabolism, Sodium and potassium help maintain fluid balance and cellular function but do not have direct effects on bone mineralization **(Humalda *et al*.,2020).**

4.5. Effect of Hypercholesterolemia diet on serum anti-oxidant:

4.5.1 Effect of Hypercholesterolemia diet on serum GSH:

The current study shows a significant decrease (P< 0.05) in the rate of GSH (19.66±1.46) compared with control groups and (23.03±2.74) respectively figure (4-9), and this result is in agreement with **(Emekli-Alturfan *et al.*, 2007 ; Cortes *et al*., 2014 and Xiao *et al*., 2024)**, who illustrated that compensatory mechanism between hypercholesterimia and hyperlipidemia. Increased lipid profile often accompanies conditions like hyperlipidemia or hypercholesterolemia, which can lead to oxidative stress. In response, the body may up regulate the production of antioxidants like GSH to counteract the increased oxidative load.

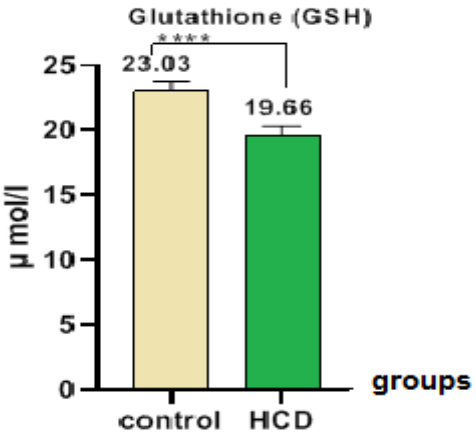


Figure (4-9): Effect of Hypercholesterolemia diet on serum GSH

Serum GSH levels differ significantly between the cholesterol and control groups.(P< 0.05):Control group rats revived normal diet daily for 8 weeks days , hypercholesteremic diet (HCD) group (rats received 0.2mg/kg BW cholesterol) daily for weeks days .

Glutathione often referred to as the body's master antioxidant, plays a crucial role in scavenging free radicals and protecting cells from oxidative damage. A decrease in the level of glutathione in the group of rats that was administrated with cholesterol indicates an increase in the amount of oxidants that are present within the body. It has been noted in one study that the amount of ROS increases by the amount of cholesterol that is ingested by the rats and thus leads to a decrease in its amount of GSH inside the body **(Pizzorno, 2014).**

Some of the studies found that High cholesterol levels have been associated with increased oxidative stress, which can impact GSH gene expression patterns throughout the body. This may include downregulation of genes involved in GSH synthesis, recycling, or utilization, thereby compromising the antioxidant capacity of cells and tissues **(Stranahan *et al* ., 2014; and Oniki *et al*.,2024).**

Also a high cholesterol levels can lead to decreased expression of genes encoding key enzymes involved in GSH synthesis, such as glutamate-cysteine ligase (GCL) and glutathione synthetize (GS). Additionally, cholesterol-induced oxidative stress may

alter the activity of transcription factors that regulate GSH-related genes, further influencing their expression levels Lipid metabolism and GSH metabolism are interconnected. Alterations in lipid metabolism can influence the availability of substrates for GSH synthesis, potentially leading to an increase in GSH levels. **(El-Demerdash *et al*., 2014).**

High cholesterol levels can increase the production of reactive oxygen species (ROS) and free radicals, leading to oxidative damage to cellular components including proteins and DNA. This oxidative damage can impair cellular function, of GSH synthesis**(Jomova *et al*., 2023)** .

4.5.2 Effect of Hypercholesterolemia diet on serum MDA:

It was found a significant (P< 0.05) increase in the rate of MDA (0.5014±0.083) concurrent in the treated rat group as compared with the control group (0.3500±0.046) as shown in figure (4-10). and this result is in agreement with **(Lecumberri *et al* ., 2007 ; Yang *et al*., 2008 and Wang *et al* ., 2024)** .

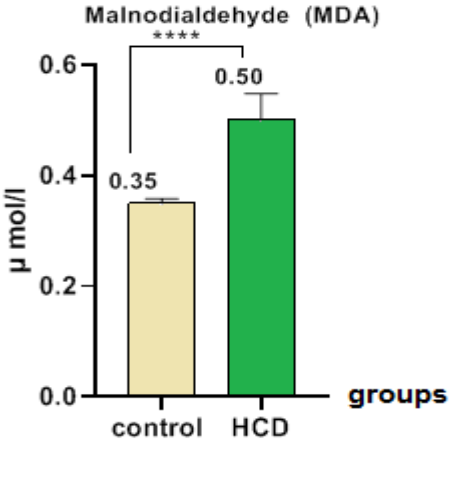


Figure (4-10): Effect of Hypercholesterolemia diet on serum MDA

Showed a significant difference in serum MDA levels between the cholesterol and control groups. (P< 0.05) :Control group rats revived normal diet daily for 8 weeks , hypercholesteremic diet (HCD) group (rats received 0.2mg/kg BW cholesterol) daily for8 weeks.

High cholesterol levels can contribute to oxidative stress by promoting the production of reactive oxygen species (ROS) which can damage cells and tissues this damage leads to the formation of MDA among other byproducts. Increased MDA levels are associated with lipid peroxidation, a process where free radicals attack lipids in cell membranes, resulting in their degradation and the release of MDA **(Peleman *et al*., 2024).** Hypercholesterolemia can trigger inflammatory processes which further enhances oxidative stress. Inflammatory processes produce ROS, leading to increased oxidative damage and MDA formation **(Wang *et al*., 2024)** . In response to high cholesterol levels and other factors, the immune system may become activated leading to the release of inflammatory molecules such as cytokines and chemokines these molecules can stimulate the production of reactive oxygen species (ROS) by various cell types, including immune cells like macrophages and endothelial cells lining the blood vessels **(Tall *et al*., 2015).** Hypercholesterolemia can impair the function of endothelial cells lining the blood vessels. Healthy endothelial cells produce antioxidants and regulate vascular tone, but dysfunction of these cells can disrupt antioxidant production and promote oxidative stress **(Queiroz *et al*., 2024)**.

4.6 Effect of Hypercholesterolemia diet on gene expiration (osterix) :

The analysis of the RT-qPCR gene expression data Osterix gene in male rats was recorded that cholersertol groups was increased of Osterix gene expression fold change than control groups figure (4-11 and 4-12)

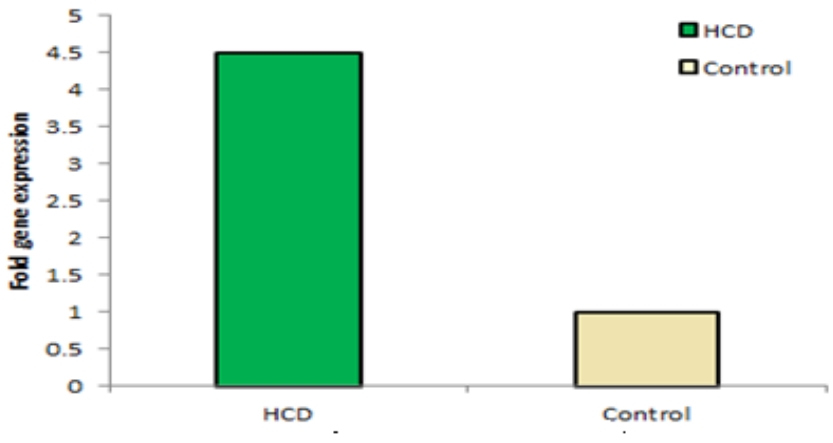


Figure (4-11): Fold change comparison between the groups expressed Osterix gene. This shows significant upregulation of the HCD group compared with control groups.

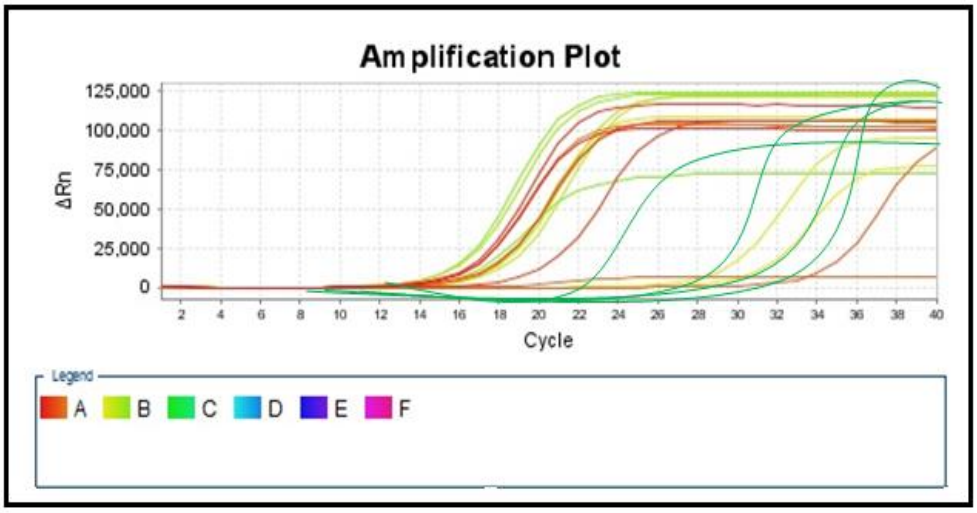


Figure (4-12): Amplification curve of the tested samples represents the Osterix gene. This indicates a successful RNA extraction and cDNA synthesis

Osterix gene transcription factor, which is critical for osteoblast bone formation and differentiation, was up-regulated in comparison with control groups **(Nordin,1997).** Osteoblasts are highly specialized cells that have the primary function of synthesizing and depositing the bone matrix. Over time, this matrix undergoes mineralization, resulting in the formation of fully mature bone tissue **(Liu *et al*., 2020).** Osterix is upregulated during osteoblast differentiation and acts downstream of other key transcription factors, such as Runx2 (“Runt-related transcription factor 2”) master controller of osteoblast differentiation, Runx2 directly activates Osterix expression in osteoblasts **(Mohamad *et al* .,2019),** Some studies **(Fu *et al*., 2007; and Matsubara *et al* ., 2008)** suggest Osterix plays a role in osteoblast development and maturation. Because of their roles in lipoprotein synthesis and cholesterol transporter control, osteoblasts are linked to cholesterol metabolism. Osterix's interaction with other genes and signalling pathways may also have indirect impacts on cholesterol metabolism **(Liu *et al*.,2020)** , finally, it has been demonstrated that Osterix interacts with Wnt

signalling, a pathway that regulates cholesterol homeostasis in different tissues **(Zhu *et al*., 2012).**

4.7 Effect of Hypercholesterolemia diet on femoral bone X- ray examination:

The results of the current study in the figure (4-13) , when using X-rays in normal male rats, showed the presence of radiopaque area, and this is due to, naturally developed calcifications as part of the aging process (1.5 months of age) as well as The attachments of certain ligaments and tendons to the pelvic bones, can become ossified (turned into bone) over time **(Iwanaga *et al*., 2016).** At the joints, the clear, sclerotic margins of the bone are typically seen extending up to the joint line, the joint spaces appear uniform and of normal width, without any signs of narrowing or irregularity **(Seifert & Marks, 1985).**

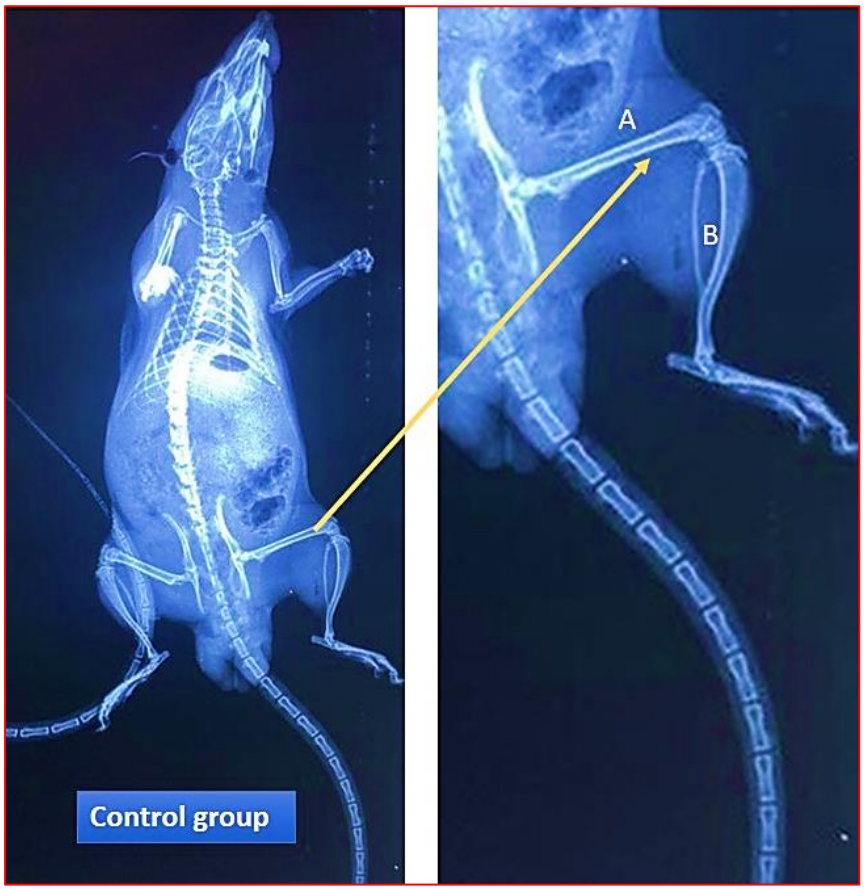


Figure (4 -13) :Radiographic image of (A)femoral bone and (B) tibial bone at the end of one - month Pre-experiment, as a control group showing a sclerotic, clear border of the bone with a radiopaque area at the pelvic bones.

The study found in the cholesterol group a radiolucent area at the pelvic bones in the figure (4-13), in the pelvic bones of rats with osteoporosis induced by a high-cholesterol diet, it would be expect to see areas of radiolucency (decreased radiodensity or "decreased whiteness") on the radiographic images, this area indicated a decrease in bone mineral density, which is a hallmark of osteoporosis **.**This study was inagreement with **(Winge *et al*., 2021)** who found the overall bone structure may appear less defined and more porous compared to healthy, control rats and the presence of radiolucent areas in the pelvic bones of rats on a high-cholesterol diet is a characteristic radiographic finding associated with osteoporosis. These results can be used to monitor the progression of bone loss and the efficacy of potential interventions or treatments aimed at preventing or reversing the osteoporotic changes.

