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Effect of Hyperhomocysteinemia on some Biomarkers and Osteoclasts Activity in Male Rabbits induce by Methionine

A THESIS

Submitted to the council of The College of Veterinary Medicine, University of Karbala in Partial Fulfillment of the Requirements for the Degree of Master of Science in Veterinary Medicine / Physiology

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بسم الله الرحمن الرحيم

قَالُوا سُبْحَانَكَ لَا عِلْمَ لَنَا إِلَّا مَا
عَلَّمْتَنَا ۖ إِنَّكَ أَنتَ الْعَلِيمُ الْحَكِيمُ

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Dedication

To those who their prayers illuminate my way

My father and mother

To my wife and children who shared me all good days and hard times

To my brothers and friends to support me in completing my research

To the souls of Iraq's martyrs who sacrificed for us

Mohammed

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Summary

This study was carries out to investigate the effect of Hyperhomocysteinemia (HHcy) on osteoclast activity that induced by methionine overload in male rabbits.

Twenty adult male rabbits were used, which were randomly divided into two groups (10/group). The first group was intubated with tap water and serve as control group. Rabbits in the second group were intubated orally with (100mg/kg BW) of methionine daily for 12 weeks.

Fasting blood samples were collected by heart puncture technique after 12 weeks of experiment to measure of the following criteria: **a-** Homocysteine (Hcy) concentration in serum. **b-** Biomarker of osteoclast activity including: Receptor Activator of Nuclear factor Kappa-b (RANK) and Receptor Activator of Nuclear factor Kappa-b Ligand (RANK-L). **c-** Measuring the level of ions and minerals including: calcium (Ca^{+2}), sodium (Na^{+}), potassium (K^{+}) and iron (Fe^{+2}) concentration in serum. **d-** Parathyroid hormone (PTH) concentration in serum. **e-** Alkaline phosphatase (ALP) enzyme. **f-** Biomarker of oxidative stress including: serum glutathione (GSH) and malondialdehyde (MDA) concentrations, in addition to studying histopathological changes of the femur bone.

The results of this study show that daily oral intubation of methionine (100mg/kg BW) to males rabbits for 12 weeks cause significant increase ($p \leq 0.001$) in the concentration of Hcy, RANK, RANK-L, PTH, ALP, Fe^{+2} and MDA, and significant decrease ($p \leq 0.001$) in serum Ca^{+2}, Na^{+} and GSH concentration, in addition to significant decrease ($p \leq 0.05$) in serum K^{+} concentration, when compared to the control group. The histological examination by lighting microscope and scanning electronic microscope showed that daily oral intubation of methionine caused increase in the numbers and activity of osteoclast cells accompanied by erosion and necrosis of the trabecular structure of the femur bone.

As for correlations there are a significant ($p \leq 0.01$) positive correlation between Hcy and all of RANK-L, Fe^{+2} and ALP. The Hcy also show significant ($p \leq 0.01$) negative correlation with Ca^{+2} and GSH. RANK-L showed a significant ($p \leq 0.01$) positive correlation with RANK. PTH showed significant positive ($p \leq 0.01$) correlation with RANK-L, while it showed significant ($p \leq 0.01$) negative correlation with Ca^{+2}.

In summary, it is concluded from the current study that HHcy that induced by excess l-methionine intubation caused a significant increased in the levels of RANK and RANK-L, which has resulted in an increase in the numbers and activity of osteoclasts resulting in necrosis and erosion in the trabecular structure of the femur bone. This shows that high Hcy in the blood is a risk factor for the occurrence of osteoporosis.

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List of Abbreviations

Abbreviations	
Abbreviation	**Meaning**
1,25D	1,25-dihydroxyvitamin D
25D	25-hydroxyvitamin D
ALP	Alkaline phosphatase
ALT	alanine aminotransferase
AP-1	activator protein-1
AST	aspartate amino transferase
BHMT	Betaine-homocysteine methyltransferase
BMD	bone menial density
Btk	Bruton's tyrosine kinase
Ca^{+2}	calcium
CBS	cystathionine β-synthase
CKD	chronic kidney disease
ClC-7	chloride channel type 7
CoA	coenzyme A
COX2	Cyclooxygenases 2
CRDs	cysteine-rich pseudo-repeat domains
CSE	cystathionine γ-lyase
DAP12	DNAX-activating protein-12
DNMT1	DNA (cytosine-5) – methyltransferase 1
EGF	epidermal growth factor
ERK	extracellular signal-regulated kinase
FcRγ	Fc receptor common γ subunit
FGF	Fibroblast growth factor
FOXO1	Forkhead Box O1
GSH	Glutathione
GSH-Px	GSH peroxidase
GSSG	glutathione disulfide
HCL	hydrochloride acid
Hcy	Homocysteine
HHcy	Hyperhomocysteinemia
HSPGs	heparin sulphate proteoglycans
IFN-γ	Interferon gamma
IGF	Insulin-like growth factor
IL	Interleukin
ITAM	immunoreceptor tyrosine-based activation motif
JNK	c-Jun N-terminal kinase
K^{+}	Potassium
MAPK	mitogen-activated protein kinase
MAT	Methionine Adenosyl Transferase
M-CSF	Macrophage - Colony Stimulating Factor
MDA	Malondialdehyde

MIP-1α	Macrophage inflammatory protein-1 alpha
MITF	Microphthalmia-associated transcription factor
MMPs	metalloproteinases
MTHFR	Methylene tetra hydrofolate reductase
N-5- MTHF	N-5-Methyltetrahydrofolate
Na^+	sodium
NAC	N-acetyl cysteine
NADPH	Nicotinamide adenine dinucleotide phosphate
NFATc1	Nuclear Factor of Activated T-cells Cytoplasmic 1
NF-kB	nuclear factor kappa-light-chain-enhancer of activated B cells
NMDA receptor	N-methyl-D-aspartate receptors
OPG	Osteoprotegerin
OSCAR	osteoclast-associated receptor
OSF	osteoclast-stimulating factor
OSM	oncostatin M
OSTM1	osteopetrosis-associated transmembrane protein 1),
PDGF	Platelet-derived growth factor
PGE2	prostaglandin E2
PI3K	phosphatidyl inositol 3-kinase
PLA2	phospholipase A2
PLC-γ	phospholipase C-γ
PP2A	protein phosphatase 2A
PTCs	proximal tubular cells
PTH	Parathyroid hormone
RAAS	renin-angiotensin-aldosterone system
RANK	Receptor Activator of Nuclear factor Kappa-b
RANK-L	Receptor Activator of Nuclear factor Kappa-b Ligand
RNS	Reactive Nitrogen Species
ROS	reactive oxygen species
SAH	S-Adenosyl- l-homocysteine
SAM	S- adenosylmethionine
Syk	Tyrosine-protein kinase
TAB2	TAK1- binding protein 2
TAK1	TGF-β activated kinase1
Tec	Tyrosine protein kinase
TGF- alpha	Transforming growth factor alpha
TGF-β	Transforming growth factor beta
THF	tetra hydrofolate
TLR	Toll like receptor
TNF	Tumor necrosis factor
TNFR	tumor necrosis factor receptor
TNFRS11B	TNF receptor superfamily member 11B
TNFSF11A	tumor necrosis factor ligand superfamily member 11A
TNF-α	Tumor necrosis factor alpha
TRAcP	tartrate resistant acid phosphatase

TRAF	TNF receptor-associated factor
TRAIL	TNF-related apoptosis-inducing ligand
TRANCE	TNF-related activation-induced cytokine (TRANCE)
TRANCE- receptor	TNF-related activation-induced cytokine receptor
TREM-2	Triggering receptor expressed on myeloid cells 2
VEGF	Vascular endothelial growth factor
VIP	Vasoactive intestinal peptide
WHO	world health organization

Chapter One

Introduction

Introduction

Methionine is an aliphatic, sulfur containing, essential amino acid and a precursor of Homocysteine (Hcy), cysteine, creatine and succinyl-CoA (**Martínez *et al*., 2017**). The methionine used at multiple levels in cellular metabolism as a protein constituent, in the initiation of mRNA translation, and as a regulatory molecule in the form of S- adenosylmethionine (SAM) which considered as the major biological methyl donor **(Ahmad *et al*., 2012; Gao *et al*., 2018).**

The high levels of this amino acid can be found in eggs, meat, and fish; sesame seeds, Brazil nuts, and some other plant seeds; and cereal grains. Also fruits and vegetables contain very little. Most legumes, though protein dense, are low in methionine. Proteins without adequate methionine are not considered to be complete proteins **(Finkelstein, 1990).** For that reason it is sometimes used as nutritional supplement **(Christiansen, 2019).**

The L - methionine is used extensively in human medicine for a verity of therapeutic purposes, including heal wounds, drug withdrawal, schizophrenia, radiation, copper poisoning, asthma, allergies, alcoholism, liver damage and those experiencing Parkinson's and depression **(Anufrieva *et al*., 2015; Zyl, 2017),** but the excessive uptake of methionine can become harmful and can increase the risk of developing heart diseases, liver disease, Type-2 diabetes, certain types of cancer, kidney disease and brain alterations such as schizophrenia, and memory impairment **(Soares *et al*., 2017; Kumar *et al*., 2020).**

The methionine overload is one of many factors responsible for causing disturbance in Hcy metabolism resulting in accumulation of Hcy with subsequent development of Hyperhomocysteinemia (HHcy) **(Milovanovic *et al*., 2017).** Hcy levels also tend to increase with age both in males and females and may vary according to different habits, such as cigarette smoking, alcohol consumption, and side effects of some drugs **(Blachier *et al*., 2013; Kim *et al*., 2018).**

The HHcy that induced by chronic administration of methionine overload lead to increase production of free radicals and inflammation markers in body tissues **(Aparna *et al*., 2010; Micovic *et al*., 2016)** Where the Hcy is considered one of the most potent oxidant agents **(Cristiana *et al*., 2012),** which has the ability to cause

oxidative stress, inflammation, endothelium dysfunction and endoplasmic reticulum stress that have been considered to play an important role in the pathogenesis of several diseases including atherosclerosis **(Lai & Kan, 2015; Moretti & Caruso, 2019; Wu *et al*., 2019),** cardiovascular disorders, myocardial infarction, stroke, dementia, Parkinson's disease, multiple sclerosis, epilepsy, eclampsia, cancer development, autoimmune disease, and kidney disease **(Zhu *et al*., 2011; Ansari *et al*., 2014).**

A studies report Hcy to be a newly recognized risk factor for osteoporosis **(Behera *et al*., 2017; Álvarez-Sánchez *et al*., 2018).** Osteoporosis is a major health problem and considers a silent disease as there are no symptoms prior to a fracture, which characterized by low bone mineral density, deterioration of bone micro architecture and an increased risk of fracture. Osteoporotic fractures are associated with increased level of morbidity and mortality and with substantial economic costs **(Morin *et al*., 2011; Ferdous *et al*., 2016).**

The bone homeostasis depends on the resorption of bone by osteoclasts and formation of bone by the osteoblasts. Imbalance of this tightly coupled process can cause diseases such as osteoporosis **(Guido *et al*., 2009; Chen *et al*., 2017).** Osteoclasts are specialized cells that resorb bone, whereas osteoblasts are cells that synthesize new bone, the Receptor Activator of Nuclear Factor Kappa-b Ligand (RANK-L), a protein expressed by the osteoblasts, plays an important role in osteoclast formation, function, and survival through its interaction with Receptor Activator of Nuclear Factor Kappa-b (RANK) on the osteoclast. Osteoprotegerin (OPG), a natural inhibitor of RANK-L, interferes with RANK-L and RANK association and thereby regulates osteoclast activity and resorption in the bone **(Matthew, 2014; Chen *et al*., 2017; Ono *et al*., 2020**).