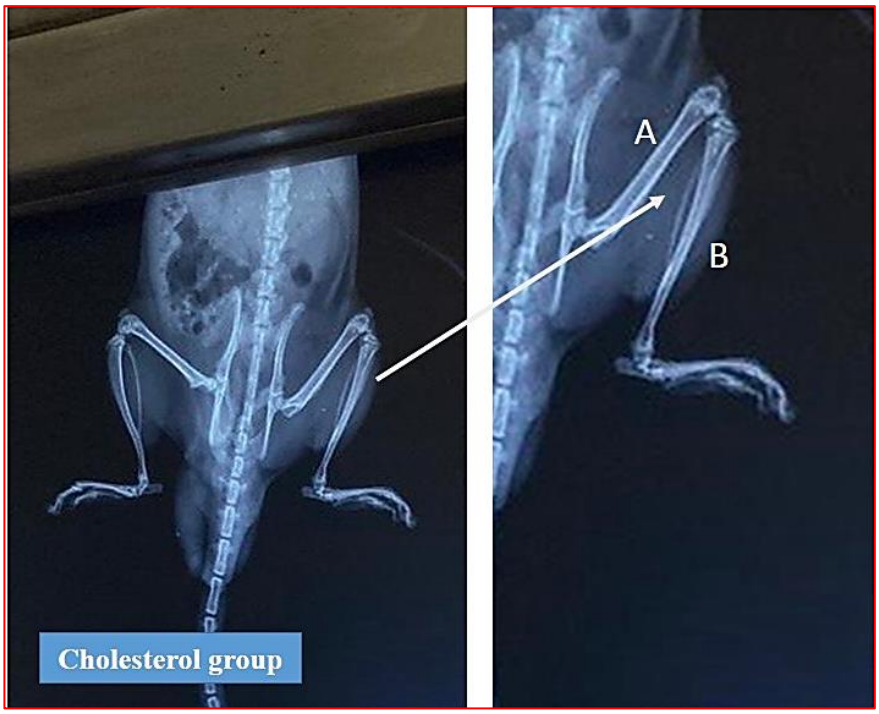


Figure (4-14): Radiographic image of(A) femoral bone and(B) tibial bone at the end of one – month age post-experiment, as cholesterol group showing a development of osteoporosis at the border of the bones with a radiolucent area at the pelvic bones.

after two months of present study, the control groups is normal, In the control group of rats in figure (4-15), our study observed a radiographic image with a more significant sclerosis and increased bone mineral density around the vertebral, femoral, and pelvic bones, with a high radiopaque area, this would be considered a normal and expected finding, this meaning, the control rats, not subjected to any interventions, would be expected to have a healthy, normal bone architecture and mineralization, and The radiographic image would show areas of increased bone density, appearing as a higher degree of radiopacity (whiteness) around the vertebral, femoral, and pelvic bones **(Gustafsson *et al*., 2006).**

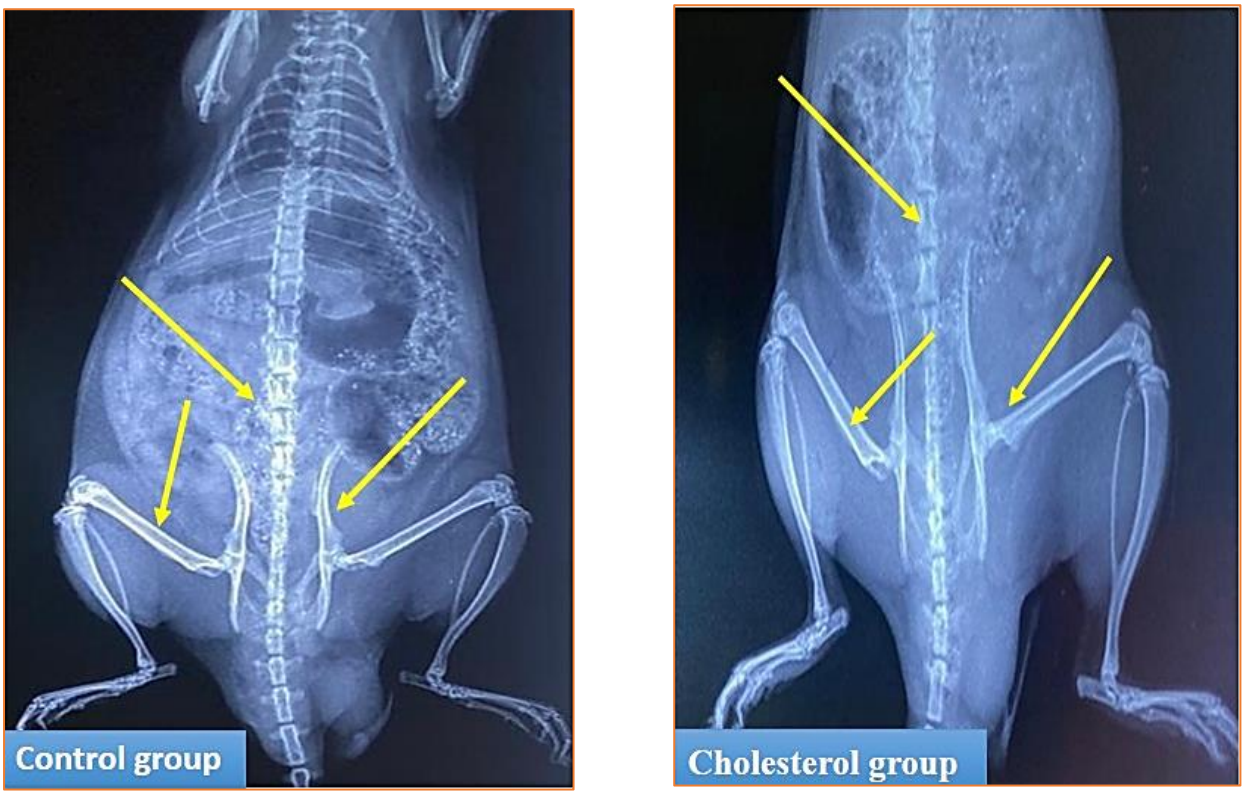


Figure (4-15): Radiographic image of the bones with yellow arrow of control group showing more significant sclerosis with increase in bones mineral density ,while Radiographic image of the bones with yellow arrow of cholesterol group showing increase osteoporosis with reduction in bones mineral density around the vertebral ,femoral and pelvic bones with a high radiopaque area, at the end of experiment (after Two month)

The results of the current study figure (4-15), when using the cholesterol group in experimental rats, showed the presence of increased osteoporosis, the radiographic image would show a reduction in bone mineral density around the vertebral, femoral, and pelvic bones, resulting in a high radiolucent area, the bone mineral density would be significantly reduced, leading to a decreased radiographic density or "radiolucency" (decreased whiteness) in the affected areas **(Thrall., 2017).** This study was agreement with **(Schulte *et al*., 2013)** who was found the ability of bone to adapt its internal microstructure in response to changing mechanical demands, through the coordinated actions of bone-forming and bone-resorbing cells, has been extensively studied in mouse models.

4.8 Effect of Hypercholesterolemia diet on histological examination of femoral bone:

In the control group the rat femoral bone was the same of the normal cells of the healthy bone, with normal osteocytes , osteoclast haversian system , while there was a significant increase in the adipocytes in bone marrow and thin bone trabeculae of the femoral bone of the hypercholesterolemia diet rats also the hypercholesterolemia diet rats shown an increase in the atrophied and necrosis of osteocytes and there were a lack of osteoblasts on borders of trabeculae with multiple multinucleated osteoclasts as compare with the control group as shown in figures(4-17) &(4-18).

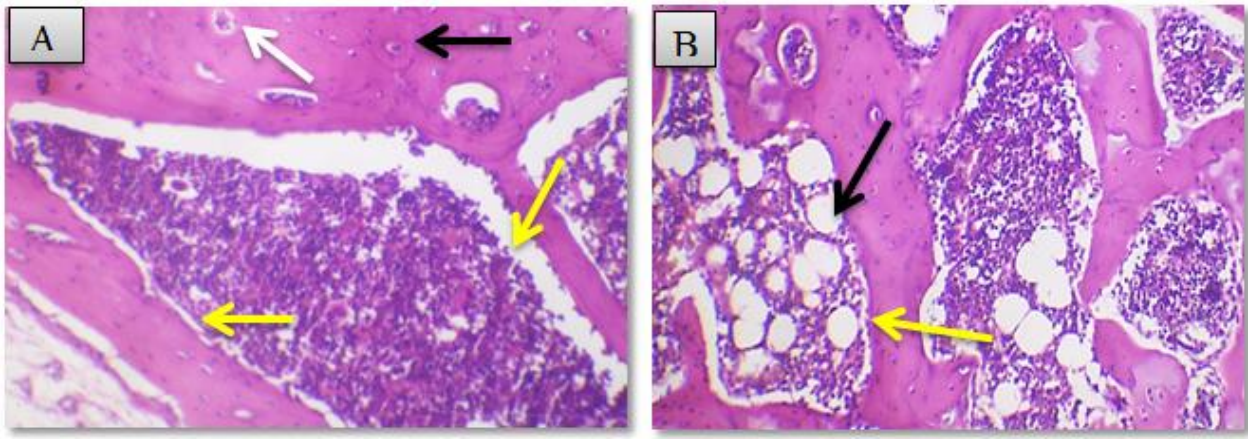


Figure (4-16) :(A) Photomicrograph for a control animal femoral epiphysis, showing remarkable normal osteocytes (black arrow), regular bone marrow cavities (yellow arrow) and haversian system(white arrow). (B) Photomicrograph for Cholesterol treated animal femoral

**epiphysis, showing characteristic histopathological changes , significant hyperlipidemia spaces (increase adipocytes in bone marrow)
(black arrow), thin bone trabeculae (yellow arrow) . (H and E,4X)**

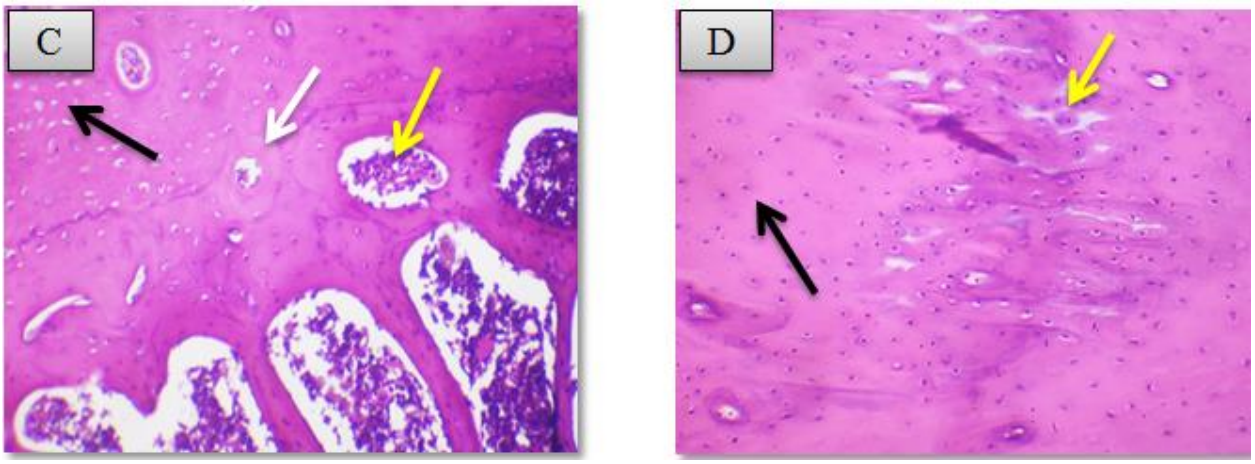


Figure (4-17): (C) Photomicrograph for a control animal femoral epiphysis, showing remarkable normal osteocytes (black arrow), regular bone marrow cavities (yellow arrow) and haversian system(white arrow).(D) Photomicrograph for Cholesterol treated animal femoral diaphysis, showing apparent osteocytes (atrophied) (black arrow), resorption and necrosis of osteoid (yellow arrow) . (H and E,10X).

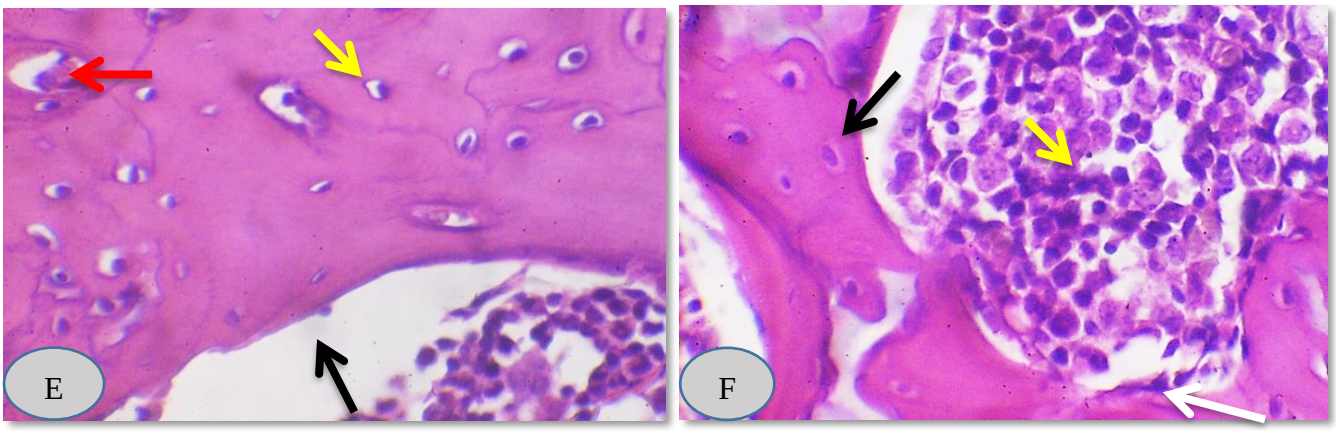


Figure (4-18) :(E) Photomicrograph for a cholesterol treated animal femoral section, showing marked lack of osteoblasts on borders of trabeculae (black arrow), necrosis of osteocytes (yellow arrow) with multiple multinucleated osteoclasts (red arrow). (F) Photomicrograph for a control animal femoral epiphysis, showing remarkable normal osteocytes in lacunae (black arrow), regular bone marrow cavities (yellow arrow) and regular osteoblasts in line on trabecular border (white arrow). (H and E,40 X)

Hypercholesterolemia and high-cholesterol diets have been associated with alterations in bone marrow composition, including increased adiposity. Excess cholesterol in the bloodstream can affect bone marrow function and lead to changes in cellular composition, favoring the accumulation of adipocytes within the marrow space **(Gomes *et al*., 2010 and Yin *et al*., 2019).**

Hypercholesterolemia is often accompanied by systemic inflammation which can influence bone marrow microenvironment. Inflammatory mediators released in response to elevated cholesterol levels may promote adipogenesis and inhibit osteogenesis, leading to an imbalance between adipocytes and osteoblasts within the bone marrow **(Zhang *et al*., 2021).**Dyslipidemia associated with hypercholesterolemia can disrupt lipid metabolism in the bone marrow microenvironment. Excessive intake of cholesterol and saturated fats may promote adipocyte differentiation and lipid accumulation in bone marrow stromal cells, contributing to marrow adiposity **(Wang *et al*., 2022).**

Elevated cholesterol levels can induce oxidative stress, which has been implicated in the pathogenesis of marrow adiposity. Oxidative stress can disrupt cellular homeostasis and signaling pathways involved in adipogenesis and osteogenesis, promoting the expansion of adipocytes within the bone marrow**(Georgiou *et al*., 2012).** Hypercholesterolemia has been associated with alterations in bone remodeling, the process by which old bone tissue is resorbed by osteoclasts and new bone tissue is formed by osteoblasts. Excess cholesterol levels can disrupt the balance between bone resorption and formation, leading to inadequate bone remodeling and thinning of trabecular bone**(Feng & McDonald, 2011 and Mandal, 2015).**

Hypercholesterolemia is often accompanied by systemic inflammation, which can adversely affect bone health. Inflammatory cytokines released in response to elevated cholesterol levels may stimulate osteoclast activity and bone resorption while inhibiting osteoblast function, resulting in thinning of trabecular bone **(Wang *et al*., 2022).** Oxidative stress disrupts the activity of bone-forming osteoblasts and promotes

the differentiation and activity of bone-resorbing osteoclasts, leading to thinning of trabecular bone in the femoral bone**(Zhang *et al*., 2013).**

Hypercholesterolemia can impair endothelial function and blood flow in the bone microvasculature, which may compromise the delivery of nutrients and oxygen to bone cells. Reduced blood supply to the femoral bone can impair bone formation and lead to thinning of trabecular bone **(Marenzanab & Arnett, 2013).**

On the other hand hypercholesterolemia can cause Oxidized lipids which have toxic effects on bone cells including osteocytes. Oxidized lipids can induce cytotoxicity and apoptosis in osteocytes, leading to their atrophy and necrosis**(Baliou *et al*., 2021).**

Chapter Five

Conclusions and Recommendations

5. Conclusions & Recommendations

5.1 Conclusions:

From the results obtained in the research ,it can concluded that:

Exposure of male rats to hypercholesterolemia (cholesterol rich meal)caused loss of osteoblast with numerous osteoclasts as documented by:

1.Dyslipidemia:change in lipid profile.

2. Oxidative stress :elevation in serum MDA and depression in serum GSH concentration,

3.Hypercalcemia :elevation in serum calcium.

4.Histopathological changes in bone.

5.Hyperthyroidism, hyperparathyroidism and increase in calcitonine levels.

6.Changes in bone metabolic pathways through increase in levels RANK-RANKL and ERK levels and no change with TRAP.

7. up-regulation in osterix gene.

8. Osteoporosis in the pelvic bones.

5.2. Recommendations:

1- Employ single-cell RNA sequencing to analyze Osterix and RANKexpression at the individual cell level within the bone microenvironment

2- Investigate the dynamic regulation of osterix and Runx2 by RANK/RANKL signaling over the course of osteoporosis development.

3- Analyze the total antioxidant capacity and oxidative stress indices in bone

4- Examine the impact of oxidative stress on osteoblast and osteoclast function, differentiation, and apoptosis

5- using other agents to induce osteoporosis Glucocorticoid like Decal in experimental animals.

6.Studing other bone parameters like cathepsin and OPG in rat exposure to hypercholesterol diet.

Chapter six

References

References

Ascone, G., Di Ceglie, I., Walgreen, B., Sloetjes, A. W., Lindhout, E., Bot, I. & van Lent, P. L. E. M. (2020). High LDL levels lessen bone destruction during antigen-induced arthritis by inhibiting osteoclast de Munter formation and function. *Bone*, *130*, 115140.

Chin, K. Y., Ng, B. N., Rostam, M. K. I., Muhammad Fadzil, N. F. D., Raman, V., Mohamed Yunus, F. & Ekeuku, S. O. (2022). A mini review on osteoporosis: From biology to pharmacological management of bone loss. Journal of Clinical Medicine, 11(21), 6434

Cortes, R., Martinez-Hervas, S., Ivorra, C., De Marco, G., Gonzalez-Albert, V., Rojo-Martínez, G. & Chaves, F. J. (2014). Enhanced reduction in oxidative stress and altered glutathione and thioredoxin system response to unsaturated fatty acid load in familial hypercholesterolemia. *Clinical Biochemistry*, *47*(18), 291-297.