It has been reported that bone resorption and turnover rate are increased among individual with HHcy **(Álvarez-Sánchez *et al*., 2018).** The molecular mechanisms of how Hcy hampered bone remodeling or how increased in bone resorption is still unclear until now**.**

Abundant researches have been published about the interaction of Hcy and bone, and several mechanisms and hypotheses have been proposed about this involvement of Hcy in bone pathology. Some of these researches have success to find association

between Hcy and bone remolding and other has failed **(Thaler *et al.*, 2011; Tyagi *et al.*, 2011; Baig *et al.*, 2015)** one of the most common assumptions is those that suggested by Herrmann *et al*. who referred to the occurrence of osteoporosis due to increase in osteoclast activity by Hcy **(Herrmann *et al.*, 2005).**

Therefore, this study aims to investigate the effect of HHcy that induces by methionine overload on osteoclast activity and it possible role of inducing osteoporosis; in addition to study the structural changes that occur in bone tissues. Thereby measuring the following parameters:

1. serum Receptor Activator of Nuclear Factor Kappa B Ligand (RANK-L)
2. serum Receptor Activator of Nuclear Factor Kappa B (RANK)
3. serum Homocysteine (Hcy)
4. serum Malondialdehyde (MDA)
5. serum reduced glutathione concentration (GSH)
6. serum minerals measurement assay
7. serum alkaline phosphatase (ALP)
8. serum parathyroid hormone (PTH)
9. Histological and longitudinal section from femur bone: head of femur and epicondyle region of femur bone were taken for lighting and dissecting microscope examination.
10. Scanning electron microscope examination for femur head.

Chapter Two

Literature review

2.1 Methionine

Chemical name are**:** - 2 amino-4-methyl thiobutyric acid. Or α-amino-γ-methyl mercaptobutyric acid.

Other name: - Met, L-methionine, D-methionine, DL-methionine., and Acimethin. Amino acid composition has an amino group (NH_2) adjacent to a carboxyl (COOH) group on carbon. Methionine: is an amino acid with chemical formula ($C_5H_{11}NO_2S$).

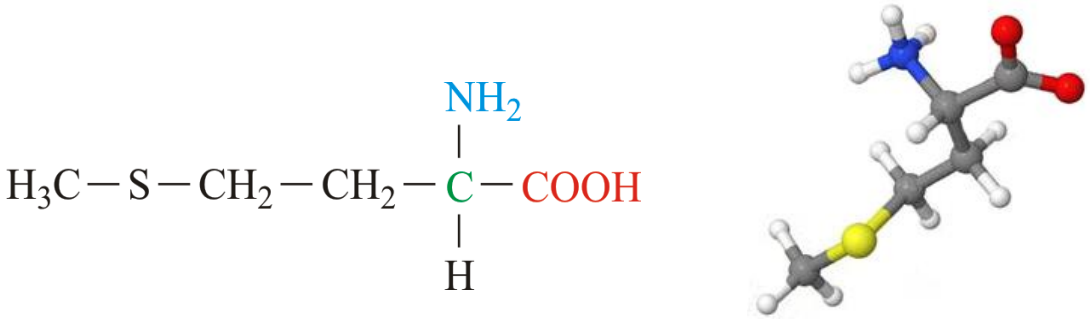

Figure 2-1: chemical structure of methionine **(Shoob *et al.*, 2001)**.

Methionine: is essential sulfur containing amino acid obtained from various proteins or prepared synthetically and used as a dietary supplement and pharmaceuticals **(Shoob *et al.*, 2001)**.

2.1.1 Properties

Methionine is colorless or a white lustrous plate, crystalline powder, has a slight characteristic odor minimally soluble in water, alkaline solutions, mineral acids, and slightly soluble in alcohol and insoluble in ether **(Hoeler & Hooge, 2003).**

2.1.2 Source of methionine

Virtually all protein-containing foods have some methionine, the amount varies widely, eggs, fish and some meats contain high amounts of this amino acid, it is estimated that around 8% of the amino acids in egg whites are sulfur-containing amino acids (methionine and cysteine), this value is about 5% in chicken and beef and 4% in dairy products, plant proteins usually have even lower quantities of these amino acids, some research has also examined the overall amount of the sulfur-containing amino acids (methionine and cysteine) in different types of diets, the highest content (6.8 grams per day) was reported in high-protein diets, while lower

intakes present for vegetarians (3.0 grams per day) and vegans (2.3 grams per day), despite the low intake among vegetarians, other research has shown that they actually have higher blood concentrations of methionine than those who eat meat and fish, this finding led the researchers to conclude that dietary content and blood concentrations of methionine are not always directly related, however, it was found that vegans have both low dietary intake and low blood concentrations of methionine **(Tinsley, 2018).** The recommended daily methionine intake is 13 mg/kg or about one gram daily for adults **(Sahi *et al*., 2006).**

2.2 Homocysteine (Hcy)

The Hcy is a sulfur-containing amino acid formed in the metabolic pathway between methionine and cysteine (**Zidan & Elnegris, 2015**). Elevated levels of Hcy known as HHcy are associated with various diseases and disorders like cardio vascular disease , Alzheimer's disease and bone abnormalities: such as osteopenia and osteoporosis **(Ciaccio & Bellia, 2010; Zidan & Elnegris, 2015).** The HHcy can be caused by genetic defects, nutritional deficiencies, renal dysfunction, alcoholism, hypothyroidism, or certain medications **(Petras *et al*., 2014**; **Škovierová *et al*., 2016).**

2.2.1 Biosynthesis and Metabolism of Homocysteine

The L- Methionine has a methyl (CH3) group that attached to its sulfur atom**,** in the Methionine Cycle, the methionine's methyl group becomes activated by ATP, with addition of adenosine to the sulfur of methionine under the action of enzyme Methionine Adenosyl Transferase (MAT) to form SAM which is the most important methyl group donor in biologic methylation and a compound with high-energy that unusual in that it contains no phosphate **(Anderson *et al*., 2012; Denise, 2014).**

After activation of methyl group it can be easily transferred by methyl transferases enzyme to a variety of acceptor molecules such as norepinphrine in the synthesis of epinephrine or to DNA methyltransferase as an intermediate acceptor in the process of DNA methylation **(Appleton & Vanbergen, 2012; Denise, 2014).**

After donate the methyl group the result of reaction product is S-Adenosyl homocysteine (SAH) **(Tehlivets *et al*., 2013).** The SAH is hydrolyzed to Hcy and

Adenosine. This reaction is reversible, although the thermodynamic equilibrium favors SAM synthesis since both Hcy and adenosine are usually quickly removed, leading the reaction to progress toward hydrolysis **(Tinelli *et al*., 2019).**

The Hcy has two pathways if there is a deficiency of methionine; Hcy may be remethylated to methionine, if methionine stores are adequate; Hcy may enter the transsulfuration pathway, where it is converted into cysteine that can be used in the formation of the anti-oxidant molecule GSH **(Dahlhoff *et al*., 2013; Denise, 2014).**

2.2.2 The Remethylation pathway

In this process Hcy converts to methionine, Hcy accepts a methyl group from N-5-Methyltetrahydrofolate (N-5- MTHF) known as folic acid or folate. N-5- MTHF in concert with vitamin B12 as a methyl-group donor involved in the conversion of the amino acid Hcy to methionine (**National Center for Biotechnology Information**, 2019). In this reaction Vitamin B12 removes the methyl group from N-5-MTHF and produces tetra hydrofolate (THF) **(Goljan, 2011).** And then the Methylated vitamin B12 transfers the methyl group to Hcy, to produces methionine with help of methionine synthase enzyme **(Mahmood, 2014),** as shown in **figure (2-2**).

The N-5- MTHF is the major source of methyl groups for the Remethylation of Hcy although, depending on the body organ, Betaine, through the enzyme involved; Betaine-homocysteine methyltransferase (BHMT) can also act as methyl group donors. The Betaine pathway is mainly restricted to the liver, kidney and lens, in which BHMT is primarily expressed and Betaine is an intermediate of choline oxidation **(Škovierová *et al*., 2016).** In fact, around 70% of generated Hcy comes from the Remethylation step depending on the content of methionine and choline in the diet **(Tinelli *et al*., 2019).**

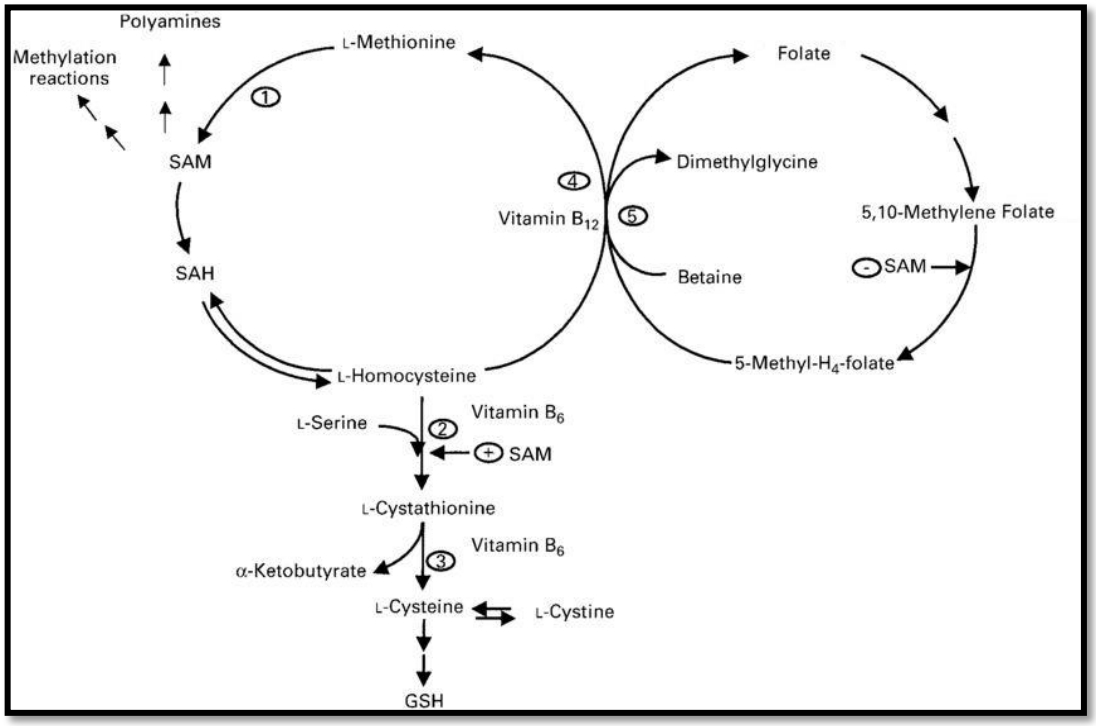

Figure 2-2 metabolic pathways describing L-methionine metabolism and the remethylation transsulfuration **(Škovierová *et al*., 2016)**