Cortez, M., Carmo, L. S., Rogero, M. M., Borelli, P., & Fock, R. A. (2013). A high-fat diet increases IL-1, IL-6, and TNF-α production by increasing NF-κB and attenuating PPAR-γ expression in bone marrow mesenchymal stem cells. *Inflammation*, *36*, 379-386.

Chung, S. L., Chu, M. F., Zhang, Y., Xie, Y., Lo, C. H., Lee, T. Y., ... & Wang, Y. (2005). Tibetan tectonic evolution inferred from spatial and temporal variations in post-collisional magmatism. Earth-Science Reviews, 68(3-4), 173-196.

Cunha, L. F., Ongaratto, M. A., Endres, M., & Barschak, A. G. (2021). Modelling hypercholesterolaemia in rats using high cholesterol diet. International Journal of Experimental Pathology, 102(2), 74-79

de Cardiologia, A. B. (2017). Atualização da Diretriz Brasileira de Dislipidemia e Prevenção da Aterosclerose. Sociedade Brasileira de Cardiologia,109(2 Supl)

de Munter, W., van den Bosch, M. H., Annet, S. W., Croce, K., Hogg, N., Vogl, T. & van Lent, P. L. (2015). SAT0006 High Systemic LDL Cholesterol Levels Lead to Synovial Activation and Strongly Accelerate Development of Ectopic Bone Formation During Experimental Osteoarthritis.

Degirmenci, U., Wang, M., & Hu, J. (2020). Targeting aberrant RAS/RAF/MEK/ERK signaling for cancer therapy. *Cells*, *9*(1), 198.

Deng, Y., Tu, Y., Lao, S., Wu, M., Yin, H., Wang, L., & Liao, W. (2022). The role and mechanism of citrus flavonoids in cardiovascular diseases prevention and treatment. Critical reviews in food science and nutrition, 62(27), 7591-7614

Di Pietro, N., Formoso, G., & Pandolfi, A. (2016). Physiology and pathophysiology of oxLDL uptake by vascular wall cells in atherosclerosis. Vascular pharmacology, 84, 1-7.

Drewes, P. A. (1972). Direct colorimetric determination of phosphorus in serum and urine. Clinica Chimica Acta, 39(1), 81-88

Duan, Y., Gong, K., Xu, S., Zhang, F., Meng, X., & Han, J. (2022). Regulation of cholesterol homeostasis in health and diseases: From mechanisms to targeted therapeutics. *Signal Transduction and Targeted Therapy*, *7*(1), 265.

El-Demerdash, F. M., & Nasr, H. M. (2014). Antioxidant effect of selenium on lipid peroxidation, hyperlipidemia and biochemical parameters in rats exposed to diazinon. Journal of Trace Elements in Medicine and Biology, 28(1), 89-93.

Emekli-Alturfan, E., Kasikci, E., & Yarat, A. (2007). Peanuts improve blood glutathione, HDL-cholesterol level and change tissue factor activity in rats fed a high-cholesterol diet. *European journal of nutrition*, *46*, 476-482.

Fan, Q., Yin, X., Rababa'h, A., Diaz Diaz, A., Wijaya, C. S., Singh, S. & McConnell, B. K. (2019). Absence of gravin-mediated signaling inhibits development of high-fat diet-induced hyperlipidemia and atherosclerosis. *American Journal of Physiology-Heart and Circulatory*

Physiology, *317*(4), H793-H810.

Feng, X., & McDonald, J. M. (2011). Disorders of bone remodeling. *Annual Review of Pathology: Mechanisms of Disease*, *6*, 121-145.

Findlay, D. M., & Atkins, G. J. (2011). Relationship between serum RANKL and RANKL in bone. Osteoporosis International, 22, 2597-2602.

Forcina, G. C., & Dixon, S. J. (2019). GPX4 at the crossroads of lipid homeostasis and ferroptosis. *Proteomics*, *19*(18), 1800311.

Fossati, P., & Prencipe, L. (1982). Serum triglycerides determined colorimetrically with an enzyme that produces hydrogen peroxide. Clinical Chemistry, 28(10), 2077–2080.

Frost, M. L., Moore, A. E., Siddique, M., Blake, G. M., Laurent, D., Borah, B. & Fogelman, I. (2013). 18F-fluoride PET as a noninvasive imaging biomarker for determining treatment efficacy of bone active agents at the hip: a prospective, randomized, controlled clinical study. *Journal of Bone and Mineral Research*, *28*(6), 1337-1347.

Fu, C., & Shi, R. (2020). Osteoclast biology in bone resorption: a review. STEMedicine, 1(4), e57-e57

Fu, H., Doll, B., McNelis, T., & Hollinger, J. O. (2007). Osteoblast differentiation in vitro and in vivo promoted by Osterix. *Journal of Biomedical Materials Research Part A: An Official Journal of The Society for Biomaterials, The Japanese Society for Biomaterials, and The Australian Society for Biomaterials and the Korean Society for Biomaterials*, *83*(3), 770-778.

Gao, Y., Xun, R., Xia, J., Xia, H., & Sun, G. (2023). Effects of phytosterol supplementation on lipid profiles in patients with hypercholesterolemia: a systematic review and meta-analysis of randomized controlled trials. *Food & Function*, *14*(7), 2969-2997.

Georgiou, K. R., Hui, S. K., & Xian, C. J. (2012). Regulatory pathways associated with bone loss and bone marrow adiposity caused by aging, chemotherapy, glucocorticoid therapy and radiotherapy. *American journal of stem cells*, *1*(3),

205.

Gibbons, G. F. (2003). Regulation of fatty acid and cholesterol synthesis: co-operation or competition?. Progress in lipid research, 42(6), 479-497

Goel, D., & Vohora, D. (2021). Liver X receptors and skeleton: Current state-of-knowledge. *Bone*, *144*, 115807.

Gomes, A. L., Carvalho, T., Serpa, J., Torre, C., & Dias, S. (2010). Hypercholesterolemia promotes bone marrow cell mobilization by perturbing the SDF-1: CXCR4 axis. *Blood, The Journal of the American Society of Hematology*, *115*(19), 3886-3894.

González-Peña, D., Giménez, L., de Ancos, B., & Sánchez-Moreno, C. (2017). Role of dietary onion in modifying the faecal bile acid content in rats fed a high-cholesterol diet. *Food & function*, *8*(6), 2184-2192.

Gugliucci, A. (2024). The chylomicron saga: time to focus on postprandial metabolism. Frontiers in Endocrinology, 14, 1322869.

Guo, D. C., Gao, J. W., Wang, X., Chen, Z. T., Gao, Q. Y., Chen, Y. X. & Zhang, H. F. (2024). Remnant cholesterol and risk of incident hypertension: a population-based prospective cohort study. Hypertension Research, 1-10.

Guo, J., Ren, R., Sun, K., Yao, X., Lin, J., Wang, G. & Guo, F. (2020). PERK controls bone homeostasis through the regulation of osteoclast differentiation and function. Cell death & disease, 11(10), 847

Guo, Y. J., Pan, W. W., Liu, S. B., Shen, Z. F., Xu, Y., & Hu, L. L. (2020). ERK/MAPK signalling pathway and tumorigenesis. *Experimental and therapeutic medicine*, *19*(3), 1997-2007.

Gustafsson, B. I., Westbroek, I., Waarsing, J. H., Waldum, H., Solligård, E., Brunsvik, A. & Syversen, U. (2006). Long-term serotonin administration leads to higher bone mineral density, affects bone architecture, and leads to higher femoral bone stiffness in rats. Journal of cellular biochemistry, 97(6), 1283-1291.

Hachani, R., Houcine, D. A. B., Sakly, M., Vicaut, E., Callebert, J., Sercombe,

R., & Kacem, K. (2010). Influence of antagonist sensory and sympathetic nerves on smooth muscle cell differentiation in hypercholesterolemic rat. *Autonomic Neuroscience*, *155*(1-2), 82-90.

Han, Y., You, X., Xing, W., Zhang, Z., & Zou, W. (2018). Paracrine and endocrine actions of bone—the functions of secretory proteins from osteoblasts, osteocytes, and osteoclasts. Bone research, 6(1), 16

Harrabi, B., Athmouni, K., Hamdaoui, L., Ben Mahmoud, L., Hakim, A., El Feki, A. & Ghozzi, H. (2017). Polysaccharides extraction from Opuntia stricta and their protective effect against HepG2 cell death and hypolipidaemic effects on hyperlipidaemia rats induced by high-fat diet. Archives of Physiology and Biochemistry, 123(4), 225-237

Hashemi, M., Hoshyar, R., R Ande, S., Chen, Q. M., Solomon, C., Zuse, A., & Naderi, M. (2017). Mevalonate cascade and its regulation in cholesterol metabolism in different tissues in health and disease. Current molecular pharmacology, 10(1), 13-26.

Hendrijantini, N., Hartono, C. K., Daniati, R. P., Hong, G., Sitalaksmi, R. M., Kuntjoro, M., & Ari, M. D. A. (2021). Human umbilical cord mesenchymal stem cell-induced osterix, bone morphogenetic protein-2, and tartrate-resistant acid phosphatase expression in osteoporotic mandibular bone. European Journal of Dentistry, 15(01), 084-089

Hernández, A., Concepción, M. T., Rodríguez, M., Salido, E., & Torres, A. (1996). High phosphorus diet increases preproPTH mRNA independent of calcium and calcitriol in normal rats. Kidney international, 50(6), 1872-1878.

Herwana, E., Setiabudy, R., Soegondo, S. I. D. H. A. R. T. A. W. A. N., Baziad, A., & Hidayat, A. (2020). Soy isoflavone supplementation reduces RANKL/OPG ratio on postmenopausal women with osteopenia. International Journal of Pharmaceutical Research, 12(4), 1820-1827

Hofmaenner, D. A., Kleyman, A., Press, A., Bauer, M., & Singer, M. (2022). The

many roles of cholesterol in sepsis: a review. *American Journal of Respiratory and Critical Care Medicine*, *205*(4), 388-396.

Hooshiar, S. H., Tobeiha, M., & Jafarnejad, S. (2022). Soy isoflavones and bone health: Focus on the RANKL/RANK/OPG pathway. BioMed Research International, 2022. .

Huff, T., Boyd, B., & Jialal, I. (2017). Physiology, cholesterol.

Hussein, A. A., & Mustafa, N. G. (2024). Impact of a high-fat diet on dyslipidemia and gene expression of low-density lipoprotein receptors in male rats. *Iraqi Journal of Veterinary Sciences*, *38*(1), 133-138.

I, K., N, M., Y-J, Y., & J-Y, L. (**2018**). Induce Hyperlipidemia in Rats Using High Fat Diet Investigating Blood Lipid and Histopathology. *Journal of Hematology and Blood Disorders*, *4*(1), 1–5.

Infante, M., Fabi, A., Cognetti, F., Gorini, S., Caprio, M., & Fabbri, A. (2019). RANKL/RANK/OPG system beyond bone remodeling: involvement in breast cancer and clinical perspectives. Journal of Experimental & Clinical Cancer Research, 38, 1-18

Iwanaga, R., Orlicky, D. J., Arnett, J., Guess, M. K., Hurt, K. J., & Connell, K. A. (2016). Comparative histology of mouse, rat, and human pelvic ligaments. International urogynecology journal, 27, 1697-1704.

Jain, R., Watson, U., Vasudevan, L., & Saini, D. K. (2018). ERK activation pathways downstream of GPCRs. *International review of cell and molecular biology*, *338*, 79-109.

Jann, J., Gascon, S., Roux, S., & Faucheux, N. (2020). Influence of the TGF-β superfamily on osteoclasts/osteoblasts balance in physiological and pathological bone conditions .International journal of molecular sciences, 21(20), 7597

Jasim, R. F., & Allwsh, T. A. (2021). Effect of plasma isolated Orexin-A on the regulation of metabolites in male rats. *Iraqi Journal of Veterinary Sciences*, *35*(3), 451-457.

Jasim, R. F., & Allwsh, T. A. (2021). Effect of plasma isolated Orexin-A on the regulation of metabolites in male rats. Iraqi Journal of Veterinary Sciences, 35(3), 451-457.

Jayalekshmi, V. S., Jaikumar, V. S., Mehra, P., Thulaseedharan, T., Vinod, V. M., & Ramachandran, S. (2023). Differential expression of lipid metabolic genes in hypercholesterolemic rabbit placenta predisposes the offspring to develop atherosclerosis in early adulthood. *Life Sciences*, *327*, 121823.

Jin, Y., Zhou, B. H., Zhao, J., Ommati, M. M., Wang, S., & Wang, H. W. (2023). Fluoride-induced osteoporosis via interfering with the RANKL/RANK/OPG pathway in ovariectomized rats: Oophorectomy shifted skeletal fluorosis from osteosclerosis to osteoporosis. Environmental Pollution, 336, 122407

Jomova, K., Raptova, R., Alomar, S. Y., Alwasel, S. H., Nepovimova, E., Kuca, K., & Valko, M. (2023). Reactive oxygen species, toxicity, oxidative stress, and antioxidants: Chronic diseases and aging. *Archives of toxicology*, *97*(10), 2499-2574.

Kadhem, M. A., Al-Rekabi, F. M., & Jassam, S. A. (2023). The Ameliorating Effects of Salvia hispanica Seeds Ethanolic Extract on Methylprednisolone-Induced Osteoporosis in Female Rabbits. The Iraqi Journal of Veterinary Medicine, 47(1), 28-37

Kara-Ali, G. H., Cano, L., Dion, S., Imerzoukene, G., Hamon, A., Simoes Eugénio, M. & Dimanche-Boitrel, M. T. (2024). Trim21 deficiency in mice increases HCC carcinogenesis in a NASH context and is associated with immune checkpoint upregulation. *International Journal of Cancer*.

Katica, M., & Tepekoy, F. (2020). The effect of Calcitriol 1, 25 (OH) 2-D3 on osteoblast-like cell proliferation during in vitro cultivation. Veterinary Journal of Mehmet Akif Ersoy University, 5(1), 11-17

Khalaf, R. M., & Almudhi, A. A. (2022). The effect of vitamin D deficiency on the RANKL/OPG ratio in rats. Journal of Oral Biology and Craniofacial Research, 12(2), 228-232.

Khosravi, M., Hosseini-Fard, R., & Najafi, M. (2018). Circulating low density lipoprotein (LDL). Hormone Molecular Biology and Clinical Investigation, 35(2), 20180024.

Kim, H., Oh, B., & Park-Min, K. H. (2021). Regulation of osteoclast differentiation and activity by lipid metabolism. Cells, 10(1), 89.

Kim, J. M., Lin, C., Stavre, Z., Greenblatt, M. B., & Shim, J. H. (2020). Osteoblast-osteoclast communication and bone homeostasis. Cells, 9(9), 2073.

Kitaura, H., Marahleh, A., Ohori, F., Noguchi, T., Shen, W. R., Qi, J. & Mizoguchi, I. (2020). Osteocyte-related cytokines regulate osteoclast formation and bone resorption. *International journal of molecular sciences*, *21*(14), 5169.

Kuo, T. R., & Chen, C. H. (2017). Bone biomarker for the clinical assessment of osteoporosis: recent developments and future perspectives. Biomarker research, 5(1), 1-9.

Lakhan, K., & Khedkar, K. M. (2022). REVIEW ON LIPID PROFILE OF CHOLESTEROL.

Lecumberri, E., Goya, L., Mateos, R., Alía, M., Ramos, S., Izquierdo-Pulido, M., & Bravo, L. (2007). A diet rich in dietary fiber from cocoa improves lipid profile and reduces malondialdehyde in hypercholesterolemic rats. *Nutrition*, *23*(4), 332-341.

Lee, J. C., Joung, K. H., Kim, J. M., Kang, S. M., Kim, H. J., & Ku, B. J. (2022). Effect of cholesterol-lowering agents on soluble epidermal growth factor receptor level in type 2 diabetes and hypercholesterolemia. Medicine, 101(34), e30287.

Lee, Y. D., Yoon, S. H., Park, C. K., Lee, J., Lee, Z. H., & Kim, H. H. (2015). Caveolin-1 regulates osteoclastogenesis and bone metabolism in a sex-dependent manner. Journal of Biological Chemistry, 290(10), 6522- 6530.

Leevers, S. J., & Marshall, C. J. (1992). Activation of extracellular signal-regulated

kinase, ERK2, by p21ras oncoprotein. The EMBO journal, 11(2), 569-574

Li, B., Wang, P., Jiao, J., Wei, H., Xu, W., & Zhou, P. (2022). Roles of the RANKL–RANK Axis in Immunity—Implications for Pathogenesis and Treatment of Bone Metastasis. *Frontiers in Immunology*, *13*, 824117.

Li, L. H., Dutkiewicz, E. P., Huang, Y. C., Zhou, H. B., & Hsu, C. C. (**2019**). Analytical methods for cholesterol quantification. *Journal of Food and Drug Analysis*, *27*(2), 375–386.

Li, X., Jie, Q., Zhang, H., Zhao, Y., Lin, Y., Du, J. & Luo, Z. (2016). Disturbed MEK/ERK signaling increases osteoclast activity via the Hedgehog-Gli pathway in postmenopausal osteoporosis. *Progress in biophysics and molecular biology*, *122*(2), 101-111.

Li, X., Wang, L., Huang, B., Gu, Y., Luo, Y., Zhi, X. & Su, J. (2020). Targeting actin-bundling protein L-plastin as an anabolic therapy for bone loss. Science advances, 6(47), eabb7135

Lian, W. S., Wu, R. W., Chen, Y. S., Ko, J. Y., Wang, S. Y., Jahr, H., & Wang, F. S. (2021). MicroRNA-29a in osteoblasts represses high-fat diet-mediated osteoporosis and body adiposis through targeting leptin. International Journal of Molecular Sciences, 22(17), 9135

Lin, X., Patil, S., Gao, Y. G., & Qian, A. (2020). The bone extracellular matrix in bone formation and regeneration. Frontiers in pharmacology, 11, 521497

Liu, Q., Li, M., Wang, S., Xiao, Z., Xiong, Y., & Wang, G. (2020). Recent advances of osterix transcription factor in osteoblast differentiation and bone formation. Frontiers in cell and developmental biology, 8, 601224.

Liu, W., & Zhang, X. (2015). Receptor activator of nuclear factor-κB ligand (RANKL)/RANK/osteoprotegerin system in bone and other tissues. Molecular Medicine Reports, 11(5), 3212-3218

Lombardi, G., Ziemann, E., & Banfi, G. (2019). Physical activity and bone health: what is the role of immune system? A narrative review of the third way. *Frontiers in endocrinology*, *10*, 443554.

López-Gómez, J. J., Pérez-Castrillón, J. L., García de Santos, I., Pérez-Alonso, M., Izaola-Jauregui, O., Primo-Martín, D., & De Luis-Román, D. A. (2022). Influence of obesity on bone turnover markers and fracture risk in postmenopausal women. Nutrients, 14(8), 1617

Ma, Q., Liang, M., Wu, Y., Luo, F., Ma, Z., Dong, S. & Dou, C. (2021). Osteoclast-derived apoptotic bodies couple bone resorption and formation in bone remodeling. Bone research, 9(1), 5

Ma, X., Su, P., Yin, C., Lin, X., Wang, X., Gao, Y. & Qian, A. (2020). The roles of FoxO transcription factors in regulation of bone cells function. *International journal of molecular sciences*, *21*(3), 692.

Mahdi, S. S., Albazi, W., & Al-ASadiq Safaaameli, M. hussain. (**2021**). The Beneficial Effect of Glutathione in Protection of the Central Nerves System from Damage Induced by D-galactose. *Proceedings of the 1st International Ninevah Conference on Medical Sciences (INCMS 2021)*, *38*(Incms), 70–74.

Mandal, C. C. (2015). High cholesterol deteriorates bone health: new insights into molecular mechanisms. *Frontiers in endocrinology*, *6*, 154102.

Mangu, S. R., Patel, K., Sukhdeo, S. V., Savitha, M. R., & Sharan, K. (2022). Maternal high-cholesterol diet negatively programs offspring bone development and downregulates hedgehog signaling in osteoblasts. *Journal of Biological Chemistry*, *298*(9).

Marenzana, M., & Arnett, T. R. (2013). The key role of the blood supply to bone. *Bone research*, *1*(1), 203-215.

Martin-Vega, A., & Cobb, M. H. (2023). Navigating the ERK1/2 MAPK cascade. Biomolecules, 13(10), 1555.

März, W., Kleber, M. E., Scharnagl, H., Speer, T., Zewinger, S., Ritsch, A. & Laufs, U. (2017). HDL cholesterol: reappraisal of its clinical relevance. *Clinical Research in Cardiology*, *106*, 663-675.

Mathis, K. M., Sturgeon, K. M., Winkels, R. M., Wiskemann, J., De Souza, M. J., & Schmitz, K. H. (2018). Bone resorption and bone metastasis

risk. *Medical Hypotheses*, *118*, 36-41.

Matsubara, T., Kida, K., Yamaguchi, A., Hata, K., Ichida, F., Meguro, H. & Yoneda, T. (2008). Bmp2 regulates osterix through msx2 and runx2 during osteoblast differentiation∗. *Journal of biological chemistry*, *283*(43), 29119-29125.

Matsushita, T., Chan, Y. Y., Kawanami, A., Balmes, G., Landreth, G. E., & Murakami, S. (2009). Extracellular signal-regulated kinase 1 (ERK1) and ERK2 play essential roles in osteoblast differentiation and in supporting osteoclastogenesis. Molecular and cellular biology, 29(21), 5843-5857.

McDonald, M. M., Kim, A. S., Mulholland, B. S., & Rauner, M. (2021). New insights into osteoclast biology. Journal of Bone and Mineral Research Plus, 5(9), e10539

Mederle, O. A., Balas, M., Ioanoviciu, S. D., Gurban, C. V., Tudor, A., & Borza, C. (2018). Correlations between bone turnover markers, serum magnesium and bone mass density in postmenopausal osteoporosis. *Clinical interventions in aging*, 1383-1389.

Minaychev, V. V., Smirnova, P. V., Kobyakova, M. I., Teterina, A. Y., Smirnov, I. V., Skirda, V. D. & Fadeeva, I. S. (2024). Low-Temperature Calcium Phosphate Ceramics Can Modulate Monocytes and Macrophages Inflammatory Response In Vitro. *Biomedicines*, *12*(2), 263.

Miranda, R. M., Fernandes, J. L., SANTOS, M. D. S., Jácome-Santos, H., Milagres, R. M. C., Pretti, H. & Macari, S. (2024). Influence of risedronate on orthodontic tooth movement in rodents: a systematic review and case report. Dental Press Journal of Orthodontics, 28, e2322280.

Mira-Pascual, L. (2019). *Role of tartrate-resistant acid phosphatase in bone remodeling* (Doctoral dissertation, Karolinska Institutet (Sweden).

Miyazaki, S., Igarashi, M., Nagata, A., Tominaga, Y., Onodera, K., & Komoda, T. (2003). Development of immunoassays for type-5 tartrate-resistant acid phosphatase in human serum. Clinica chimica acta, 329(1-2), 109-115.

Mizoguchi, T., & Ono, N. (2021). The diverse origin of bone-forming osteoblasts. *Journal of Bone and Mineral Research*, *36*(8), 1432-1447

Mohamad, N., Nabih, E. S., Zakaria, Z. M., Nagaty, M. M., & Metwaly, R. G. (2019). Insight into the possible role of miR-214 in primary osteoporosis via osterix. *Journal of Cellular Biochemistry*, *120*(9), 15518-15526.

Molagoda, I. M. N., Athapaththu, A. M. G. K., Park, E. K., Choi, Y. H., Jeon, Y. J., & Kim, G. Y. (2022). Fermented oyster (Crassostrea gigas) extract cures and prevents prednisolone-induced bone resorption by activating osteoblast differentiation. Foods, 11(5), 678

Morello, F., Saglio, E., Noghero, A., Schiavone, D., Williams, T. A., Verhovez, A. & Mulatero, P. (2009). LXR-activating oxysterols induce the expression of inflammatory markers in endothelial cells through LXR-independent mechanisms. *Atherosclerosis*, *207*(1), 38-44

Morgan, A. E., Mooney, K. M., Wilkinson, S. J., Pickles, N. A., & Mc Auley, M. T. (2016). Cholesterol metabolism: A review of how ageing disrupts the biological mechanisms responsible for its regulation. Ageing research reviews, 27, 108-124.

Mustofa, S., Adjeng, A. N. T., Kurniawaty, E., Ramadhita, L., & Tamara, T. (2024). Influence of Rhizophora apiculata barks extract on Cholesterol, Triglyceride, LDL, and HDL Levels of Rattus norvegicus (Sprague Dawley) fed high-cholesterol diet. *Research Journal of Pharmacy and Technology*, *17*(1), 396-400.

Mutemberezi, V., Guillemot-Legris, O., & Muccioli, G. G. (2016). Oxysterols: From cholesterol metabolites to key mediators. *Progress in lipid research*, *64*, 152-169.

Nabi, S., & Nabi, S. (2014). Biochemical Estimation Techniques. Toxic Effects of Mercury, 77-83

Naito, H. K., & Kaplan, A. (1984). Cholesterol. Clinical chemistry, 119, 4-1213.

Nakamura, M., Aoyama, N., Yamaguchi, S., & Sasano, Y. (2021). Expression of tartrate-resistant acid phosphatase and cathepsin K during osteoclast differentiation in developing mouse mandibles. Biomedical

Nakashima, K., Zhou, X., Kunkel, G., Zhang, Z., Deng, J. M., Behringer, R. R., & De Crombrugghe, B. (2002). The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation. Cell, 108(1), 17-29

Nessler, K., Windak, A., Grzybczak, R., Nessler, M., Siniarski, A., & Gajos, G. (2018). High-density lipoprotein (HDL) cholesterol: more complicated than we think?. Annals of Agricultural and Environmental Medicine, 25(3).

Nordin, B. C. (1997). Calcium and osteoporosis. *Nutrition*, *13*(7-8), 664-686.

Omi, M., & Mishina, Y. (2022). Roles of osteoclasts in alveolar bone. remodeling. genesis, 60(8-9), e23490.

Oniki, K., Ogura, M., Matsumoto, E., Watanabe, H., Imafuku, T., Seguchi, Y. & Saruwatari, J. (2024). Impaired Cholesterol Efflux Capacity rather than Low HDL-C Reflects Oxidative Stress under Acute Myocardial Infarction. *Journal of Atherosclerosis and Thrombosis*, 64691.

Pacifico, L., Andreoli, G. M., D'Avanzo, M., De Mitri, D., & Pierimarchi, P. (2018). Role of osteoprotegerin/receptor activator of nuclear factor kappa B/receptor activator of nuclear factor kappa B ligand axis in nonalcoholic fatty liver disease. *World Journal of Gastroenterology*, *24*(19), 2073.

Pan, X. F., Wang, L., & Pan, A. (2021). Epidemiology and determinants of obesity in China. The lancet Diabetes & endocrinology, 9(6), 373-392

Papadopoulou, A., Bountouvi, E., & Karachaliou, F. E. (2021). The molecular basis of calcium and phosphorus inherited metabolic disorders. *Genes*, *12*(5), 734.

Park, J. I. (2023). MAPK-ERK Pathway. International Journal of Molecular

Sciences, 24(11), 9666.

Peleman, C., Francque, S., & Berghe, T. V. (2024). Emerging role of ferroptosis in metabolic dysfunction-associated steatotic liver disease: revisiting hepatic lipid peroxidation. *EBioMedicine*, *102*.

Peng, T. C., Gitelman, H. J., & Garner, S. C. (1979). Acute lead-induced increase in serum calcium in the rat without increased secretion of calcitonin. Proceedings of the Society for Experimental Biology and Medicine, 160(1), 114-117.

Pirih, F., Lu, J., Ye, F., Bezouglaia, O., Atti, E., Ascenzi, M. G. & Tintut, Y. (2012). Adverse effects of hyperlipidemia on bone regeneration and strength. *Journal of bone and mineral research*, *27*(2), 309-318.

Pizzorno, J. (2014). Glutathione!. *Integrative Medicine: A Clinician's Journal*, *13*(1), 8.

Prideaux, M., Findlay, D. M., & Atkins, G. J. (2016). Osteocytes: the master cells in bone remodelling. Current opinion in pharmacology, 28, 24-30

Purva, A., Sharma, K., & Khan, M. S. (2020). A review on dyslipidemia: types, risk factors and management. *Asian Journal of Pharmaceutical Research and Development*, *8*(2), 96-98.

Qin, S. (2020). LDL and HDL oxidative modification and atherosclerosis. Lipid Transfer in Lipoprotein Metabolism and Cardiovascular Disease, 157-169.

Qin, X. F., Zhao, L. S., Chen, W. R., & Wang, H. (2015). Effects of vitamin D on plasma lipid profiles in statin-treated patients with hypercholesterolemia: a randomized placebo-controlled trial. *Clinical Nutrition*, *34*(2), 201-206.

Qu, S., Wu, J., Bao, Q., Yao, B., Duan, R., Chen, X. & Ma, C. (2019). Osterix promotes the migration and angiogenesis of breast cancer by upregulation of S100A4 expression. Journal of cellular and molecular medicine, 23(2), 1116-1127

Queiroz, M. I., Lazaro, C. M., Dos Santos, L. M., Rentz, T., Virgilio-da-Silva, J. V., Moraes-Vieira, P. M. & Oliveira, H. C. (2024). In vivo chronic exposure

to inorganic mercury worsens hypercholesterolemia, oxidative stress and atherosclerosis in the LDL receptor knockout mice. *Ecotoxicology and Environmental Safety*, *275*, 116254.

Raheem, H. A., Albazi, W., Altaee, R., Al-Thuwaini, T. M., & Jhoni, G. H. (2023). Effect of hypercholestermic diet on the β-amyloid deposition and microglial cells with some biomarkers alterations in male rats. Iraqi Journal of Veterinary Sciences, 37(Supplement I-IV), 251-257

Reiss, A. B., Miyawaki, N., Moon, J., Kasselman, L. J., Voloshyna, I., D'Avino Jr, R., & De Leon, J. (2018). CKD, arterial calcification, atherosclerosis and bone health: Inter-relationships and controversies. *Atherosclerosis*, *278*, 49-59.

Research, 42(1), 13-21 .**Aguilar-Ballester, M., Herrero-Cervera, A., Vinué, Á., Martínez-Hervás, S., & González-Navarro, H. (2020).** Impact of cholesterol metabolism in immune cell function and atherosclerosis. *Nutrients*, *12*(7), 2021.

Rezatabar, S., Karimian, A., Rameshknia, V., Parsian, H., Majidinia, M., Kopi, T. A. & Yousefi, B. (2019). RAS/MAPK signaling functions in oxidative stress, DNA damage response and cancer progression. *Journal of cellular physiology*, *234*(9), 14951-14965.

Riggs, B. L., & Parfitt, A. M. (2005). Drugs used to treat osteoporosis: the critical need for a uniform nomenclature based on their action on bone remodeling. Journal of bone and mineral research, 20(2), 177-184.

Robertson, W. G., Marshall, R. W., & Walser, M. (1979). Calcium measurements in serum and plasma—total and ionized. CRC Critical Reviews in Clinical Laboratory Sciences, 11(3), 271-304

Santos-López, J. A., Garcimartin, A., López-Oliva, M. E., Bautista-Avila, M., González-Muñoz, M. J., Bastida, S., ... & Sánchez-Muniz, F. J. (2017). Chia oil–enriched restructured pork effects on oxidative and inflammatory status of aged rats fed high cholesterol/high fat diets. Journal of medicinal

food, 20(5), 526-534

Sawale, P. D., Pothuraju, R., Abdul Hussain, S., Kumar, A., Kapila, S., & Patil, G. R. (2016). Hypolipidaemic and anti-oxidative potential of encapsulated herb (Terminalia arjuna) added vanilla chocolate milk in high cholesterol fed rats. Journal of the Science of Food and Agriculture, 96(4), 1380-1385. https://doi.org/10.1002/jsfa.7234

Sawy, H. A. E., Hassan, D. F., & Rizk, A. I. (2022). Effect of natural sources rich in calcium on treated rats induced osteoporosis. Discover Food, 2(1), 23.

Schade, D. S., Shey, L., & Eaton, R. P. (2020). Cholesterol review: a metabolically important molecule. Endocrine Practice, 26(12), 1514-1523.

Schulte, F. A., Ruffoni, D., Lambers, F. M., Christen, D., Webster, D. J., Kuhn, G., & Müller, R. (2013). Local mechanical stimuli regulate bone formation and resorption in mice at the tissue level. PloS one, 8(4), e62172.

Seifert, M. F., & Marks Jr, S. C. (1985). Morphological evidence of reduced bone resorption in the osteosclerotic (oc) mouse. American journal of anatomy, https://doi.org/10.1002/aja.1001720204172(2), 141-153.

Shaik, F., Cuthbert, G. A., Homer-Vanniasinkam, S., Muench, S. P., Ponnambalam, S., & Harrison, M. A. (2020). Structural basis for vascular endothelial growth factor receptor activation and implications for disease therapy. *Biomolecules*, *10*(12), 1673.