2.2.3 Transsulfuration pathway

Transsulfuration of Hcy to cysteine is catalyzed by two vitamin B6-dependent enzymes, cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE), as shown in Figure 2-3, CBS catalyzes the condensation of Hcy and serine to form cystathionine, cystathionine is then hydrolyzed by CSE to form cysteine and α-ketobutyrate plus ammonia, α-ketobutyrate can be further catabolized by oxidative decarboxylation to propionyl-coenzyme A (CoA), which enters the tricarboxylic acid cycle at the level of succinyl-CoA. Thus, the transsulfuration pathway is responsible for catabolism of the carbon chain of methionine, release of the amino nitrogen in a form that can be funneled into pathways of nitrogen excretion, and transfer of methionine sulfur to serine to synthesize cysteine, where cysteine is a precursor for the synthesis of proteins: coenzyme A, sulfates and GSH. The last one is a tripeptide that reduces reactive oxygen species, thereby protecting cells from oxidative stress **(Richard, 2011; Stipanuk & Ueki, 2011; Belalcázar *et al*., 2014; Kumar *et al*., 2017).**

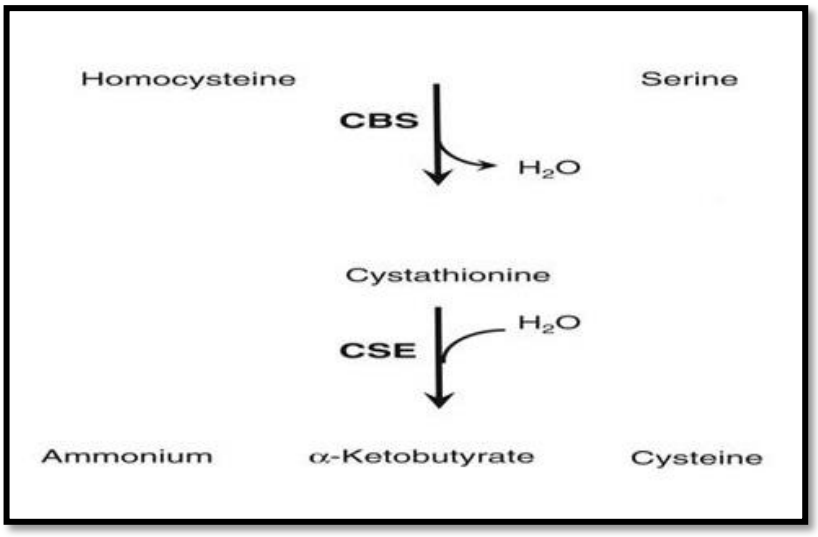

Figure (2-3) Transsulfuration pathway of homocysteine **(Stipanuk, & Ueki, 2011).**

2.3 Hyperhomocysteinemia (HHcy) causes and risk factors:

2.3.1 Causes of Hyperhomocysteinemia (HHcy)

The major cause of HHcy is the genetic defects of the transcription of enzymes responsible for the Hcy metabolism **(Horigan *et al.*, 2010).** The main enzymes involved in Hcy metabolism such us Methylene tetra hydrofolate reductase (MTHFR), CBS, Methionine synthase, Methionine synthetase reductase, and MAT **(Ambrosino *et al.*, 2015).**

One of the most studied polymorphisms is C677T, about gene encoding for the folate-metabolizing enzyme, MTHFR. It has been estimated that 10% of the worldwide population is homozygous (TT genotype) for the common C677T polymorphism, but the frequency can rise up to 25% in southern Italy and to 32% in some areas in Mexico, The TT genotype is responsible for the reduced activity of the MTHFR enzyme, which in turn leads to an increase of Hcy concentrations, molecular studies pursued on individuals carrying the TT genotype have shown that mutated MTHFR enzymes have a decreased affinity for riboflavin cofactor **(Horigan *et al.*, 2010; Tinelli *et al.*, 2019),** it has been shown to be an important modulator of Hcy concentration, especially in individuals with TT genotype **(Wilson *et al.*, 2010).**

The MTHFR gene C677T strongly influences the levels of the Hcy and cardiovascular risk **(Santilli *et al.*, 2016).** So the close relationship between MTHFR polymorphisms and folate levels in the serum of the mothers raises a question about

the use of dietary supplements containing folic acid by pregnant women. Another important polymorphism is T833C which is present, as a mutation, on the gene encoding for CBS, an enzyme that takes part in the transsulfuration pathway in Hcy metabolism converting Hcy to cystathionine, and this mutation in turn lead to increasing Hcy levels (**Tinelli *et al*., 2019)**. Evidence has shown that the genetic polymorphism of CBS: T833C an enzyme involved in HHcy was associated with an increased risk for developing stroke **(Ding *et al*., 2012).** In addition to genetic causes there are some studies shows HHcy may be the consequence of immune system activation. In fact the increase of reactive oxygen spices (ROS) production, induced by immune system activation, involves a greater demand for antioxidants, such as vit B12 and folate, and in case of a non-sufficient dietary intake this could lead to HHcy **(Schroecksnadel *et al*., 2004; Ientile *et al*., 2010).**

Others causes depend mainly on habits and lifestyle have been identified as being responsible for HHcy, For example, nutritional deficiencies of some of the cofactors involved in Hcy metabolism such as folic acid, vit B6, vit B12, and betaine are undoubtedly responsible for the development of HHcy, folic acid consumption is reduced especially in those countries in which the fortification of cereal-grain products is absent or rare, for example, it has been reported that 33.8% of preschool-age children in Venezuela contain a folate deficit, compared with 48.8% of pregnant woman in Costa Rica and 25.5% in Venezuela. Moreover, up to 61% of the Latin American and Caribbean population showed a reduced concentration of vitamin B12, which is caused by nutritional deficits affecting a large sector of the population, including vegetarians **(McLean *et al*., 2008).**

Indeed, a report provided evidence of a low plasmatic level of vit B6 in the 40% of women from 21 to 44 years old **(Ho *et al*., 2016).** Prevalence of high Hcy>14 μmol/L was found in 29.3% of subjects and was greatest among the subjects with low folate level **(Tinelli *et al*., 2019).** Patients with Renal failure show extremely high Hcy levels due to less efficient renal clearance of Hcy **(Martella *et al*., 2018).** Males and females may vary according to different habits, such as cigarette smoking, alcohol consumption, and sedentary lifestyle **(Cohen *et al*., 2019).**

2.3.2 Hyperhomocysteinemia as a risk factor

Elevated concentrations of Hcy, referred to as HHcy that recognized as a risk factor for different types of diseases and prolonged exposure to this condition can lead to the onset of several pathological conditions **(Tinelli *et al*., 2019).** One of these pathological conditions is cardiovascular diseases; where HHcy considered an important and independent risk factor for atherosclerosis, cardiovascular disease **(Gorial *et al*., 2013; Ganguly & Alam, 2015),** and ischemic stroke **(Banecka-Majkutewicz *et al*., 2012).**

Also elevated Hcy has been reported to be associated with atrial fibrillation **(Yao *et al*., 2017).** Cross-sectional and case control study have pointed towards a clear correlation between serum Hcy and the incidence of coronary, carotid, and peripheral vascular disease **(Okura *et al*., 2014).** The WHO agreed to consider HHcy a strong contributor for cardiovascular disease **(Tinelli *et al*., 2019).**

It has found that HHcy also implicated in pathogenesis of various diseases affecting the nervous system, such as stroke, Parkinson's disease, Alzheimer's disease, multiple sclerosis and epilepsy **(Ientile *et al*., 2010; Nevmerzhytska *et al*., 2019).** Elevated concentrations of Hcy are indeed implicated in an augmented risk of dementia, in particular Alzheimer's disease **(Smith *et al*., 2010; Smith *et al*., 2018).** High levels of Hcy were also found in the blood of Parkinson's and epileptic patients **(Belcastro & Striano, 2012; Ni *et al*., 2014).**

The HHcy also play an important role during pregnancy which can cause damage to the vascular system that support the placental function, and this damage might lead to miscarriage and other adverse pregnancy outcome **(Humadi, 2016).** It has been reported that HHcy as a risk factor in recurrent and early pregnancy losses **(Nelen *et al*., 2000; Osunkalu *et al*., 2015),** and late pregnancy complications such as: preeclampsia, eclampsia **(Qureshi *et al*., 2010),** preterm birth **(Dhobale *et al*., 2012),** intrauterine growth retardation **(Furness *et al*., 2013),** low birth weight, placental abruption, and intrauterine fetal death **(Bergen *et al*., 2012).**

It has found that HHcy induce congenital defects include Down syndrome, congenital heart defect, neural tube defect and nonsyndromic oral clefts **(Perna & Ingrosso, 2016; Škovierová *et al*., 2016).** Folate deficiency and HHcy are also

effects on oocyte quality and maturation, implantation, placentation, fetal growth, and organ development, correlating with sub fertility and negatively correlated with embryo quality **(Boxmeer *et al*., 2009).** The HHcy is recognized as risk factor for osteoporosis **(Behera *et al*., 2017),** which is the main topic that we will discuss.

2.4 Bone

Bone is a rigid organ that constitutes part of the vertebrate skeleton in animals, bones come in a variety of shapes and sizes and have a complex internal and external structure, they are lightweight yet strong and hard, and serve multiple functions like; protect the various organs of the body, produce red and white blood cells, store minerals particularly calcium (Ca^{+2}), provide structure and support for the body, and enable mobility **(Newman, 2018).**

The bone tissue is a hard tissue, a type of dense connective tissue, it has a honeycomb-like matrix internally, which helps to give the bone rigidity, bone tissue is made up of different types of bone cells, osteoblasts and osteocytes are involved in the formation and mineralization of bone; osteoclasts are involved in the resorption of bone tissue, modified (flattened) osteoblasts become the lining cells that form a protective layer on the bone surface, the mineralised matrix of bone tissue has an organic component of mainly collagen called ossein and an inorganic component of bone mineral including hydroxyapatite and other salts, such as Ca^{+2} and phosphate, bone tissue is a mineralized tissue of two types, cortical bone and cancellous bone, other types of tissue found in bones include bone marrow, endosteum, periosteum, nerves, blood vessels and cartilage **(Marshall Cavendish Corporation, 2010; Newman, 2018),** as explained in the figure 2-4 and 2-5.

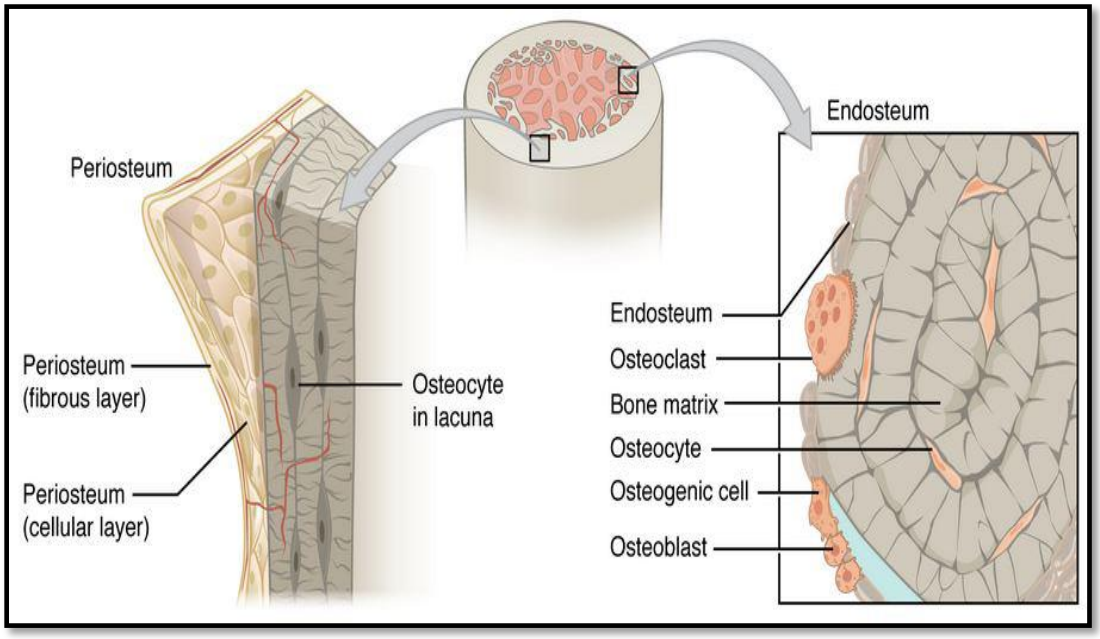

Figure (2-4) Periosteum and Endosteum: The periosteum forms the outer surface of bone, and the endosteum lines the medullary cavity **(Biga *et al.*, 2019).**

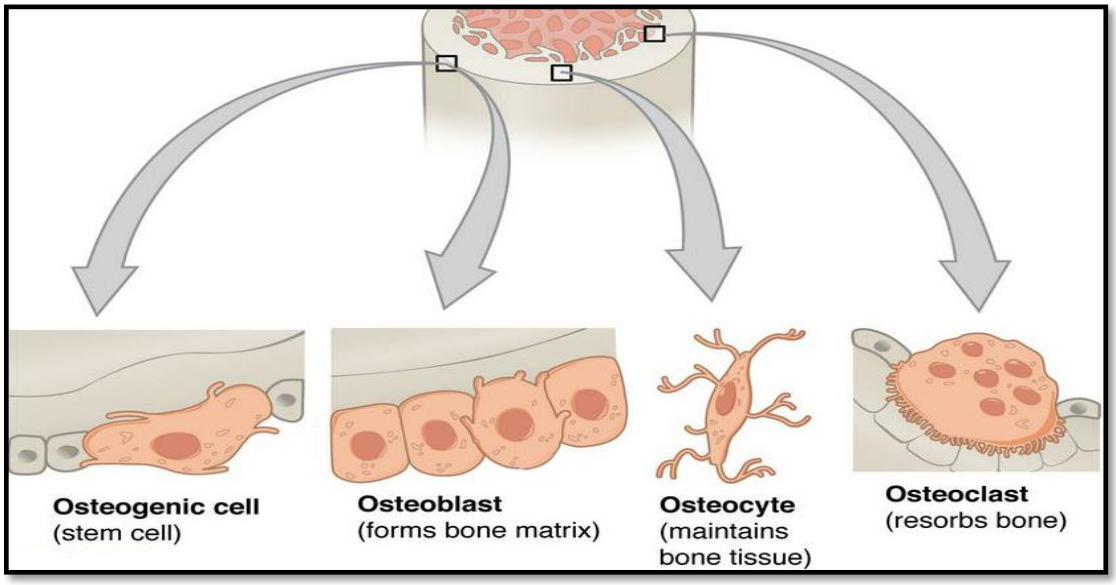

Figure (2-5) Bone Cells: Four types of cells are found within bone tissue (**Biga *et al.*, 2019**).

2.4.1 Bone remodeling

Bone formation and bone modeling is necessary for growth of the skeleton, and determines the size, shape and density of bone, a second process, bone remodeling, is crucial for mineral homeostasis, renewal of bone and repair of microdamage that can occur in response to mechanical stress (**Dole, 2015)**.Bone remodeling, performed by teams of osteoblasts and osteoclasts, occurs in small packets known as

basic multicellular units, and the cycle consists of 4 distinct phases: activation, resorption, reversal and formation **(Raisz, 1999; Raggatt & Partridge, 2010).**

Remodeling can be triggered either in response to microdamage or by hormones and local factors in response to fluctuations in Ca^{+2} and phosphate homeostasis. Microdamage-induced bone remodeling is initiated by osteocytes (mineralized osteoblasts) in proximity to the micro crack locus. This frequently results in localized osteocyte apoptosis, activation of osteoblasts and recruitment of osteoclast precursors to the resorption site, activated osteoblasts express key cytokines that promote osteoclast precursors to differentiate into mature bone resorbing osteoclasts. In addition, osteoblasts also secrete matrix metalloproteinases (MMPs), including MMP-13 that degrades osteoid on the bone surface, thereby providing surface domains for osteoclast attachment (**Dole**, 2015).

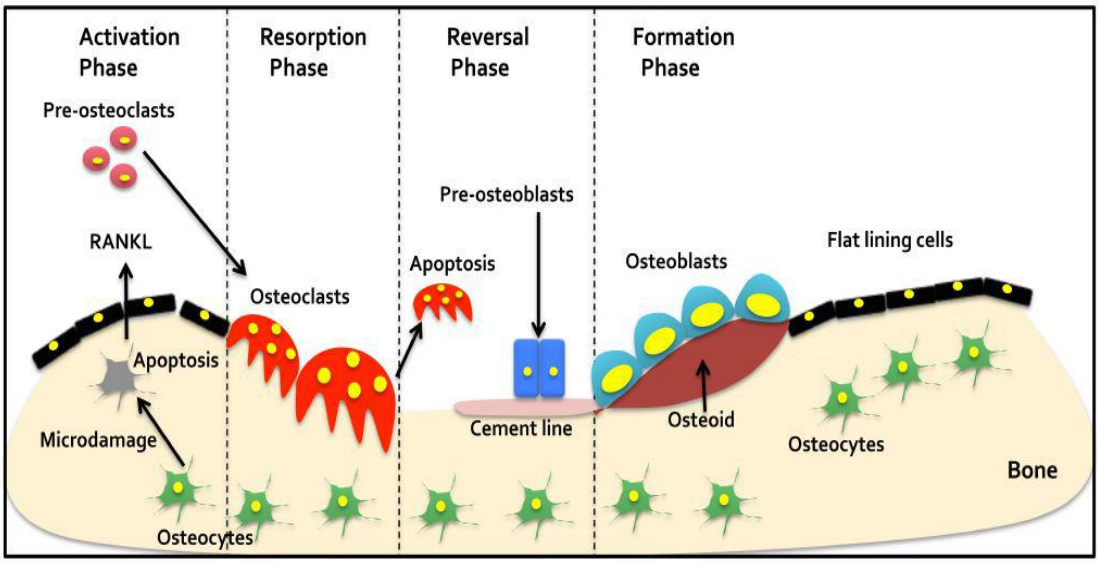

Figure 2-6 Schematic of bone remodeling (**Dole**, 2015).

During the bone resorption phase, osteoclasts attach to the bone surface through sealing zone and enclose a resorption pit **(Takito *et al*., 2018).** Osteoclasts generate H^+ and HCO^{3-} ions through carbonic anhydrase II enzyme activity; the H^+ ions are secreted into the resorption pit through the H^+ATPase proton pump, HCO^{3-} ions are pumped out of the cell by Cl^-/HCO^{3-} exchanger leading to an increase in intracellular Cl^-, which is secreted into the resorption pit by chloride channel type 7(ClC-7), the acidified environment created in the resorption pit leads to the demineralization of bone matrix. Subsequently, proteolytic enzymes like Cathepsin-K and matrix MMP-9 degrade the organic phase of bone matrix, following the

completion of resorption, osteoclasts undergo apoptosis, which marks the reversal phase, during the final "formation" phase of the remodeling cycle, local osteoblast precursors are recruited to the resorbed site. Recruited precursors undergo osteogenic differentiation and active mature osteoblasts deposit newly synthesized osteoid at the resorbed site (Figure 2-6) (**Raggatt & Partridge, 2010; Dole, 2015**).

2.4.2 Osteoclasts

Osteoclasts: are multinucleated cells that resorb the bone matrix keeping the skeleton healthy by ensuring bone turnover **(Cappariello *et al*., 2014).** They arise from the monocyte-macrophage lineage under the stimulation of two pivotal cytokines: Macrophage-Colony Stimulating Factor (M-CSF) and RANK-L, both mainly produced by osteoblasts in the bone, thus actively participating in the regulation of osteoclast formation, osteoclast differentiation, it appear to be a complex process that can be divided in three major stages: (1) the commitment of the hematopoietic stem cells towards the macrophages lineage; (2) the acquisition of the positivity for the tartrate resistant acid phosphatase (TRAcP) enzyme and calcitonin receptor, thus giving rise to an osteoclast precursor and (3) the fusion of the osteoclast precursors eventually forming to mature poly nucleated osteoclasts (Figure 2-7) (**Maurizi & Rucci, 2018).**

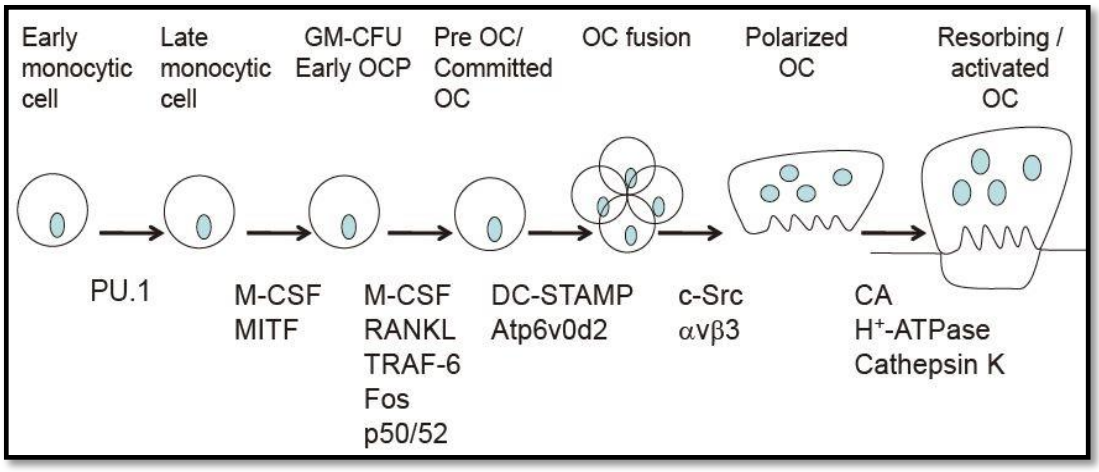

Figure 2-7 Regulation of osteoclast formation and differentiation (**Maurizi & Rucci, 2018**).