Shaker, J. L., & Deftos, L. (2023). Calcium and phosphate homeostasis. Endotext [Internet]

Shankaran, V., Wisinski, K. B., Mulcahy, M. F., & Benson, A. B. (2008). The role of molecular markers in predicting response to therapy in patients with colorectal cancer. Molecular diagnosis & therapy, 12, 87-98.

Si, J., Wang, C., Zhang, D., Wang, B., Hou, W., & Zhou, Y. (2020). Osteopontin in bone metabolism and bone diseases. Medical science monitor: international medical journal of experimental and clinical research, 26, e919159-1

Sims, N. A., & Martin, T. J. (2020). Osteoclasts provide coupling signals to

osteoblast lineage cells through multiple mechanisms. Annual review of physiology, 82, 507-529

Soffientini, U., & Graham, A. (2016). Intracellular cholesterol transport proteins: roles in health and disease. Clinical Science, 130(21), 1843-1859.

Srivastava, R. K., Sapra, L., & Mishra, P. K. (2022). Osteometabolism: metabolic alterations in bone pathologies. *Cells*, *11*(23), 3943.

Šromová, V., Sobola, D., & Kaspar, P. (2023). A Brief Review of Bone Cell Function and Importance. Cells, 12(21), 2576.

Stranahan, A. M., Cutler, R. G., Button, C., Telljohann, R., & Mattson, M. P. (2011). Diet-induced elevations in serum cholesterol are associated with alterations in hippocampal lipid metabolism and increased oxidative stress. *Journal of neurochemistry*, *118*(4), 611-615.

Su, N., Yang, J., Xie, Y., Du, X., Chen, H., Zhou, H., & Chen, L. (2019). Bone function, dysfunction and its role in diseases including critical illness. International journal of biological sciences, 15(4), 776

Sul, O. J., Kim, J. E., Ke, K., Suh, J. H., & Choi, H. S. (2020). Atherogenic diet-induced bone loss is primarily due to increased osteoclastogenesis in mice. *The Journal of Nutritional Biochemistry*, *79*, 108337.

Sun, Y., Li, J., Xie, X., Gu, F., Sui, Z., Zhang, K., & Yu, T. (2021). Recent advances in osteoclast biological behavior. Frontiers in Cell and Developmental Biology, 9, 788680

Suske, G., Bruford, E., & Philipsen, S. (2005). Mammalian SP/KLF transcription factors: bring in the family. Genomics, 85(5), 551-556.

Szekanecz, Z., Raterman, H. G., Pethő, Z., & Lems, W. F. (2019). Common mechanisms and holistic care in atherosclerosis and osteoporosis. Arthritis research & therapy, 21, 1-10.

Tall, A. R., & Yvan-Charvet, L. (2015). Cholesterol, inflammation, and innate immunity. Nature Reviews Immunology, 15(2), 104-116

Tamiya, Y., Hamba, H., Mitomo, K., Furusawa, M., & Muramatsu, T. (2021).

High-cholesterol condition promotes apical periodontitis and bone resorption in rats. *Journal of Hard Tissue Biology*, *30*(2), 199-204.

Thrall, D. E. (2017). Introduction to radiographic interpretation. Textbook of Veterinary Diagnostic Radiology-E-Book, 110.

Tian, L., & Yu, X. (2015). Lipid metabolism disorders and bone dysfunction-interrelated and mutually regulated. Molecular medicine reports, 12(1), 783-794

Ticho, A. L., Malhotra, P., Dudeja, P. K., Gill, R. K., & Alrefai, W. A. (2019). Intestinal absorption of bile acids in health and disease. Comprehensive Physiology, 10(1), 21.

Trajkovska, K. T., & Topuzovska, S. (2017). High-density lipoprotein metabolism and reverse cholesterol transport: strategies for raising HDL cholesterol. Anatolian journal of cardiology, 18(2), 149.

Tsukasaki, M., & Takayanagi, H. (2022). Osteoclast biology in the single-cell era. Inflammation and Regeneration.

Tu, C. L., & Chang, W. (2024). Vitamin D and the calcium-sensing receptor. In Feldman and Pike's Vitamin D (pp. 331-363). Academic Press.

Tuzcu, Z., Orhan, C., Sahin, N., Juturu, V., & Sahin, K. (2017). Cinnamon polyphenol extract inhibits hyperlipidemia and inflammation by modulation of transcription factors in high-fat diet-fed rats. Oxidative medicine and cellular longevity, 2017

Udagawa, N., Koide, M., Nakamura, M., Nakamichi, Y., Yamashita, T., Uehara, S. & Tsuda, E. (2021). Osteoclast differentiation by RANKL and OPG signaling pathways. Journal of bone and mineral metabolism, 39, 19-26

Valverde, A., Serafín, V., Montero-Calle, A., González-Cortés, A., Barderas, R., Yáñez-Sedeño, P. & Pingarrón, J. M. (2020). Carbon/Inorganic Hybrid Nanoarchitectures as Carriers for Signaling Elements in Electrochemical Immunosensors: First Biosensor for the Determination of the Inflammatory

and Metastatic Processes Biomarker RANK-ligand. ChemElectroChem, 7(3), 810-820.

Vancea, A., Serban, O., & Fodor, D. (2021). Relationship between osteopontin and bone mineral density. Acta Endocrinologica (Bucharest), 17(4), 509

Wang, B., Wang, H., Li, Y., & Song, L. (2022). Lipid metabolism within the bone micro-environment is closely associated with bone metabolism in physiological and pathophysiological stages. *Lipids in Health and Disease*, *21*(1), 5.

Wang, W., Ye, J., Xu, L., Mo, D. G., Chen, C., Li, T., & Yao, H. C. (2024). The effects of IGF-1 and IGFBP-2 treatments on the atherosclerosis in the aorta and the coronary arteries of the high cholesterol diet-fed rabbits. *International Immunopharmacology*, *127*, 111409.

Wang, Z. (2016). Transactivation of epidermal growth factor receptor by G protein-coupled receptors: recent progress, challenges and future research. *International journal of molecular sciences*, *17*(1), 95.

Wang, Z., Liu, L., Li, C. Y., Zhao, Y. W., Tong, X. Y., Cheng, X. Y., & Ouyang, J. M. (2024). Carboxymethylated Rhizoma alismatis polysaccharides reduces the risk of calcium oxalate stone formation by reducing cellular inflammation and oxidative stress. *Urolithiasis*, *52*(1), 63.

Wawrzyniak, A., & Balawender, K. (2022). Structural and metabolic changes in bone. Animals, 12(15), 1946

Weissel, M., Kainz, H., Tyl, E., Ogunyemi, E., & Woloszczuk, W. (1991). Clinical evaluation of new assays for determination of serum calcitonin concentrations. European Journal of Endocrinology, 124(5), 540-544

Weivoda, M. M., Chew, C. K., Monroe, D. G., Farr, J. N., Atkinson, E. J., Geske, J. R., ... & Khosla, S. (2020). Identification of osteoclast-osteoblast coupling factors in humans reveals links between bone and energy metabolism. Nature communications, 11(1), 87

Winge, M. I., Reikerås, O., & Røkkum, M. (2021). Calcium phosphate bone cement: a possible alternative to autologous bone graft. A radiological and biomechanical comparison in rat tibial bone. Archives of orthopaedic and trauma surgery, 131, 1035-1041.

Wong, S. T., Spoo, J., Kerst, K. C., & Spring, T. G. (1985). Colorimetric determination of potassium in whole blood, serum, and plasma. Clinical chemistry, 31(9), 1464-1467

Wongdee, K., Rodrat, M., Teerapornpuntakit, J., Krishnamra, N., & Charoenphandhu, N. (2019). Factors inhibiting intestinal calcium absorption: hormones and luminal factors that prevent excessive calcium uptake. *The journal of physiological sciences*, *69*, 683-696.

Wu, Y., Liu, M., Zhou, H., He, X., Shi, W., Yuan, Q. & Xie, Y. (2023). COX-2/PGE2/VEGF signaling promotes ERK-mediated BMSCs osteogenic differentiation under hypoxia by the paracrine action of ECs. Cytokine, 161, 156058.

Xiao, W., Wang, Y., Pacios, S., Li, S., & Graves, D. T. (2016). Cellular and molecular aspects of bone remodeling. Tooth movement, 18, 9-16

Xiao, Y., Wang, J., Zhang, Y., Zhang, T., Qi, X., Hou, L. & Xu, F. (2024). Hepatic polypeptide nutrient solution improves high-cholesterol diet-induced rats with nonalcoholic fatty liver disease by activating AMP-activated protein kinase signaling pathway. *Food Science & Nutrition*.

Xie, J., Guo, J., Kanwal, Z., Wu, M., Lv, X., Ibrahim, N. A., ... & Sun, Q. (2020). Calcitonin and bone physiology: in vitro, in vivo, and clinical investigations. International Journal of Endocrinology, 2020.

Xie, Y., Ma, J., Yang, M., Fan, L., & Chen, W. (2021). Extracellular signal-regulated kinase signaling pathway and silicosis. Toxicology research, 10(3), 487-494

Xing, W., Godwin, C., Pourteymoor, S., & Mohan, S. (2019). Conditional disruption of the osterix gene in chondrocytes during early postnatal growth

impairs secondary ossification in the mouse tibial epiphysis. Bone research, 7(1), 24

Yan, W., Li, D., & Zhou, X. (2017). Pravastatin attenuates the action of the ETS domain-containing protein ELK1 to prevent atherosclerosis in apolipoprotein E-knockout mice via modulation of extracellular signal-regulated kinase 1/2 signal pathway. *Clinical and Experimental Pharmacology and Physiology*, *44*(3), 344-352.

Yang, D. K., Shin, E. K., Oh, Y. I., Lee, K. W., Lee, C. S., Kim, S. Y., ... & Song, J. Y. (2012). Comparison of four diagnostic methods for detecting rabies viruses circulating in Korea. Journal of Veterinary Science, 13(1), 43.

Yang, R. L., Shi, Y. H., Hao, G., Li, W., & Le, G. W. (2008). Increasing oxidative stress with progressive hyperlipidemia in human: relation between malondialdehyde and atherogenic index. *Journal of clinical biochemistry and nutrition*, *43*(3), 154-158.

Yao, Y., Cai, X., Ren, F., Ye, Y., Wang, F., Zheng, C. & Zhang, M. (2021). The macrophage-osteoclast axis in osteoimmunity and osteo-related diseases. *Frontiers in Immunology*, *12*, 664871.

Yari, Z., Tabibi, H., Najafi, I., Hedayati, M., & Movahedian, M. (2020). Effects of isoflavones on bone turnover markers in peritoneal dialysis patients: a randomized controlled trial. International Urology and Nephrology, 52, 1367-1376

Yasuda, H. (2021). Discovery of the RANKL/RANK/OPG system. Journal of bone and mineral metabolism, 39(1), 2-11

Yin, W., Li, Z., & Zhang, W. (2019). Modulation of bone and marrow niche by cholesterol. *Nutrients*, *11*(6), 1394.

You, L., Sheng, Z. Y., Tang, C. L., Chen, L., Pan, L., & Chen, J. Y. (2011). High cholesterol diet increases osteoporosis risk via inhibiting bone formation in rats. *Acta Pharmacologica Sinica*, *32*(12), 1498-1504.

Yu, W., Zhong, L., Yao, L., Wei, Y., Gui, T., Li, Z. & Qin, L. (2021). Bone marrow

adipogenic lineage precursors promote osteoclastogenesis in bone remodeling and pathologic bone loss. The Journal of clinical investigation, 131(2)

Zerwekh, J. E. (2008). Blood biomarkers of vitamin D status. The American journal of clinical nutrition, 87(4), 1087S-1091S

Zhang, J. K., Yang, L., Meng, G. L., Yuan, Z., Fan, J., Li, D. & Liu, J. (2013). Protection by salidroside against bone loss via inhibition of oxidative stress and bone-resorbing mediators. *PloS one*, *8*(2), e57251.

Zhang, Y., Chen, C. Y., Liu, Y. W., Rao, S. S., Tan, Y. J., Qian, Y. X. & Xie, H. (2021). Neuronal induction of bone-fat imbalance through osteocyte neuropeptide Y. *Advanced Science*, *8*(24), 2100808.

Zhang, Y., Liang, J., Liu, P., Wang, Q., Liu, L., & Zhao, H. (2022). The RANK/RANKL/OPG system and **tumor** bone metastasis: Potential mechanisms and therapeutic strategies. *Frontiers in Endocrinology*, *13*, 1063815.

Zhou, R., Guo, Q., Xiao, Y., Guo, Q., Huang, Y., Li, C., & Luo, X. (2021). Endocrine role of bone in the regulation of energy metabolism. Bone research, 9(1), 25

Zhu, F., Friedman, M. S., Luo, W., Woolf, P., & Hankenson, K. D. (2012). The transcription factor osterix (SP7) regulates BMP6-induced human osteoblast differentiation. *Journal of cellular physiology*, *227*(6), 2677-2685.

Appendixes

`

Appendixes I

Determination of Serum Total Cholesterol (TC) Concentration:

Total Cholesterol (TC) was detected by use Enzymatic method described by **(Allain and al. Allain C. C. et al., Clin. Chem. (1974), 20/4, p.470-475).** which reaction scheme is as follows:

Cholesterol esters cholesterol +free fatty acids

Cholesterol +O2 cholesten4 one3 +H2O2

2H2O2+phenol+PAP quinoneimine (pink) +4H2O2

Procedure:

At room temperature Add 1000 µL of reagent and after that add 10 µL of Blank, Standard, Control or specimen

Let them stand at 37 C for 5 min record absorbance at 500nm against reagent blank.

Calculation:

The result was calculated according to this equation

$$\textbf{Total Cholesterol (TC)} = \frac{abs(assay)}{abs(standerd)} \times \textbf{standerd concentration}$$

$abs(assay) = absorbance\ of\ samble$

$abs(standerd) = absorbance\ of\ stander$

`

Appendix II

Determination of serum Triglycerides (TG):

Total Serum Triglyceride was detected by use Fossati and Prencipe method associated with Trinder reaction. Reaction scheme (Fossati P., Prencipe L., Clin. Chem. (1982), 28, p.2077-2080.), (Trinder P. Ann. Clin. Biochem. (1969), 6, p.27-29.).it is as follows:

Triglycerides lipase ⟶ Glycerol + free fatty acids

GK

Glycerol + ATP ⟶ Glycerol 3 Phosphate + ADPGlycerol 3

GPO

Phosphate + O2 ⟶ DihydroxyacetonePhosphate + H2O2 POD

H2O2 + 4 Chlorophenol + PAP ⟶ Quinoneimine (pink) + H2O

The absorbance of the coloured complex (quinoneimine), proportional to the amount of triglycerides in the specimen, is measured at 500 nm.

REAGENTS PREPARATION

Add promptly the contents of vial R2 (Enzymes), into vial R1 (Buffer).

Mix gently and wait for complete dissolution before using reagent (approximately 2 minutes).

Procedure:

The procedure was done as shown in the table below:

Pipette into well identified test tubes	blank	Standard	assay
Regent	1ml	1ml	1ml
Demineralized water	10ul		
Standard		10ul	
Specimen			10ul

`

Calculation

The result was calculated according to this equation

Serum Triglyceride (TAG) = $\frac{abs(assay)}{abs(standerd}$ ×standerd concentration

Appendixes III

Determination of serum low-density lipoprotein (LDL):

PRINCIPLE:

Direct method using selective detergents without specimen pretreatment. During the first phase, only non-LDL lipoproteins are solubilised by detergent 1. Such generated Cholesterol, subjected to Cholesterol oxidase (CO) and Cholesterol esterase (CE) actions, produces a colourless compound. During the second phase, detergent 2 solubilises LDL-Cholesterol. The chromogenic coupler allows for colour formation that is proportional to the concentration of LDLCholesterol. The absorbance is measured at 546 nm (520-580).

PROCEDURE

The procedure was done as shown in the table below:

Tubes	Blank	Calibrator	assay
Regent 1	300 µL	300 µL	300 µL
Calibrator		3 µL	
specimen			3 µL

Let stand in 37°C for 5 min and Record absorbance of them at 546 nm against reagent blank. Add 100 µL of regent 2 Let stand in 37°C for 5 min and Record absorbance of them at 546 nm against reagent blank.

Calculation

`

The result was calculated according to this equation

$$\text{HDL-C-} = \frac{\Delta\, \boldsymbol{abs.assay} \times \text{calibrator concentration}}{\Delta \boldsymbol{abs.calibrator}}$$

Δ abs. assay = the different between the two record for the assay

Δabs. calibrator= the different between the two record for the calibrator.

Appendixes IV

Determination of serum high-density lipoprotein (HDL):

Accelerator selective detergent methodology. Direct method, without specimen pre-treatment. During the first phase, LDL, VLDL particles and Chylomicrons generate free Cholesterol, which through an enzymatic reaction, produce Hydrogen peroxide. The generated peroxide is consumed by a peroxidase reaction with DSBmT yielding a colourless product. During the second phase, specific detergent solubilizes HDL-Cholesterol. In conjunction with CO and CE action, POD + 4-AAP develop a colored reaction which is proportional to HDL-Cholesterol concentration. The absorbance is measured at 600.