The proliferation and survival of preosteoclasts enhances by M-CSF **(Xu & Teitelbaum, 2013).** The understanding of its role became clear after the finding that mice lacking functional M-CSF, or mice knockout for the M-CSF receptor, displayed an osteopetrotic phenotype due to the complete lack of osteoclasts

(Wiktor-Jedrzejczak, 1990). Another important function for M-CSF is to elicit RANK receptor expression via osteoclast precursors **(Soysa *et al.*, 2012)** figure 2-8**.**

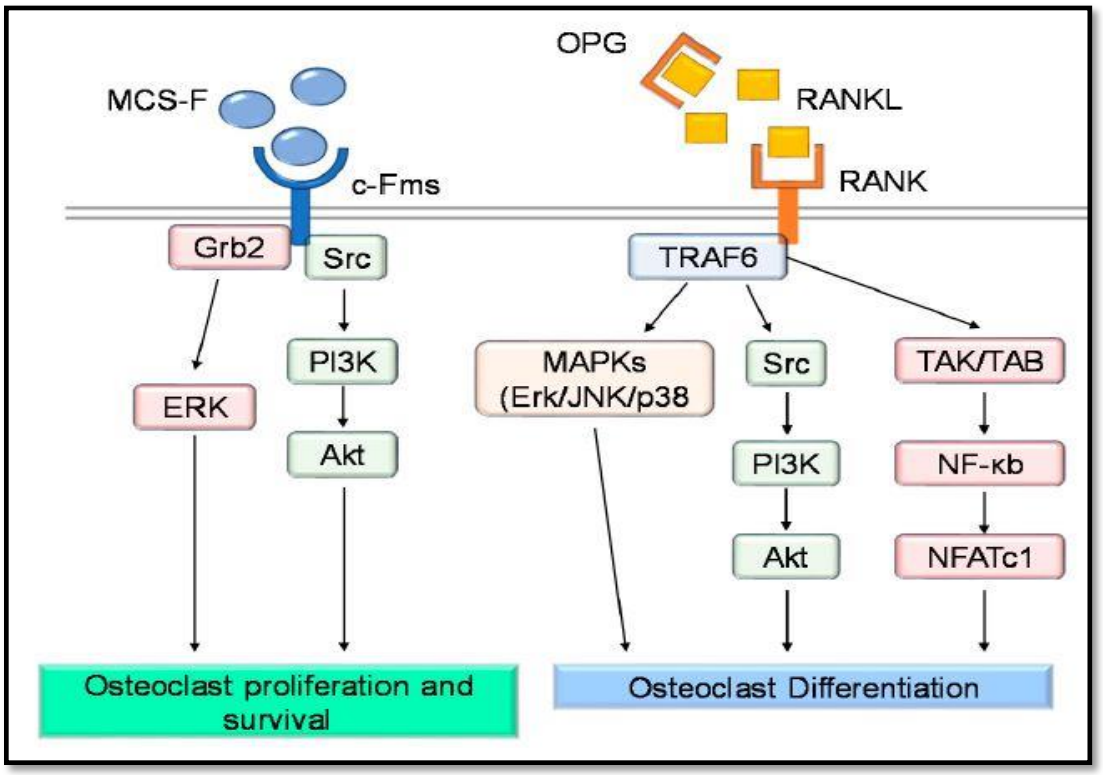

Figure 2-8 Molecular pathways involved in osteoclast proliferation, differentiation and survival (**Maurizi & Rucci, 2018)**.

2.4.3 Osteoclast function

A mature osteoclast is a multinucleated and polarized cell, in which it is possible to identify specific membrane domains reflecting specific functions (Figure 2-9). In the apical membrane there is a "sealing zone" that contains two distinct areas called the "sealing membrane" and the "clear zone" **(Cappariello *et al.*, 2014).** The "sealing membrane" is a bone-facing membrane portion including adhesion structures called podosomes **(Marchisio *et al.*, 1984),** these are made by actin microfilaments, adhesion molecules, adapter and signaling proteins that during bone resorption are essential for the formation of peripheral hoops called actin rings **(Schachtner *et al.*, 2013).**

Moreover, the integrin receptors alpha2 beta1, alpha V beta 3 and alpha V beta 5, anchor the osteoclast to the extracellular matrix, this tight adhesion is mandatory for osteoclast activity, thus, sealing the portion of the bone matrix to be degraded. Adjacent to the "sealing zone" is the "ruffled board": a peculiar membrane structure

consisting of several membrane expansions **(Siddiqui & Partridge, 2016).** Here, the lysosomal membranes fuse with the osteoclast membrane allowing the release of lysosomal enzymes that will digest the organic part of the matrix. Moreover, the ruffled board contains transporters essential for the release of ions, mainly chloride and protons, and for the reuptake of the resorbed materials from the resorption lacuna. Moving further from bone surface encounter the basolateral membrane domain that is in contact with the vascular compartment and present several ion transporters **(Baron, 1989).** In particular, the ruffled board presents a "fusion zone" and an "uptake zone" that is involved in the endo lysosome vesicular fusion, the re-uptake of digested bone matrix and recycling of the lysosomal enzymes **(Mulari *et al*., 2003; Hirvonen *et al*., 2013).**

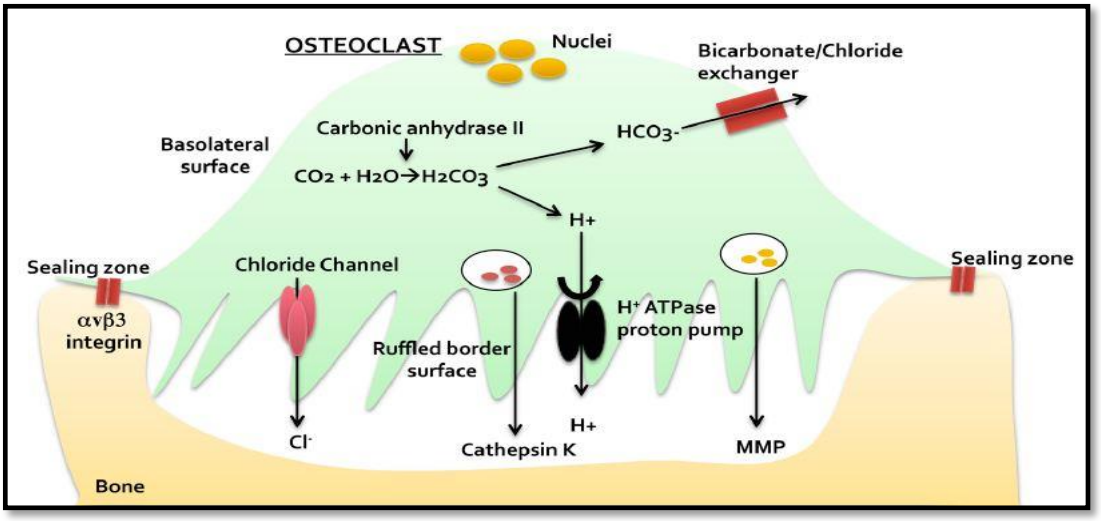

Figure 2-9 Mechanism of bone resorption by osteoclasts **(Dole, 2015).**

Moving to the basolateral membrane, a "functional secretory domain" is present, which is essential for releasing the digested matrix components into the blood stream **(Hirvonen *et al*., 2013).** Once adhered to the bone surface, the osteoclast can start the process of bone resorption. This process firstly requires the acidification of the area targeted for digestion, called the resorption lacuna or Howship lacuna, since it only becomes possible to dissolve the hydroxyapatite crystals with this chemical reaction **(Blair *et al*., 1986).**

On the intracellular side, type II carbonic anhydrase catalyzes the hydration of carbonic anhydride (CO2) thus forming carbonic acid (H_2CO_3), which in turn dissociates and releases protons (H^+) and carbonate ions (HCO^{3-}). The former is pumped outside the osteoclast into the Howship lacuna by the Vacuolar-ATPase (V-

ATPase) proton pump inserted in the ruffled border. At the same time, the chloride ion Cl^-/ HCO^{3-} exchanger allows the Cl^- to enter and HCO^{3-} to leave the osteoclast. Then, the proton/chloride antiporter ClC-7 also inserted in the ruffled border, together with its ß-subunit OSTM1 (osteopetrosis-associated transmembrane protein 1), allows the release of Cl^- into the Howship lacuna **(Baron, 1989; Cappariello *et al.*, 2014).**

Disorder in any one of the members of this molecular machinery induces the osteoclast-rich-osteopetrosis characterized by a high osteoclast number which however are unable to resorb bone **(Sobacchi *et al.*, 2013)**. We now have hydrochloride acid (HCL) inside the resorption lacuna, which allows the dissolution of the inorganic matrix and the subsequent exposure of collagen fibers, which can now be digested by lysosomal enzymes, mainly acid hydrolases, released into the lacuna by the osteoclasts **(Baron, 1989; Cappariello *et al.*, 2014).**

Cathepsin K and MMP-9 are crucial enzymes involved in this process **(Colnot *et al.*, 2003).** The resulting byproducts of degradation enter the osteoclast through endocytosis and are transported to the basolateral region in TRAcP rich vesicles and released to the exterior through exocytosis **(Arboleya & Castaneda, 2013).**

2.4.4 Receptor Activator of Nuclear factor Kappa-b (RANK)

RANK also known as TNF-related activation-induced cytokine receptor (TRANCE- receptor) or TNFRSF11A, is a member of the tumor necrosis factor receptor (TNFR) molecular sub-family (**Hai *et al.*, 2019**). RANK is the receptor for RANK-L and part of the RANK/RANK-L/OPG signaling pathway that regulates osteoclast differentiation and activation**,** it is associated with bone remodeling and repair, immune cell function, lymph node development, thermal regulation, and mammary gland development, RANK is encoded on human chromosome 18q22.1, it shows 85% homology between mouse and human homologues **(Liu *et al.*, 2010; Walsh & Choi, 2014).**

The human RANK Consist from 616 amino acids type I glycoprotein transmembrane, these amino acids divided into a C-terminal cytoplasmic domain of 383 amino acid, an N-terminal extracellular domain of 184 amino acid, a signal peptide of 28 amino acid and a transmembrane domain of 21 amino acid which

contains four cysteine rich pseudo repeats and two N-glycosylation sites **(Wright *et al.*, 2009; Schramek & Penninger, 2011)** figure 2-10**.**

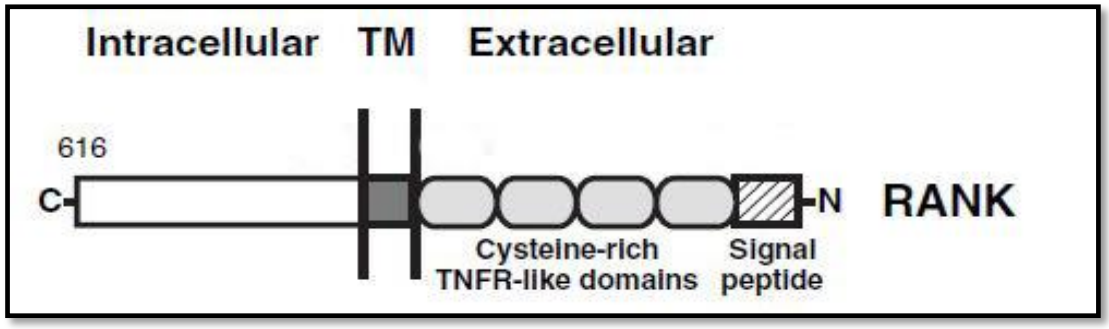

Figure 2-10 Protein structure of RANK **(Kearns *et al.*, 2008).**

Like other members of the TNFR family, it has four extracellular cysteine-rich pseudo-repeat domains (CRDs) spanning a length of 100 Angstroms which makes it the longest member of the TNFR family to date, the binding of RANK-L to RANK trimerizes the receptor and activates a signaling pathway, the RANK-RANK-L complex forms a heterohexameric complex, only two of the four RANK CRDs are in direct contact with the RANK-L (**Liu *et al.*, 2010)**.