PROCEDURE

The procedure was done as shown in the table below:

Tubes	**Blank**	**Calibrator**	**Assay**
Regent 1 300 µL	**300 µL**	**300 µL**	**300 µL**
Calibrator		**3 µL**	
Specimen			**3 µL**

Let stand in 37°C for 5 min and Record absorbance of them at 600 nm against reagent blank. Add 100 µL of regent 2 Let stand in 37°C for 5 min and Record absorbance of them at 600 nm against reagent blank.

Calculation

`

The result was calculated according to this equation

HDL-C- = $\Delta \frac{abs.assay}{\Delta abs.calibrator}$ × calibrator concentration

Δ abs .assay = the different between the two record for the assay

Δabs. calibrator= the different between the two record for the calibrator

Appendixes V

Determination of serum Biomarker of bone:

Determination of serum RANK:

Protocol:

Reagent Preparation:

1. Bring all kit components and samples to room temperature (18-25°C) before use Make sure all

components are dissolved and mixed well before using the kit.

2. If the kit will not be used up in 1 time, please only take out strips and reagents for present

experiment, and save the remaining strips and reagents as specified.

3. Dilute the 25× Wash Buffer into 1× Wash Buffer with double-distilled Water.

4. Standard Working Solution - Centrifuge the Standard at 1000 × g for 1 minute. Reconstitute the Standard with 1.0 mL of Standard Diluent Buffer, kept for 10 minutes at room temperature, shake gently (not to foam). The concentration of the Standard in the stock solution is 10 ng/mL. Pleas prepare 7 tubes containing 0.5 mL Standard Diluent Buffer and use the Diluted Standard to produce a double dilution series according to the picture shown below. To mix each tube thoroughly before the next transfer, pipette the solution up and down several times. Set up 7 points of Diluted Standard such as 10 ng/mL, 5 ng/mL, 2.5 ng/mL, 1.25 ng/mL, 0.63 ng/mL, 0.32 ng/mL,

`

0.16 ng/mL, and the last EP tubes with Standard Diluent is the Blank as 0 ng/mL. In order to guarantee the experimental results validity, please use the new Standard Solution for each experiment. When diluting the Standard from high concentration to low concentration, replace the pipette tip for each dilution. Note: the last tube is regarded as a Blank and do not pipette solution into it from the former tube.

5. Biotinylated Antibody and 1× Streptavidin-HRP - Briefly spor centrifuge the stock Biotinylated Antibody and Streptavidin-HRP before use. Dilute them to working concentration 100-fold with Biotinylated Antibody Diluent and HRP Diluent, respectively.

Samples Preparation:

1. Equilibrate all materials and prepared reagents to room temperature prior to use. Prior to use, mix all reagents thoroughly taking care not to create any foam within the vials.

2. The user should calculate the possible amount of the samples used in the whole test. Please reserve sufficient samples in advance.

3. Please predict the concentration before assaying. If values for these are not within the range of the Standard curve, users must determine the optimal sample dilutions for their particular experiments.

Assay Procedure:

1. Determine wells for Diluted Standard, Blank and Sample. Prepare 7 wells for Standard, 1 well for Blank. Add 100 μL each of Standard Working Solution (please refer to Reagent Preparation), or 100 μL of samples into the appropriate wells. Cover with the Plate Cover. Incubate for 80 minutes at 37°C. Note: solutions should be added to the bottom of the micro ELISA plate well, avoid touching the inside wall and causing foaming as much as possible.

2. Pour out the liquid of each well. Aspirate the solution and wash with 200 μL of 1× Wash Solution to each well and let it sit for 1-2 minutes. Remove the remaining liquid

`

from all wells completely by snapping the plate onto absorbent paper. Totally wash 3 times. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against absorbent

paper. Notes: (a) When adding Washing Solution, the pipette tip should not touch the wall of the wells to a void contamination. (b) Pay attention to pouring the washing liquid directly to ensure that the washing liquid does not contaminate other wells.

3. Add 100 μL of Biotinylated Antibody Working Solution to each well, cover the wells with the Plate http://www.elkbiotech.com elkbio@elkbiotech.com10 Cover and incubate for 50 minutes at 37°C.

4. Repeat the aspiration,wash process for total 3 times as conducted in step 2.

5. Add 100 μL of Streptavidin-HRP Working Solution to each well, cover the wells with the plate sealer and incubate for 50 minutes at 37°C.

6. Repeat the aspiration, wash process for total 5 times as conducted in step 2.

7. Add 90 μL of TMB Substrate Solution to each well. Cover with a new Plate Cover. Incubate for 20 minutes at 37°C (Don't exceed 30 minutes) in the dark. The liquid will turn blue by the addition of TMB Substrate Solution. Preheat the Microplate Reader for about 15 minutes before OD measurement.

8. Add 50 μL of Stop Reagent to each well. The liquid will turn yellow by the addition of Stop Reagent. Mix the liquid by tapping the side of the plate. If color change does not appear uniform, gently tap the plate to ensure thorough mixing. The insertion order of the Stop Reagent should be the same as that of the TMB Substrate Solution.

9. Wipe off any drop of water and fingerprint on the bottom of the plate and confirm there is no bubble on the surface of the liquid. Then, run the microplate reader and conduct measurement at 450 nm immediately.

Calculation of Results :

`

Average the duplicate readings for each Standard, Control, and Samples and subtract the average zero Standard optical density. Construct a Standard curve with the Rat RANK concentration on the y-axis and absorbance on the x-axis, and draw a best fit curve through the points on the graph. If samples have been diluted, the concentration read from the Standard curve must be multiplied by the dilution.

Appendix VI

Detection of serum RANKL

Reagent Preparation:

1. Bring all kit components and samples to room temperature (18-25°C) before use. Make sure all

components are dissolved and mixed well before using the kit.

2. If the kit will not be used up in 1 time, please only take out strips and reagents for present

experiment, and save the remaining strips and reagents as specified.

3. Dilute the 25× Wash Buffer into 1× Wash Buffer with double-distilled Water.

4. Standard Working Solution - Centrifuge the Standard at 1000 × g for 1 minute. Reconstitute the Standard with 1.0 mL of Standard Diluent Buffer, kept for 10 minutes at room temperature, shake gently (not to foam). The concentration of the Standard in the stock solution is 10 ng/mL. Pleas prepare 7 tubes containing 0.5 mL Standard Diluent Buffer and use the Diluted Standard to produce a double dilution series according to the picture shown below. To mix each tube thoroughly before the next transfer, pipette the solution up and down several times. Set up 7 points of Diluted Standard such as 10 ng/mL, 5 ng/mL, 2.5 ng/mL, 1.25 ng/mL, 0.63 ng/mL, 0.32 ng/mL, 0.16 ng/mL, and the last EP tubes with Standard Diluent is the Blank as 0 ng/mL. In order to guarantee the experimental results validity, please use the new Standard Solution for each experiment. When diluting the Standard from high concentration to low concentration, replace the pipette tip for each dilution. Note: the last tube is regarded as a Blank and do not pipette solution into it from the former tube.

`

5- Biotinylated Antibody and 1× Streptavidin-HRP - Briefly spor centrifuge the stock Biotinylated Antibody and Streptavidin-HRP before use. Dilute them to working concentration 100-fold with Biotinylated Antibody Diluent and HRP Diluent, respectively.

6. TMB Substrate Solution - Aspirate the needed dosage of the solution with sterilized tips and do not dump the residual solution into the vial again.

Samples Preparation:

1. Equilibrate all materials and prepared reagents to room temperature prior to use. Prior to use, mix all reagents thoroughly taking care not to create any foam within the vials.

2. The user should calculate the possible amount of the samples used in the whole test. Please reserve sufficient samples in advance.

3. Please predict the concentration before assaying. If values for these are not within the range of the Standard curve, users must determine the optimal sample dilutions for their particular experiments.

Assay Procedure

1. Determine wells for Diluted Standard, Blank and Sample. Prepare 7 wells for Standard, 1 well for Blank. Add 100 μL each of Standard Working Solution (please refer to Reagent Preparation), or 100 μL of samples into the appropriate wells. Cover with the Plate Cover. Incubate for 80 minutes at 37°C. Note: solutions should be added to the bottom of the micro ELISA plate well, avoid touching the inside wall and causing foaming as much as possible.

2. Pour out the liquid of each well. Aspirate the solution and wash with 200 μL of 1× Wash Solution to each well and let it sit for 1-2 minutes. Remove the remaining liquid from all wells completely by snapping the plate onto absorbent paper. Totally wash 3 times. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against absorbent paper. Notes: (a) When adding

`

Washing Solution, the pipette tip should not touch the wall of the wells to a void contamination. (b) Pay attention to pouring the washing liquid directly to ensure that the washing liquid does not contaminate other wells.

3. Add 100 μL of Biotinylated Antibody Working Solution to each well, cover the wells with the Plate http://www.elkbiotech.com elkbio@elkbiotech.com10 Cover and incubate for 50 minutes at 37°C.

4. Repeat the aspiration,wash process for total 3 times as conducted in step 2.

5. Add 100 μL of Streptavidin-HRP Working Solution to each well, cover the wells with the plate sealer and incubate for 50 minutes at 37°C.

6. Repeat the aspiration, wash process for total 5 times as conducted in step 2.

7. Add 90 μL of TMB Substrate Solution to each well. Cover with a new Plate Cover. Incubate for 20 minutes at 37°C (Don't exceed 30 minutes) in the dark. The liquid will turn blue by the addition of TMB Substrate Solution. Preheat the Microplate Reader for about 15 minutes before OD measurement.

8. Add 50 μL of Stop Reagent to each well. The liquid will turn yellow by the addition of Stop Reagent. Mix the liquid by tapping the side of the plate. If color change does not appear uniform, gently tap the plate to ensure thorough mixing. The insertion order of the Stop Reagent should be the same as that of the TMB Substrate Solution.

9. Wipe off any drop of water and fingerprint on the bottom of the plate and confirm there is no bubble on the surface of the liquid. Then, run the microplate reader and conduct measurement at 450 nm immediately.

Calculation of Results:

Average the duplicate readings for each Standard, Control, and Samples and subtract the average zero Standard optical density. Construct a Standard curve with the Rat RANK concentration on the y-axis and absorbance on the x-axis, and draw a best fit curve through the points on the graph. If samples have been diluted, the concentration read from the Standard curve must be multiplied by the dilution.

`

Appendix VII

Determination of serum extra cellular regulates kinas (ERK):

Reagent Preparation:

1. Bring all kit components and samples to room temperature (18-25°C) before use.

2. If the kit will not be used up in 1 time, please only take out strips and reagents for present experiment, and save the remaining strips and reagents as specified.

3. Dilute the 25×Wash Buffer into 1×Wash Buffer with double-distilled Water.

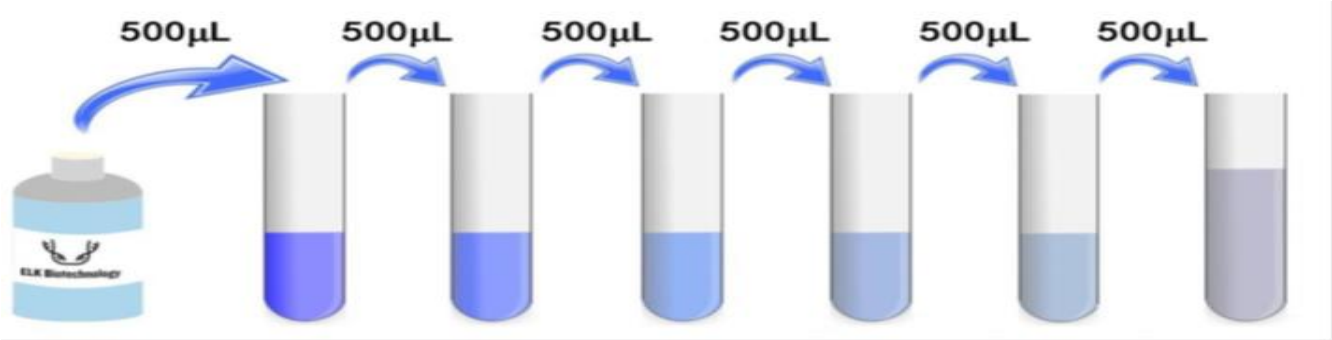
4. Standard Working Solution - Centrifuge the Standard at 1000 × g for 1 minute. Reconstitute the

Standard with 1.0 mL of Standard Diluent Buffer, kept for 10 minutes at room temperature, shake

gently (not to foam). The concentration of the Standard in the stock solution is 40 ng/mL. Please

prepare 7 tubes containing 0.5 mL Standard Diluent Buffer and use the Diluted Standard to produce a double dilution series according to the picture shown below. To mix each tube thoroughly before the next transfer, pipette the solution up and down several times. Set up 7 points of Diluted Standard such as 40 ng/mL, 20 ng/mL, 10 ng/mL, 5 ng/mL, 2.5 ng/mL, 1.25 ng/mL, 0.63 ng/mL, and the last EP tubes with Standard Diluent is the Blank as 0 ng/mL. In order to guarantee the experimental results validity, please use the new Standard Solution for each experiment. When diluting the Standard from high concentration to low concentration, replace the pipette tip for each dilution. Note: the last tube is regarded as a Blank and do not pipette solution into it from the former tube.

`



40 20 10 5 2.5 1.25 0.63

5. 1×Biotinylated Antibody and 1×Streptavidin-HRP - Briefly spin or centrifuge the stock Biotinylated Antibody and Streptavidin-HRP before use. Dilute them to the working concentration 100-fold with Biotinylated Antibody Diluent and HRP Diluent, respectively.

6.TMB Substrate Solution - Aspirate the needed dosage of the solution with sterilized tips and do not dump the residual solution into the vial again.

Samples Preparation:

1. Equilibrate all materials and prepared reagents to room temperature prior to use. Prior to use, mix all reagents thoroughly taking care not to create any foam within the vials.

2. The user should calculate the possible amount of the samples used in the whole test. Please reserve sufficient samples in advance.

3. Please predict the concentration before assaying. If values for these are not within the range of the Standard curve, users must determine the optimal sample dilutions for their particular experiments.

Assay Procedure

1. Determine wells for Diluted Standard, Blank and Sample. Prepare 7 wells for Standard, 1 well for Blank. Add 100 µL each of Standard Working Solution (please

`

refer to **Reagent Preparation**), or 100μL of samples into the appropriate wells. Cover with the Plate Cover. Incubate for 80 minutes at 37°C. Note: solutions should be added to the bottom of the micro ELISA plate well, avoid touching
the inside wall and causing foaming as much as possible.

2. Pour out the liquid of each well. Aspirate the solution and wash with 200 μL of 1×Wash Solution to each well and let it sit for 1-2 minutes. Remove the remaining liquid from all wells completely by snapping the plate onto absorbent paper. Totally wash 3 times. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against absorbent paper.

Notes: (a) When adding Washing Solution, the pipette tip should not touch the wall of the wells to a void contamination.

(b) Pay attention to pouring the washing liquid directly to ensure that the washing liquid does not contaminate other wells.

3. Add 100 μL of Biotinylated Antibody Working Solution to each well, cover the wells with the Plate Cover and incubate for 50 minutes at 37°C

4. Repeat the aspiration,wash process for total 3 times as conducted in step 2.

5. Add 100 μL of Streptavidin-HRP Working Solution to each well, cover the wells with the plate sealer and incubate for 50 minutes at 37°C.

6. Repeat the aspiration, wash process for total 5 times as conducted in step 2.

7. Add 90 μL of TMB Substrate Solution to each well. Cover with a new Plate Cover. Incubate for 20 minutes at 37°C (Don't exceed 30 minutes) in the dark. The liquid will turn blue by the addition of

TMB Substrate Solution. Preheat the Microplate Reader for about 15 minutes before OD measurement.

8. Add 50 μL of Stop Reagent to each well. The liquid will turn yellow by the addition of Stop Reagent. Mix the liquid by tapping the side of the plate. If color change does not appear uniform, gently tap the plate to ensure thorough mixing. The insertion order of the Stop Reagent should be the same as that of the TMB Substrate Solution.

`

9. Wipe off any drop of water and fingerprint on the bottom of the plate and confirm there is no bubble on the surface of the liquid. Then, run the microplate reader and conduct measurement at 450 nm immediately.

Calculation of Results:

Average the duplicate readings for each Standard, Control, and Samples and subtract the average

zero Standard optical density. Construct a Standard curve with the Rat ERK1 concentration on the y-axis

and absorbance on the x-axis, and draw a best fit curve through the points on the graph. If samples have

been diluted, the concentration read from the Standard curve must be multiplied by the dilution factor.

Appendix VIII

Determination of serum tartrate-resistant acid phosphatase (TRAP):

Reagent Preparation:

1. Bring all kit components and samples to room temperature (18-25°C) before use.

2. If the kit will not be used up in 1 time, please only take out strips and reagents for present experiment, and save the remaining strips and reagents as specified.

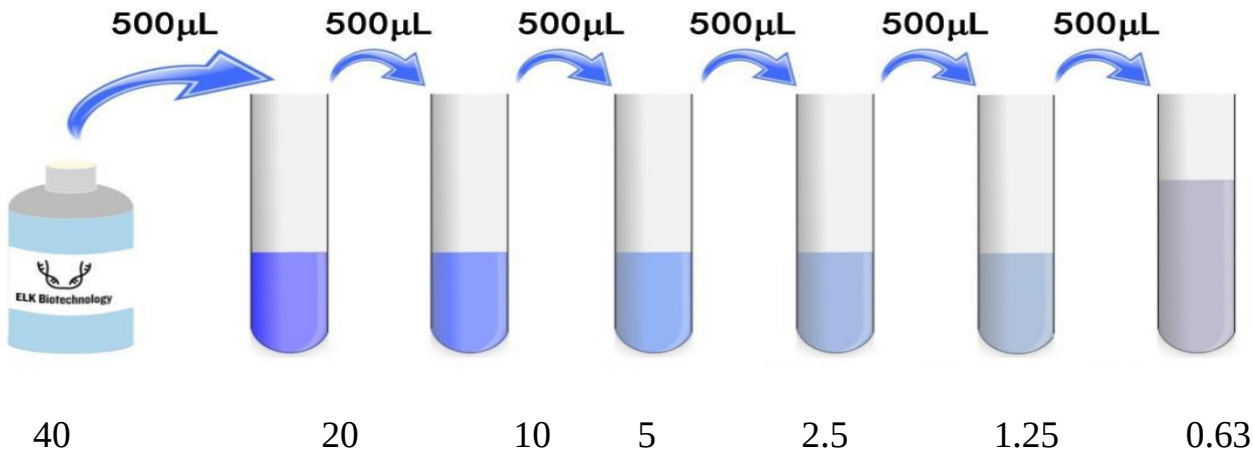
3. Dilute the 25×Wash Buffer into 1×Wash Buffer with double-distilled Water.

4. Standard Working Solution - Centrifuge the Standard at 1000 × g for 1 minute. Reconstitute the Standard with 1.0 mL of Standard Diluent Buffer, kept for 10 minutes

`

at room temperature, shake gently (not to foam). The concentration of the Standard in the stock solution is 40 ng/mL. Please prepare 7 tubes containing 0.5 mL Standard Diluent Buffer and use the Diluted Standard to produce a double dilution series according to the picture shown below. To mix each tube thoroughly before

the next transfer, pipette the solution up and down several times. Set up 7 points of Diluted Standard such as 40 ng/mL, 20 ng/mL, 10 ng/mL, 5 ng/mL, 2.5 ng/mL, 1.25 ng/mL, 0.63 ng/mL, and the last EP tubes with Standard Diluent is the Blank as 0 ng/mL. In order to guarantee the experimental results validity, please use the new Standard Solution for each experiment. When diluting the Standard from high concentration to low concentration, replace the pipette tip for each dilution. Note: the last tube is regarded as a Blank and do not pipette solution into it from the former tube.



5. 1×Biotinylated Antibody and 1×Streptavidin-HRP - Briefly spin or centrifuge the stock

Biotinylated Antibody and Streptavidin-HRP before use. Dilute them to the working concentration

100-fold with Biotinylated Antibody Diluent and HRP Diluent, respectively.

`

6. TMB Substrate Solution - Aspirate the needed dosage of the solution with sterilized tips and do not dump the residual solution into the vial again.

Assay Procedure:

1. Determine wells for Diluted Standard, Blank and Sample. Prepare 7 wells for Standard, 1 well for Blank. Add 100 μL each of Standard Working Solution (please refer to Reagent Preparation), or 100 μL of samples into the appropriate wells. Cover with the Plate Cover. Incubate for 80 minutes at 37°C. Note: solutions should be added to the bottom of the micro ELISA plate well, avoid touching the inside wall and causing foaming as much as possible.

2. Pour out the liquid of each well. Aspirate the solution and wash with 200 μL of 1×Wash Solution to each well and let it sit for 1-2 minutes. Remove the remaining liquid from all wells completely by snapping the plate onto absorbent paper. Totally wash 3 times. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against absorbent paper.

Notes: (a) When adding Washing Solution, the pipette tip should not touch the wall of the wells to a

void contamination.

(b) Pay attention to pouring the washing liquid directly to ensure that the washing liquid does not contaminate other wells.

3. Add 100 μL of Biotinylated Antibody Working Solution to each well, cover the wells with the Plate Cover and incubate for 50 minutes at 37°.

4. Repeat the aspiration,wash process for total 3 times as conducted in step 2.

5. Add 100 μL of Streptavidin-HRP Working Solution to each well, cover the wells with the plate sealer and incubate for 50 minutes at 37°C.

6. Repeat the aspiration, wash process for total 5 times as conducted in step 2.

`

7. Add 90 μL of TMB Substrate Solution to each well. Cover with a new Plate Cover. Incubate for 20minutes at 37°C (Don't exceed 30 minutes) in the dark. The liquid will turn blue by the addition of TMB Substrate Solution. Preheat the Microplate Reader for about 15 minutes before OD measurement.

8. Add 50 μL of Stop Reagent to each well. The liquid will turn yellow by the addition of Stop Reagent.

Mix the liquid by tapping the side of the plate. If color change does not appear uniform, gently tap the plate to ensure thorough mixing. The insertion order of the Stop Reagent should be the same as that of the TMB Substrate Solution.

9. Wipe off any drop of water and fingerprint on the bottom of the plate and confirm there is no bubble on the surface of the liquid. Then, run the microplate reader and conduct measurement at 450 nm immediately.

Calculation of Results:

Average the duplicate readings for each Standard, Control, and Samples and subtract the average zero Standard optical density. Construct a Standard curve with the Rat TRACP-5b concentration on they-axis and absorbance on the x-axis, and draw a best fit curve through the points on the graph. If samples have been diluted, the concentration read from the Standard curve must be multiplied by the dilution factor.

Appendix IX

Determination of serum Calcitonin

Reagent Preparation:

1. Bring all kit components and samples to room temperature (18-25°C) before use. Make sure all components are dissolved and mixed well before using the kit.

2. If the kit will not be used up in 1 time, please only take out strips and reagents for present experiment, and save the remaining strips and reagents as specified.

`

3. Dilute the 25× Wash Buffer into 1× Wash Buffer with double-distilled Water.

4. Standard Working Solution - Centrifuge the Standard at 1000 × g for 1 minute. Reconstitute the Standard with 1.0 mL of Standard Diluent Buffer, kept for 10 minutes at room temperature, shake gently (not to foam). The concentration of the Standard in the stock solution is 1000 pg/mL. Please prepare 7 tubes containing 0.5 mL Standard Diluent Buffer and use the Diluted Standard to produce a double dilution series according to the picture shown below. To mix each tube thoroughly before the next transfer, pipette the solution up and down several times. Set up 7 points of Diluted Standard such as 1000 pg/mL, 500 pg/mL, 250 pg/mL, 125 pg/mL, 62.5 pg/mL, 31.25 pg/mL, 15.63 pg/mL, and the last EP tubes with Standard Diluent is the Blank as 0 pg/mL. In order to guarantee the experimental results validity, please use the new Standard Solution for each experiment. When diluting the Standard from high concentration to low concentration, replace the pipette tip for each dilution. Note: the last tube is regarded as a Blank and do not pipette solution into it from the former tube

5- Biotinylated Antibody and 1× Streptavidin-HRP - Briefly spor centrifuge the stock Biotinylated Antibody and Streptavidin-HRP before use. Dilute them to working concentration 100-fold with Biotinylated Antibody Diluent and HRP Diluent, respectively.

6. TMB Substrate Solution - Aspirate the needed dosage of the solution with sterilized tips and do not dump the residual solution into the vial again.

Assay Procedure:

1. Determine wells for Diluted Standard, Blank and Sample. Prepare 7 wells for Standard, 1 well for Blank. Add 100 μL each of Standard Working Solution (please refer to Reagent Preparation), or 100 μL of samples into the appropriate wells. Cover with the Plate Cover. Incubate for 80 minutes at 37°C. Note: solutions should be added to the bottom of the micro ELISA plate well, avoid touching the inside wall and causing foaming as much as possible.

`

2. Pour out the liquid of each well. Aspirate the solution and wash with 200 μL of 1× Wash Solution to each well and let it sit for 1-2 minutes. Remove the remaining liquid from all wells completely by snapping the plate onto absorbent paper. Totally wash 3 times. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against absorbent paper.

Notes: (a) When adding Washing Solution, the pipette tip should not touch the wall of the wells to a void contamination.

(b) Pay attention to pouring the washing liquid directly to ensure that the washing liquid does not contaminate other wells.

3. Add 100 μL of Biotinylated Antibody Working Solution to each well cover the wells with the Plate http://www.elkbiotech.com elkbio@elkbiotech.com10 Cover and incubate for 50 minutes at 37°C.

4. Repeat the aspiration,wash process for total 3 times as conducted in step2.

5. Add 100 μL of Streptavidin-HRP Working Solution to each well, cover the wells with the plate sealer and incubate for 50 minutes at 37°C.

6. Repeat the aspiration, wash process for total 5 times as conducted in step 2.

.7Add 90 μL of TMB Substrate Solution to each well. Cover with a new Plate Cover. Incubate for 20 minutes at 37°C (Don't exceed 30 minutes) in the dark. The liquid will turn blue by the addition of TMB Substrate Solution. Preheat the Microplate Reader for about 15 minutes before OD measurement.

Appendix X

Determination of serum parathyroid hormone (PTH)

Reagent Preparation:

1. Bring all kit components and samples to room temperature (18-25°C) before use.Make sure all components are dissolved and mixed well before using the kit.

`

2. If the kit will not be used up in 1 time, please only take out strips and reagents for present experiment, and save the remaining strips and reagents as specified.

3. Dilute the 25× Wash Buffer into 1× Wash Buffer with double-distilled Water.

4. Standard Working Solution - Centrifuge the Standard at 1000 × g for 1 minute. Reconstitute the Standard with 1.0 mL of Standard Diluent Buffer, kept for 10 minutes at room temperature, shake gently (not to foam). The concentration of the Standard in the stock solution is 800 pg/mL. Please prepare 7 tubes containing 0.5 mL Standard Diluent Buffer and use the Diluted Standard to produce a double dilution series according to the picture shown below. To mix each tube thoroughly before the next transfer, pipette the solution up and down several times. Set up 7 points of Diluted Standard such as 800 pg/mL, 400 pg/mL, 200 pg/mL, 100 pg/mL, 50 pg/mL, 25 pg/mL, 12.5 pg/mL, and the last EP tubes with Standard Diluent is the Blank as 0 pg/mL. In order to guarantee the experimental results validity, please use the new Standard Solution for each experiment. When diluting the Standard from high concentration to low concentration, replace the pipette tip for each dilution. Note: the last tube is regarded as a Blank and do not pipette solution into it from the former tube

5- Biotinylated Antibody and 1× Streptavidin-HRP - Briefly spor centrifuge the stock Biotinylated Antibody and Streptavidin-HRP before use. Dilute them to working concentration 100-fold with Biotinylated Antibody Diluent and HRP Diluent, respectively.

6. TMB Substrate Solution - Aspirate the needed dosage of the solution with sterilized tips and do not dump the residual solution into the vial again.

Assay Procedure:

1. Determine wells for Diluted Standard, Blank and Sample. Prepare 7 wells for Standard, 1 well for Blank. Add 100 μL each of Standard Working Solution (please refer to Reagent Preparation), or 100 μL of samples into the appropriate wells. Cover with the Plate Cover. Incubate for 80 minutes at 37°C. Note: solutions should be added

`

to the bottom of the micro ELISA plate well, avoid touching the inside wall and causing foaming as much as possible.

2. Pour out the liquid of each well. Aspirate the solution and wash with 200 μL of 1× Wash Solution to each well and let it sit for 1-2 minutes. Remove the remaining liquid from all wells completely by snapping the plate onto absorbent paper. Totally wash 3 times. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against absorbent paper.

Notes: (a) When adding Washing Solution, the pipette tip should not touch the wall of the wells to a void contamination.

(b) Pay attention to pouring the washing liquid directly to ensure that the washing liquid does not contaminate other wells.

3. Add 100 μL of Biotinylated Antibody Working Solution to each well cover the wells with the Plate http://www.elkbiotech.com elkbio@elkbiotech.com10 Cover and incubate for 50 minutes at 37°C.

4. Repeat the aspiration,wash process for total 3 times as conducted in step2.

5. Add 100 μL of Streptavidin-HRP Working Solution to each well, cover the wells with the plate sealer and incubate for 50 minutes at 37°C.

6. Repeat the aspiration, wash process for total 5 times as conducted in step 2.

7. Add 90 μL of TMB Substrate Solution to each well. Cover with a new Plate Cover. Incubate for 20 minutes at 37°C (Don't exceed 30 minutes) in the dark. The liquid will turn blue by the addition of TMB Substrate Solution. Preheat the Microplate Reader for about 15 minutes before OD measurement.

8. Add 50 μL of Stop Reagent to each well. The liquid will turn yellow by the addition of Stop Reagent. Mix the liquid by tapping the side of the plate. If color change does not appear uniform, gently tap the plate to ensure thorough mixing. The insertion order of the Stop Reagent should be the same as that of the TMB Substrate Solution.

`

9. Wipe off any drop of water and fingerprint on the bottom of the plate and confirm there is no bubble on the surface of the liquid. Then, run the microplate reader and conduct measurement at 450 nm immediately.

Calculation of Results:

Average the duplicate readings for each Standard, Control, and Samples and subtract the average zero Standard optical density. Construct a Standard curve with the Rat CT concentration on the y-axis and absorbance on the x-axis, and draw a best fit curve through the points on the graph. If samples have been diluted, the concentration read from the Standard curve must be multiplied by the dilution factor. Using some plot software, for instance, curve expert.

Appendix XI

Determination of serum Vit D

Reagent Preparation:

1. Bring all kit components and samples to room temperature (18-25°C) before use make sure all components are dissolved and mixed well before using the kit.

2. If the kit will not be used up in 1 time, please only take out strips and reagents for present experiment, and save the remaining strips and reagents as specified.

3. Dilute the 25× Wash Buffer into 1× Wash Buffer with double distilled water.

4. Standard Working Solution - Centrifuge the Standard at 1000 × g for 1 minute. Reconstitute the Standard with 1.0 mL of Standard Diluent Buffer, kept for about 10 minutes at room temperature, shake gently (not to foam). The concentration of the standard in the stock solution is 200 ng/mL. Please prepare 7 tubes containing 0.5 mL Standard Diluent Buffer and use the Diluted Standard to produce a double dilution

`

series according to the picture shown below. To mix each tube thoroughly before the next transfer, pipette the solution up and down several times. Set up 7 points of Diluted Standard such as 200 ng/mL, 100 ng/mL, 50 ng/mL, 25 ng/mL, 12.5 ng/mL, 6.25 ng/mL, 3.13 ng/mL, and the last EP tubes with Standard Diluent is the Blank as 0 ng/mL. In order to guarantee the experimental results validity, please use the new Standard Solution for each experiment. When diluting the Standard from high concentration to low concentration, replace the pipette tip for each dilution. Note: the last tube is regarded as the Blank and do not pipette solution into it from the former tube.

5. 1× Biotinylated-Conjugate and 1× Streptavidin-HRP Working Solution - Briefly spin or centrifuge the stock Biotinylated-Conjugate and Streptavidin-HRP before use. Dilute them to the working concentration 100-fold with Biotinylated-Conjugate Diluent and HRP Diluent, respectively. For http://www.elkbiotech.com elkbio@elkbiotech.com8 example, 10 μL of Streptavidin-HRP with 990 μL of HRP Diluent.

6. TMB Substrate Solution - Aspirate the needed dosage of the solution with sterilized tips and do not dump the residual solution into the vial again.

Assay Procedure

1. Determine wells for Diluted Standard, Blank and Sample. Prepare 7 wells for Standard, 1 well for Blank. Add 50 μL of Standard Working Solution (please refer to Reagent Preparation) or Sample into each well (Blank is Standard Diluent). Then, add 50 μL of Biotinylated-Conjugate (1×) to each well immediately. Mix well, cover with the Plate Cover. Incubate for 1 hour at 37°C. Note: solutions should be added to the bottom of the micro ELISA plate well, avoid touching the inside wall and causing foaming as much as possible.