The expression of RANK has been found on the surface of a wide variety of cells such as; mature osteoclasts and their precursors, fibroblasts, T and B lymphocytes, chondrocytes, endothelial cells, dendritic cells, mammary gland epithelial cells **(Czupkallo *et al.*, 2016),** and in malignant cells as breast cancer cells (**Rahnama, 2009),** and Prostate cancer cells **(Stanisławowski & Kmieć, 2009).**

Increased RANK expression is due to the activity of RANK-L, Interleukin-1 (IL-1),Macrophage inflammatory protein-1 alpha (MIP-1α), Vasoactive intestinal peptide (VIP), oncostatin M (OSM), vitamin D3, Transforming growth factor beta (TGF-β) and Interferon gamma (IFN-γ), whereas the reduction of the number of RANK molecules on the surface of osteoclasts may be induced by Interleukin-4 (IL-4) **(Vitoratos *et al.*, 2011; Dorota *et al.*, 2012).**

2.4.5 Receptor activator of nuclear factor kappa-B ligand (RANK-L)

The RANK-L is known as tumor necrosis factor ligand superfamily member 11(TNFSF11), TRANCE, Osteoprotegerin ligand, and also as osteoclast differentiation factor **(Wong *et al.*, 1997).**

RANKL is a type II homotrimeric transmembrane protein that is expressed as a membrane bound and a secreted protein, which is derived from the membrane as a result of either proteolytic cleavage or alternative splicing **(Patil & Desai, 2013).**

Three isoforms of RANK-L are described in figure (2-11) **(Kearns *et al*., 2008).** Each of which possesses similar C-terminal TNF-homology extracellular domains that are required for RANK activation **(Luan *et al*., 2012).** RANKL**1** and RANKL**2** isoforms possess a transmembrane domain and a stalk region that contains a proteolytic site that allows for cleavage of RANK-L from cell surfaces **(Lum *et al*., 1999),** the intracellular domains are absent in RANK-L2 and RANK-L3 **(Suzuki *et al*., 2004),** RANK-L3contains a truncated stalk region that possesses a proteolytic cleavage site, the function of which is unclear because this isoforms lacks a transmembrane domain and therefore secreted in the absence of proteolysis **(Theoleyre *et al*., 2004).**

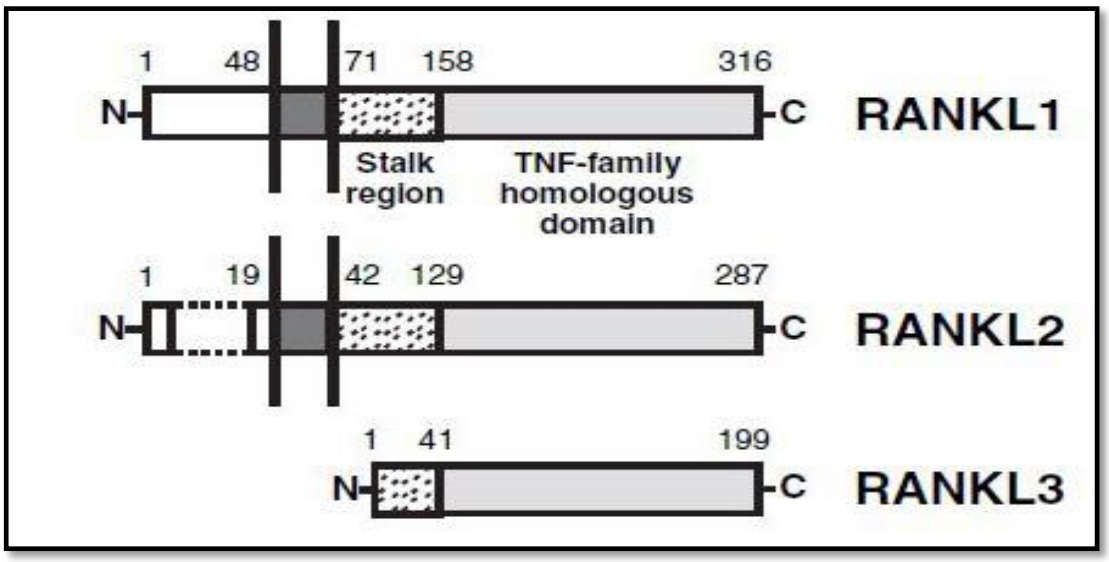

Figure 2-11 three isoforms of RANK-L **(Kearns *et al*., 2008).**

RANK-L is also cleaved to release soluble form (soluble RANK-L is sRANK-L) and circulates in the blood **(Schlöndorff *et al*., 2001).** RANK-L can be presented in a membrane or soluble form **(Sigl *et al*., 2016).** Both the membrane-spanning and soluble forms of RANK-L are assembled into functional homotrimers like other members of the TNFSF **(Liu *et al*., 2010).**

The binding of RANK-L to RANK causes trimerisation of the receptor, which triggers activation of nuclear factor kappa-light-chain-enhancer of activated B cells

(NF-kB) (**Rochette *et al.*, 2019)**. The Signaling pathway results in osteoclastogenesis from progenitor cells and the activation of mature osteoclasts **(Liu *et al.*, 2010).** The RANK-L primarily expressed on the surface of activated T-cells, bone marrow stromal cells and osteoblasts (**Dougall & Chaisson, 2006)**. Also it produces by fibroblasts, chondrocytes, endothelium, malignant cells, B lymphocytes **(Eghbali-Fatourechi, 2003)**, and megakaryocyte **(Bord *et al.*, 2004).**

The RANK-L also can be produced by osteocytes, within the bone matrix, that sense changes in load and microdamage that are thought to stimulate osteoclastogenesis via production of RANK-L at the initiation of the bone remodeling cycle **(Nakashima *et al.*, 2011; Xiong *et al.*, 2015).**

The following factors promote an increase in RANK-L gene expression: PTH, 1,25 (OH)2 vitD3, prostaglandin E2 (PGE2), inflammatory cytokines involves IL-1β, IL-6, IL-7, IL-11, IL-17, TNF, IFN-γ and glycocorticosteroids **(Stanisławowski & Kmieć, 2009).**

Over expression of RANK-L can cause an overproduction and activation of osteoclasts, which break down bone **(Schlöndorff *et al.*, 2001)**. RANK-L gene expression is blocked by 17β estradio1 and by IL-13 or TGF-β1 as well (**Stanisławowski & Kmieć, 2009**). RANK-L can be produced by a variety of hematopoietic (e.g. T and B-cell) and mesenchymal (osteoblast lineage, Chondrocyte) cell types **(Streicher *et al.*, 2017).**

2.4.6 Osteoprotegerin (OPG)

Osteoprotegerin (OPG) is a cytokine of the TNF receptor superfamily, It was named OPG because of its protective effects in bone (in Latin, “os” is bone and “protegere” is to protect). OPG is also known as osteoclastogenesis inhibitory factor or TNF receptor superfamily member 11B: (TNFRS11B). OPG is encoded by the TNFRSF11B gene, OPG is a soluble glycoprotein which can be exists either as a free monomer of 60 KD and 120-kD dimer linked by disulfide bonds or as OPG bound to its ligands RANK-L or TNF-related apoptosis-inducing ligand (TRAIL) (**Schramek & Penninger, 2011)**.

Unlike RANK and RANK-L, OPG does not have a transmembrane domain or cytoplasmic domain **(Wright, *et al*., 2009),** and composed of 401 amino acids which form seven functional domains, Domains 1-4 are CRDs N-terminal domains that interact with RANK-L during binding through form an elongated structure and binds to one of the grooves of the active RANK-L trimer, domains 5-6 are death domains that contribute to the dimerisation of OPG, domain 7 is a C-terminal heparin-binding domain ending with a cysteine (Cys-400) which also plays an important role in the dimerisation of OPG, (figure 2-12). The dimerisation of OPG is necessary for RANK/RANK-L inhibition as dimerisation increases the affinity of OPG for RANK-L **(Kearns *et al*., 2008; Baud'huin *et al*., 2013).**

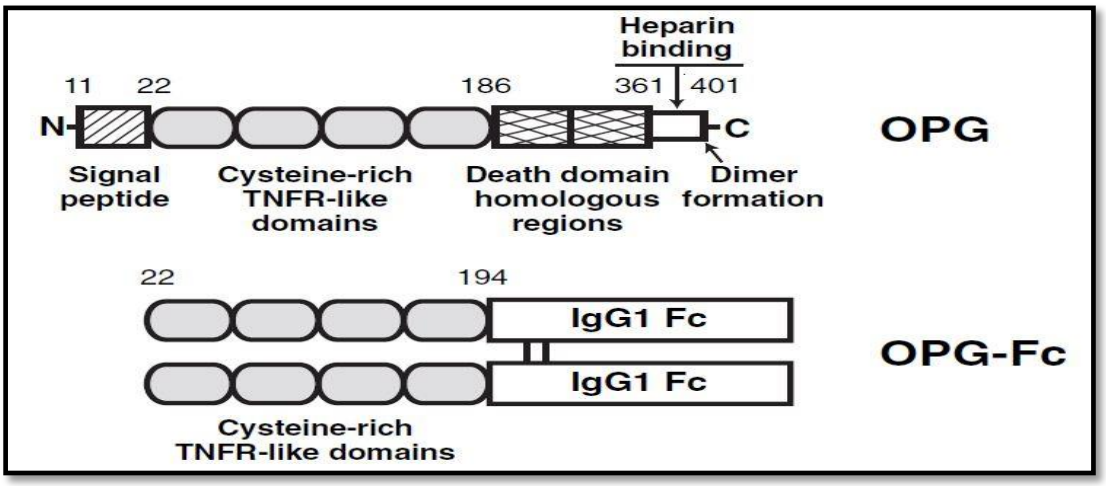

Figure 2-12 Protein structures of osteoprotegerin (OPG) **(Kearns *et al*., 2008).**

As a monomer, OPG would have insufficient affinity for RANK-L to compete with RANK and effectively suppress RANK-RANK-L interactions. Prior to secretion of both the monomeric and dimeric forms of OPG, the signal peptide is cleaved from the N-terminal giving rise to a 380 amino acid mature OPG protein. OPG is largely expressed by osteoblast lineage cells of bone, epithelial cells of the gastrointestinal tract, lung, breast, skin **(Fortner *et al*., 2017),** vascular endothelial cells as well as B-cells and dendritic cells in the immune system (**Sordillo & Pearse, 2003**).