2. Pour out the liquid of each well. Aspirate the solution and wash with 200 μL of 1× Wash Solution to each well and let it sit for 1-2 minutes. After the liquid has been decanted, completely remove the remaining liquid from all wells by snapping the

`

plate onto absorbent paper. Totally wash 3 times. Complete removal of liquid at each step is essential for good performance. After the last wash invert the plate and blot it against clean paper towels to remove excess liquid. Notes: (a) When adding Washing Solution, the pipette tip should not touch the wall of the wells to a void contamination. http://www.elkbiotech.com elkbio@elkbiotech.com10 (b) Pay attention to pouring the washing liquid directly to ensure that the washing liquid does not contaminate other wells.

3. Add 100 μL of Streptavidin-HRP Woking Solution (1×) to each well, cover the wells with the Plate Cover and incubate at 37°C for 60 minutes.

4. Repeat the aspiration, wash process for total 5 times as conducted in step 2.

5. Add 90 μL of TMB Substrate Solution to each well. Cover with a new Plate Cover. Incubate for 20 minutes at 37°C (Don't exceed 30 minutes) in the dark. The liquid will turn blue by the addition of TMB Substrate Solution. Preheat the Microplate Reader for about 15 minutes before OD measurement. Avoid placing the plate in direct light.

6. Add 50 μL of Stop Reagent to each well. The liquid will turn yellow by the addition of Stop Reagent. Mix the liquid by tapping the side of the plate. The insertion order of the Stop Reagent should be the same as that of the TMB Substrate Solution.

7. Wipe off any drop of water and fingerprint on the bottom of the plate and confirm there is no bubble on the surface of the liquid. Then, run the microplate reader and conduct measurement at 450 nm immediately.

Appendixes XII

Determination of serum Calcium (Ca):

CLINICAL SIGNIFICANCE

`

Total Calcium exists in 3 physiochemical states in plasma, of which approximately 50 % is free or ionised calcium, 40 % is bound to plasma proteins, and 10 % are bound with small anions. The level of serum calcium may be affected by intestinal malabsorption, by alterations in plasma proteins level, especially albumin, which should be measured concurrently with calcium .Hyperkalemia is found in hyperparathyroidism, multiple myeloma, bone and Para thyroidal neoplasms and in states with bones demineralisation. Hypocalcemia is encountered in hypoparathyroïdism and in several cases of necrosis and acute pancreatitis.

PRINCIPLE

Moore head and Briggs derived CPC (O-Cresol Phtalein Complexone) method allows to determinate total Calcium concentration in serum, plasma or urines.

In alkaline solution CPC reacts with calcium to form a dark-red coloured complex which absorbance measured at 570 nm is proportional to the amount of calcium in the specimen.

PROCEDURE

Detailed Kenza 240TX procedure is available on request Wavelength: 570 nm

Temperature: 37°CTemperature should be held constant as the absorbance of the dye is temperature sensitive.

	Automated Analyzer	Manual procedure
Reagents	120 µL R1 120 µL R2	WR :1000 µL
Standard, Controls, Specimen	6 µL	25 µL
Mix well. Incubate for 5 minutes at room temperature.Read absorbance at 570 nm (550-590) against reagent blank**.**The coloration is stable for 1 hour away from light.		

Appendixes XIII

`

Determination of serum phosphorus (P):

PRINCIPLE

Inorganic phosphate reacts with molybdic acid forming a phosphomolybdic complex. Its subsequent reduction in alkaline medium originates a blue molybdenum colour which intensity is proportional to the amount of phosphorus present in the sample.1

Pi

PO 3- + H+ + (NH) Mo O Phosphomolybdic Complex

pH > 10

Phosphomolybdic Complex Molybdenum blue

REDUCTANT

REAGENT COMPOSITION

Molybdate Reagent. Ammonium molybdate 7 mmol/L, sulphuric acid 0.8 mol/L. Xi R:36/37/38

Reducing solution. Hydroxylamine 0.64 mol/L. Catalyzers.

Color developer. Sodium hydroxide 3 mol/L. Stabilizers. C R:35

Chloride / Phosphorus standard. Chloride 100 mEq/L / Phosphorus 5 mg/dL.

Organic matrix based primary standard.

PROCEDURE

1. Bring reagents and samples to room temperature.
2. Pipette into labeled test tubes:

TUBES	Blank	Sample	CAL. Standard
Working Reagent	1.0 mL	1.0 mL	1.0 mL
Sample	□	50 □L	□
CAL.Standard	□	□	50 □L

3. Mix, let stand the tubes for 1 minute and then pipette:

`

R3. Developer	0,5 mL	0,5 mL	0,5 mL

3. Mix and let the tubes stand 10 minutes at room temperature.
4. Read the absorbance (A) of the sample and the standard at 740 nm against the reagent blank.

The color is stable for at least 30 minutes protected from light.

CALCULATIONS

Serum, plasma A Sample
———————— x C Standard = mg/dL phosphorus A Standard

Samples with concentrations higher than 15 mg/dL (4.8 mmol/L) should be diluted 1:2 with saline and assayed again. Multiply the results by 2.

Urine

A Sample
———————— x 100 = mg/24-hours phosphorus A Standard

If results are to be expressed as SI units apply: mg/dL x 0.323 = mmol/L

Appendixes XVI

Determination of serum sodium (Na):

Procedure for Measuring Sodium using Kenza Analyzer :

1. Sample Collection:

- Blood samples were collected in tubes without anticoagulant for serum or in tubes with anticoagulant for plasma.

2. Centrifugation:

- The blood samples were centrifuged at an appropriate speed to separate the serum or plasma from the blood cells.

3. Sample Transfer:

- The separated serum or plasma was transferred to clean analysis tubes.

4. Device Preparation:

`

- The Kenza Analyzer was turned on and allowed to initialize, ensuring no system errors were present.

5. Calibration:

- Calibration of the device was performed using standard solutions with known concentrations of sodium and potassium, following the manufacturer's instructions.

6. Sample Analysis:

- The prepared samples were placed into the designated slots on the Kenza Analyzer.

- The specific program for measuring sodium and potassium was selected on the device.

- The analysis process was initiated by pressing the start button.

7. Result Reading:

- After the analysis was complete, the concentrations of sodium and potassium were displayed by the device.

- The results were saved or printed as supported by the device.

8. Precautions:

- All tools and tubes were ensured to be clean to avoid sample contamination.

- The manufacturer's instructions were closely followed to accommodate specific model variations of the Kenza Analyzer.

- Regular maintenance of the device was performed to ensure accuracy and reliability of results.

Appendixes XVII

Determination of serum potassium (K):

INTENDED USE

This reagent is designated for professional use in laboratory (manual or automated method).

It allows the quantitative determination of potassium ions in human serum and plasma to determine if there is a problem with electrolyte balance. As part of a routine health check-up, results may be used as a screening test, in conjunction with other clinical signs and laboratory data.

PRINCIPLE

`

Potassium is determined spectrophotometrically through a kinetic coupling assay system using potassium dependent pyruvate kinase. Pyruvate generated is converted to lactate accompanying conversion of NADH in NAD+ + H+. The corresponding decrease of optical density at 380 nm is proportional to the potassium concentration in the serum.

PROCEDURE

Manual method

Let stand reagent and specimens at room temperature.

Reagent 1	800 µL
Blank, Standards, control or specimen	20 µL
Mix well. Let stand for 5 minutes at 37°C	
Reagent 2	200 µL
Mix well. Read at 380 nm absorbance A1 after 60 sec and A2 after 240 sec . Calculate □Abs (Abs A2 – Abs A1) for Blank, Standards and Assays.	

1-Performances with manual procedure should be validated by user.

2-KENZA applications and other applications proposal are available on request.

CALCULATION

Serum or plasma:

$$\frac{\square\text{Abs (Assay)} - \square\text{Abs (Blank)}}{\square\text{Abs (Standard)} - \square\text{Abs (Blank)}}$$

Interpolate the □A in the Calibration Curve

`

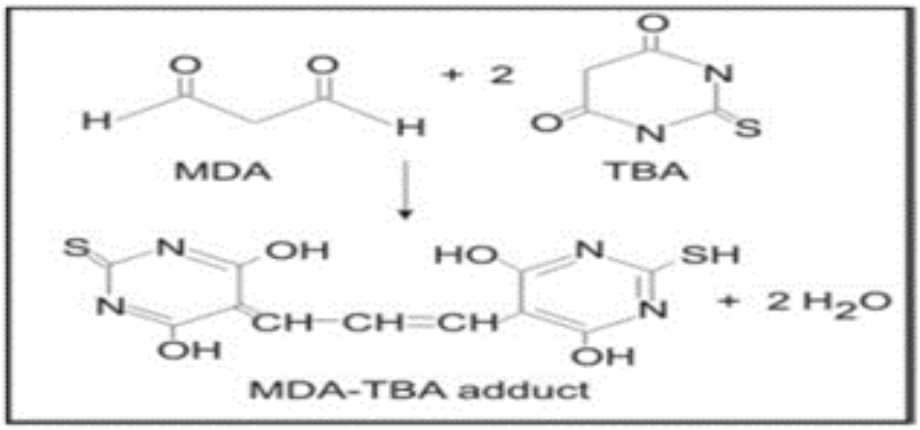
Appendixes XVIII

Determination of Serum Malondialdehyde (MDA) concentration (µ mol /L):

This method quantifies lipid peroxides by measuring aldehyde breakdown products of lipid peroxidation. Basic principle of the method is the reaction of one molecule of malondialdehyde and two molecules of thiobarbituric acid to form a red MDA-TBA complex which can be measure at 535 nm.

Stock TCA – TBA – HCl Reagent:

It was prepared by dissolving 15% W/V trichloroacetic acid and



0.375% W/V thiobarbituric acid and 0.25N HCl to make 100 ml (2.1 ml of concentrated HCl in 100 ml). This solution was mildly heated to assist in the dissolution of TBA. Dissolved 15 gm TCA and 0.375 mg thiobarbituric acid in 0.25 N HCl and volume was made up to 100 ml with 0.25 N HCl.

Procedure:

To 0.4 ml of serum, 0.6 ml TCA-TBA-HCl reagents were added. It was mixed well and kept in boiling water bath for 10 minutes. After cooling 1.0 ml freshly prepared 1N NaOH solution was added to eliminate centrifugation. This absorbance of pink colour was measured at 535 nm against blank which contained distilled water in place of serum. In blank 0.4 ml distilled water and 0.6 ml TCA-TBA-HCl reagent was mixed and

`

boiled. Blank was always taken.

Calculation:

extinction coefficient of MDA at 535 nm is = 1.56 × 105

MDA concentration = χ / 0.0624 nmol / ml

Appendixes XIX

Determination of serum Reduced Glutathione (GSH) concentration:

First, 3.0 mL precipitating solution containing metaphosphoric acid, Na2EDTA and NaCl was added to 2.0 mL of the sample. The mixture was centrifuged at 4500 × g for 10 min. 1.0 mL of supernatant was added to 4.0 mL of 0.3 M Na2HPO4 solution and 0.5 mM DTNB (5,5 -dithiobis-2-nitrobenzoic acid) was then added to this solution. Reduced glutathione was measured as the difference in the absorbance values of samples in the presence and the absence of DTNB at 412 nm. GSH value was calculated as nmol GSH/mg protein in the tissues and mmol GSH/g Hb in whole blood using the reduced glutathione as a reference (hemoglobin levels were estimated in whole blood using the Drabkins' solution).

`

الخلاصة

كان الهدف من الدراسة هو دراسة تأثير النظام الغذائي عالي الكوليسترول على هشاشة العظام من خلال استكشاف كيف يمكن لفرط كوليستيرول الدم أن يعزز تمايز ونشاط الخلايا الآكلة للعظم، مما يؤدي إلى زيادة ارتشاف العظم وفقدان العظام الصافي لاحقًا. أجريت الدراسة التجريبية على عشرين جرذاً ذكراً بأعمار (1.5-2) شهر قسمت على النحو التالي إلى مجموعتين: (10) فئران غذيت على نظام غذائي عادي، (10) فئران غذيت على نظام غذائي عالي الكولسترول (2%) لمدة 8 أسابيع كانت بمثابة مجموعة HCD، حساب المعلمات الفسيولوجية والبيولوجية RANK، RANKL، كيناز منظم للإشارة خارج الخلية (ERK)، فوسفات حمض طرطرات المقاومة (TRAP)، ملف تعريف الدهون (TC، TG، LDL، HDL)، مضادات الأكسدة (MDA، GSH)، المعادن (NaCa تم استئصال الهرمونات (كالسيتونين، هرمون الغدة الدرقية، فيتامين د) وعظام الفخذ لقياس التعبير الجيني للأوستريكس والفحص النسيجي المرضي بعد نهاية التجربة (8 أسابيع) والصورة الشعاعية قبل التجربة وبعد 4 أسابيع. من التجريبية وبعد انتهاء التجربة (8 أسابيع).

أظهرت الدراسة الحالية زيادة معنوية (P<0.05) في الكولسترول الكلي (TC)، الدهون الثلاثية (TG)، البروتين الدهني منخفض الكثافة (LDL)، في مجموعة HCD مقارنة بمجموعة السيطرة. وفي المقابل حدث انخفاض معنوي (P<0.05) في مستوى (HDL) في مجموعة HCD مقارنة بمجموعة السيطرة.

أظهرت النتائج زيادة كبيرة (P <0.05) في مصل RANK (منشط مستقبل العامل النووي (Kb، RANKEL (منشط مستقبل العامل النووي κB يجند)، كيناز ينظم الإشارة الخلوية الإضافية (ERK) في مجموعة HCD مقارنة في حين لم يكن هناك تغير معنوي (P> 0.05) في مستويات حامض الفوسفات المقاوم للطرطرات (TRAP) في مجموعة HCD مقارنة بمجموعة السيطرة.

أظهرت النتائج ارتفاع معنوي (P<0.05) في مستوى هرمون الغدة الدرقية والكالسيتونين وفيتامين د في مجموعة الكولسترول مقارنة بمجموعة السيطرة، كما أظهرت هذه الدراسة ارتفاع معنوي (P<0.05) في مصل الكالسيوم في مجموعة HCD مقارنة مع مجموعة السيطرة. إلى المجموعة الضابطة. في المقابل لم يكن هناك فرق معنوي (P> 0.05) في مصل الصوديوم والبوتاسيوم والفوسفور في مجموعة HCD مقارنة بمجموعة السيطرة.

`

أشارت نتائج الدراسة الحالية إلى وجود انخفاض معنوي ($P<0.05$) في مستوى هرمون GSH في مجموعة HCD مقارنة مع مجموعة السيطرة. في المقابل، لوحظ وجود زيادة معنوية ($P<0.05$) في Malnodialdhyde(MDA) في مجموعة HCD مقارنة بالمجموعة الضابطة.

من ناحية أخرى، أظهر جين Osterix ارتفاعًا كبيرًا في التنظيم في مجموعة HCD مقارنة بالمجموعة الضابطة. و أظهر الفحص النسيجي المرضي للأنسجة العظمية في دراستنا عدم وجود خلايا عظمية عظمية على حدود الترابيق، ونخر الخلايا العظمية ذات الخلايا العظمية متعددة النوى في مجموعة HCD مقارنة بالقسم النسيجي الطبيعي للمجموعة الضابطة، والتي تظهر خلايا عظمية طبيعية في الثغرات والعظام العادية تجاويف النخاع والخلايا العظمية المنتظمة في خط على الحدود التربيقية.

في نهاية الدراسة التجريبية، وجدت الدراسة في مجموعات الكوليسترول وجود منطقة شفافة للأشعة في عظام الحوض، في عظام الحوض و عظم الفخذ والعمود الفقري لدى الفئران المصابة بهشاشة العظام الناجمة عن اتباع نظام غذائي عالي الكوليسترول.

في الختام: يعزز أوستريكس تمعدن مصفوفة العظام عن طريق تعديل التعبير عن الجينات المعنية، وقد ارتبطت هذه الزيادة الكبيرة بتركيز الكالسيوم في مصل ذكور الجرذان المصابة بفرط كوليسترول الدم، ويمكن أن توفر مراقبة العلامات الحيوية ERK قيمة معلومات حول تطور المرض، والاستجابة للعلاج، والأهداف العلاجية المحتملة في إدارة هشاشة العظام.

`

جمهوريةٌ العراق

وزارة التعليم والبحث العلمي

جامعة كربلاء/كلية الطب البيطري

قسم الفسيولوجيا والكيمياء الحيوية والادوية

تمايز وتفعيل مسار بانيات العظم- ناقضات العظم على فقدان العظام الناجم عن النظام الغذائي المفرط في الكوليسترول في ذكور الجرذان

رسالة مقدمة إلى

مجلس كلية الطب البيطٌري جامعة كربلاء في استيفاء جزء من متطلبات درجة ماجستيرٌ في الطٌب البيطري / الفسلجة

بواسطة

دعاء رعد عبد الامير

اشراف

ا . د وفاق جبوري البازي

أ. م. د حيدر علي محمد

1446ه

2024م