The function of OPG are plays an important role in bone metabolism as a decoy receptor for RANK-L in the RANK/RANK-L/OPG axis, inhibiting osteoclastogenesis and bone resorption **(Czupkallo *et al*., 2016).** OPG has also been shown to bind and inhibit TRAIL which is responsible for inducing apoptosis in

tumour, infected and mutated cells **(Bernardi *et al*., 2016).** TRAIL is a 33–34 KD cell associated ligand which mediates apoptotic cell death particularly in cancer cells. TRAIL binds to specific cell surface receptors which contain cytoplasmic death domains, following this binding; TRAIL transduces an apoptotic signal **(Gochuico *et al*., 2000).** The binding of OPG to TRAIL was found to inhibit TRAIL-induced apoptosis of Jurkat cells in culture **(Emery *et al*., 1998).**

The OPG also has a basic heparin binding domain making interactions with heparin and heparin sulphates possible. Heparin sulphates are expressed on the cell surface as heparin sulphate proteoglycans (HSPGs). HSPGs are involved in cell-surface signaling, controlling cell behavior, actin cytoskeleton regulation, cell adhesion and migration **(Mosheimer *et al.*, 2005).**

The OPG expression is promoted by many factors, such as interleukins (IL-1α, IL-11, IL-13 and IL-18), growth factors TGF-β1, 17β-estradiol, OSM or mechanical loads exerted on bone (**Rahnama**, **2009**). In contrast, OPG inhibitors are: PTH, PGE2, immunosuppressive drugs, glucocorticoids, vitamin D3 and its derivatives (**Rogers & Eastell, 2005**).

2.4.7 Regulation of Osteoclast Differentiation and Activity

There is a network of paracrine and systemic factors regulating osteoclast differentiation involve M-CSF and RANK-L **(Soysa *et al*., 2012).** Among these, the proinflammatory cytokines play a crucial role in the enhancement of osteoclast differentiation. Indeed, TNF-α induces osteoclast differentiation directly by activating NF-κB and JNK (c-Jun N-terminal kinases) in a RANK-L-independent manner **(Kobayashi *et al*., 2000),** and indirectly by stimulating the osteoblasts to express RANK-L **(Lam *et al*., 2000).** In the same way, IL-1, IL-6 and IL-11 are potent osteoclastogenic factors **(Yao *et al*., 2008).**

Other molecules, such as EGF (epidermal growth factor), TGF alpha, OSF (osteoclast-stimulating factor), ECF-L (eosinophil chemiotactic factor-L) and Activin A increase both osteoclast differentiation and activity **(Kurihara *et al*., 2001).** In contrast, in addition to OPG, cytokines such us IL-3, IL-4, IL-10 and IL-12 inhibit osteoclast differentiation and bone resorption **(Khapli *et al*., 2003),** Figure 2-14. Several hormones regulate osteoclast fate, including estrogen and testosterone, PTH,

calcitonin, glucocorticoids, thyroid hormone and serotonin (**Siddiqui & Partridge, 2016**).

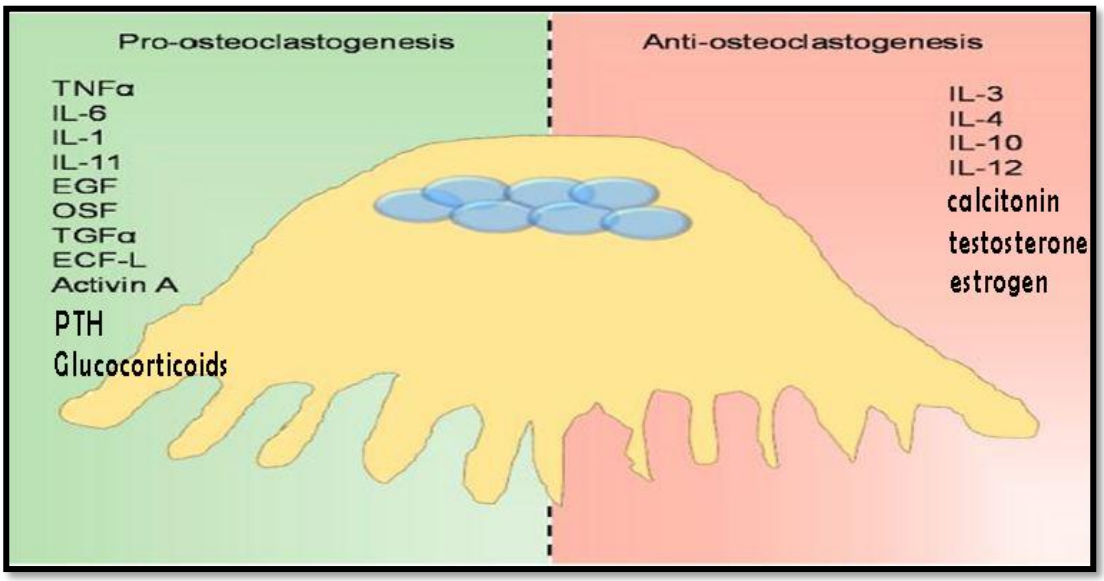

Figure 2-13 Osteoclast regulation. Cartoon illustrating the main molecules involved in the regulation of osteoclast differentiation with a pro- (green side) and anti- (red side) osteoclastogenic effect (**Maurizi & Rucci, 2018)**.

2.4.8 Osteoblasts

Arise from multipotent mesenchymal stem cells, and are responsible for the secretion of the organic components of the bone matrix, or collagen (type I) and proteoglycans, as well as for the synthesis of osteocalcin, osteopontin and proteinases **(Stanisławowski & Kmieć, 2009).** Depending on the developmental stage, the extracellular matrix proteins synthesized by osteoblasts differ, for example, the pre-osteoblasts for the most part synthesize and secrete type I collagen and fibronectin, while the immature osteoblasts secrete ALP. The more mature osteoblasts secrete bone sailoprotein followed by osteocalcin that associates with mineralized matrix **(Neve *et al*., 2011).**

Their functions are also regulated by PTH and 1,25(OH)2D3. The differentiation of osteoclasts is due to modification of osteoblasts through expression of RANK-L that activates the development of osteoclasts and to secretion of OPG that blocks the effect of RANK-L (**Stanisławowski & Kmieć, 2009)**. The proliferation and maturation of osteoblasts is influenced by many other growth factors (IGF1, IGF2, TGF-β1, TGF-β2, FGF, VEGF and PDGF). Pro-inflammatory cytokines, such as IL-

1 or TNF, play a role in the induction of osteogenic cells and increase RANK-L expression, thus causing disorders of bone metabolism (**Czupkallo *et al*., 2016**).

2.4.9 RANKL-RANK signaling

The interaction between RANK and RANK-L conceder necessary for osteoclast formation, both RANK and RANK-L deficient mice are severely osteopetrotic without osteoclast **(Lacey *et al*., 1998).**

The OPG is also produced by osteoblast, acts as a decoy receptor for RANK-L. The over expression of OPG prevents osteoclast formation resulting in osteopetrosis in mice, whereas deletion results in osteoporosis **(Simonet *et al*., 1997).** The binding of RANK-L to RANK initiates an internal signaling cascade via the cytoplasmic adaptor proteins called TNF receptor-associated factor (TRAF) adaptor proteins including TRAFs 1, 2, 3, 5, and 6 **(Wright *et al*., 2009).**

RANK has three binding domains for TRAFs, each of which has a different binding affinity for either TRAF 2, 5 or 6 which relay the RANK stimulation signal and activate downstream pathways (**Dehm & Bonham, 2004)**. Only TRAF6 has been found to be essential for osteoclast function **(Maxhimer *et al*., 2015).** TRAF6 deficient mice show severe osteopetrosis due to impaired osteoclastogenesis **(Kobayashi *et al*., 2003).** Despite the activation of TRAF6 by other receptors, such as CD40 and the IL-1R / Toll like receptor family members (TLR), only RANK-L can induce osteoclastogenesis (**Ye *et al*., 2002**).

By binding RANK-L to RANK will recruits TRAF-6 to activate all three MAPK pathways, namely ERK (extracellular signal-regulated kinase), JNK ,and p38 as well as PI3K and NFκB family of transcription factors (Figure 2-15). TRAF-6 deficient mice are severely osteopetrotic either with abundant, dysfunctional osteoclast **(Lomaga *et al*., 1999)** or without osteoclast **(Naito *et al*., 1999).**

MAPK-related TGF-β activated kinase1 (TAK1) along with TAK1- binding protein 2 (TAB2), are detected in activated receptor complexes. A dominant-negative form of TAK1 is able to abolish the RANK-induced activation of AP-1 and JNK suggesting that TAK1 is important in activation of NF-κB and AP-1**(Lee *et al*., 2002).**

Therefore the formation of the TRAF-6-TAB2-TAK1 complex is involved in the RANK signaling pathway and may regulate the formation and function of osteoclast. Gab2 is also identified as a crucial adapter that couples RANK to downstream signaling pathways required for osteoclastogenesis. The loss of Gab2 results in markedly reduced RANK-L/RANK-induced osteoclast differentiation, decreased bone resorption, and osteopetrosis, further clarifying its role in osteoclast formation **(Wada *et al*., 2005).**

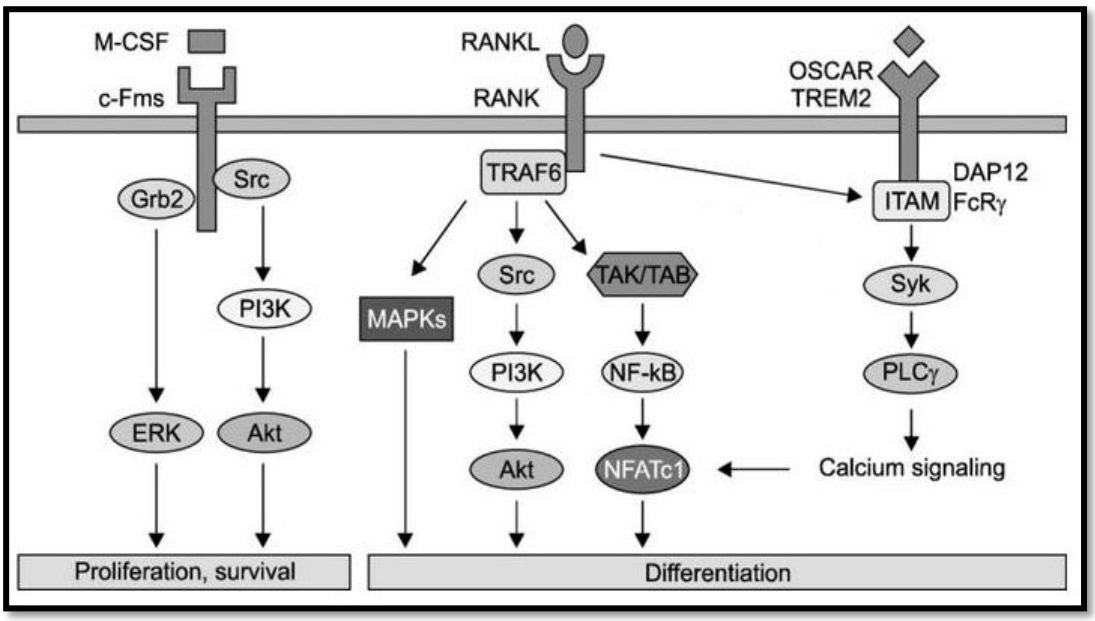

Figure 2-14. Osteoclast differentiation is stimulated by M-CSF and RANKL. M-CSF induces the proliferation and survival of osteoclast precursor cells through activation of ERK and Akt. RANKL recruits TRAF6 to activate MAPKs family proteins, Akt, and NFATc1 to promote differentiation of osteoclast precursors to osteoclasts **(Soysa *et al*., 2012).**

Activation of RANK/RANK-L signaling induces the recruitment of TRAF6, which in turn allows the activation of the NF-kB transcription factor, which translocates into the nucleus and promotes the transcription of genes regulating osteoclast differentiation **(Maurizi & Rucci, 2018).**

The inhibition of the NF-κB pathway by various inhibitors prevents the osteoclastogenesis and related bone resorption **(Alles *et al*., 2010).** The NF-κB is required for the expression of a variety of cytokines including IL-1, TNF-α, IL-6, M-CSF, RANK-L and other growth factors. The stress activated protein kinase, p38, is also involved in mediating signals induced by RANK **(Yamada *et al*., 2002).** The stimulation of p38 results in the downstream activation of the transcriptional

regulator, Microphthalmia-associated transcription factor (MITF). MITF is required for the terminal differentiation of osteoclast and mice lacking MITF gene developed osteopetrosis. MITF resides downstream of the M-CSF and RANK-L signaling pathways and controls the expression of genes encoding TRAcP, Cathepsin K **(Mansky *et al.*, 2002),** and osteoclast-associated receptor (OSCAR) **(So *et al.*, 2003).**

RANK-L induces the expression of the AP-1 family of transcription factors. AP-1 refers to a collection of proteins of the FOS family (c-Fos, Fra1, Fra 2 and Fos B) and Jun (c-Jun, Jun B and Jun D) families. Fos deficient mice are devoid of osteoclast with normal macrophage differentiation, thus, suggesting a block in differentiation at the branch point between monocyte-macrophage and osteoclast **(Wagner & Eferl, 2005).** RANK-L / RANK interaction activates many transcription factors including Nuclear Factor of Activated T-cells Cytoplasmic 1 (NFATc1), a calcineurin and Ca^{+2} regulated transcription factor. The presence of NFATc1 in precursor cells prompts them to undergo osteoclastogenesis in the absence of RANK-L NFATc1 expression is dependent on the TRAF-6-NF-KB and c-Fos pathways, which are activated by RANK-L and Ca^{+2} signaling as well (**Takayanagi *et al***., 2002).

2.4.10 Co-stimulatory signals

Certain co-stimulatory receptor signals of RANK namely, Triggering receptor expressed on myeloid cells 2 (TREM-2) and OSCAR are also required for the efficient osteoclast differentiation. For effective osteoclastogenesis, phosphorylation of immunoreceptor tyrosine-based activation motif (ITAM) of the adaptor proteins of TREM- 2 and OSCAR, DNAX-activating protein-12 (DAP12) and Fc receptor common γ subunit (FcRγ), respectively are necessary. Mice lacking the adaptor proteins DAP12 and the FcRγ are severely osteopetrotic **(Mócsai *et al*., 2004).**

Bone marrow cells of DAP12 /FcRγ/ fail to differentiate into osteoclast with impaired phosphorylation of the Tyrosine-protein kinase (Syk) showing the functional link between the RANK-L and co-receptors (Figure 2-15), the activation of the tyrosine kinase Syk results in activating the phospholipase C-γ (PLC-γ) which is necessary to increase the intracellular Ca^{+2} levels, and activating the NFATc1,

activation of tyrosine kinases Tec and Btk by RANK-RANK-L signaling also activates the PLC-γ (**Shinohara *et al***., 2008) (Figure 2-15)."

2.4.11 Osteoporosis

It's a metabolic bone problem, also recognized as “the silent thief” because there is slow bone loss, generally occurs over the years, and without any symptoms until the bone becomes so brittle that suddenly fracture occurs. Osteoporosis is the most common disorder related to age **(Lippuner *et al*., 2005).**

Increased plasma Hcy may be an independent risk factor for osteoporotic fractures and therefore may also adversely affect bone metabolism. It is a leading cause of fractures in old age: causing pain, affliction, hospitalization, financial burden, poor life quality leading to early death **(Burge *et al*., 2007).** Osteoporosis is usually considered a normal part of the ageing process and an unavoidable consequence of growing older. However, osteoporosis is a detectable, preventable, and treatable disease.

Two types of osteoporosis have been identified:-

1. Primary osteoporosis involves:

Type I also called postmenopausal osteoporosis occurs after menopause when the estrogen levels drop in the body. It typically involves the trabecular bone.

Type II also called senile osteoporosis takes place after 70 years of age, involving both trabecular and cortical bone.

2. Secondary osteoporosis it is due to the effect of medications like steroids or certain medical conditions **(Baig *et al*., 2015).**

2.5 Hcy and bone interaction evidences

The link between Hcy and bone disease was first made in 1966, when McKusick reported a disturbed collagen cross-linking in patients with homocystinuria **(Lubec *et al*., 1996).** During the last few years, abundant literature has been published about the interaction of HHcy and bone. Several mechanisms have been proposed about the involvement of Hcy in bone pathology **(Baig *et al*., 2015).** An elevated level of Hcy has been proposed as a new threat for primary osteoporosis **(Van Meurs *et al*., 2004).** Hcy showed up-regulates the formation of osteoclasts and suppresses apoptosis in these cells due to higher production of ROS. In patients with HHcy, the elevated activity of osteoclasts will lead to increase in bone resorption followed by

higher risk of fractures and decrease in bone mineral density (**Schalinske & Smazal, 2012)**.

Elevated level of Hcy activates caspase-dependent apoptosis in human bone marrow stromal cells, resulted in impairing of bone repair **(Kim *et al*., 2006).** Hcy may cause reduced blood flow in bone as it stimulates the atherogenic process, promotes platelet adhesion and has also been recognized as a potent thrombogenic compound that might contribute to compromised bone biomechanical properties **(Tyagi *et al*., 2011).**

A study by Enneman *et al*., reported inverse correlation of plasma Hcy levels with bone menial density (BMD) **(Enneman *et al*., 2014).** In the early years of Hcy and bone research, it seemed that Hcy directly affects biomechanical properties because deposition of Hcy in bone coupled with a decrease in cancellous bone **(Herrmann *et al*., 2008).** Another study found that the interaction of Hcy molecule with protein (collagen) in bone matrix takes place through thiol group and an amino group. This study also demonstrated that bone strength is reduced because most of the Hcy (65%) attaches with collagen **(Herrmann *et al*., 2009).**

A study proposed four mechanisms by which modification in bone remodeling takes place by Hcy: (i) enhancement in osteoclastic activity, (ii) reduction in osteoblastic activity, (iii) reduction in blood flow in bone, (iv) direct interaction of Hcy and bone matrix **(Herrmann *et al*., 2005).**

A study illustrated that Hcy inhibits lysyl oxidase also known as protein-lysine 6-oxidase, thus, interferes with post-translational changes of collagen and this leads to decrease in bone quality **(Liu *et al*., 1997).** The Hcy stimulated IL-6 production in osteoblasts, affects metabolism of bone by osteoclast. Janus kinase 2 (JAK2), DNA (cytosine-5) – methyltransferase 1 (DNMT1) stimulate IL-6, which affects bone matrix formation **(Thaler *et al*., 2011).**

A study documented that the skeleton and muscles movements reduce Hcy level and this link was not dependent on vitamin supplements, vegetables and fruits intake. Thus, it was suggested that levels of Hcy are also mainly affected by physical activity, though, nutritional condition also contribute significantly **(McLean *et al*.,**

2004). Thus, it seems that, regular exercise may decrease the Hcy level in HHcy individuals while it may help in maintaining Hcy level in normal subjects **(Baig *et al.*, 2015).**

A study on rats demonstrated that HHcy causes impairment in fracture repair. In their study, a closed femoral fracture was induced in rats after feeding them Hcy for 3-weeks, and biomechanical parameters were monitored after 4-weeks of healing. They found that hyperhomocysteinemic rats with fracture have reduced bending rigidity of femora and smaller callus diameter with no change in tissue composition and consequently it impaired fracture repair and reduced bone quality **(Claes *et al.*, 2009).**

A study tried to devise some likely therapeutic options for reducing increased plasma levels of Hcy that may cause damaging effect on bone. They used strontium ranelate 2g/day to lessen increased plasma levels of Hcy, because such material found to extensively reduce Hcy concentrations in osteoporotic women and were suggested to be employed as a mean to lessen fracture risk by reducing Hcy levels **(Bayhan *et al.*, 2009).**

The Hcy impairs the cross-linking of collagen and for the solidity and potency of the collagen network, these cross-links is essential. Any meddling in cross-link formation would therefore, affect the quality of bone matrix, which in turn causes the fragility of bones due to defective collagen formation **(Saito *et al.*, 2006).** It was found in an animal study that elevated levels of Hcy for three months increase femoral neck fragility by 18% in methionine-fed rats, and two-fold in Hcy fed rats. Lumbar spine and femoral neck also demonstrated reduce biomechanical characteristics, however, more in methionine- versus Hcy-fed rats **(Herrmann *et al.*, 2007).**

A meta-analysis provided a proof on Hcy and fracture risk, displaying that HHcy augments the fracture risk **(Yang *et al.*, 2012).** A very important evidence of effect of HHcy on fracture risk was provided by another meta-analysis including more than ten thousand subjects, demonstrated 4% augmented risk of fracture by per micro liter raise in Hcy level **(Van Wijngaarden *et al.*, 2013).**

Kim *et al*. investigated the effects of Hcy on human bone marrow stromal cells. They observed that Hcy induces apoptosis in primary human bone marrow stromal cells via the ROS-mediated mitochondrial pathway and NF-κB activation in human bone marrow stromal cells. Hcy was found to contribute to the development of osteoporosis by reducing bone formation **(Kim *et al*., 2006).** Koh *et al*. performed a similar study on osteoclasts and suggested that Hcy directly activates formation of osteoclasts by generation of intracellular ROS **(Koh *et al*., 2006).** Based on evidences, provided in the literature, it seems that HHcy may have negative role in bone metabolism generally and in the process of osteoporosis particularly **(Baig *et al*., 2015).**

Chapter three

Materials & Methods

3. Materials and Methods

3.1 Materials

3.1.1 Equipments and Apparatus:

The following Equipments and Apparatus were used throughout this study

Table (3-1) Equipments and Apparatus

Equipment	Company (origin)
Automated Microtome	Leica (Germany)
Binocular Stereo Microscope	Am Scope (U.S.A)
Centrifuge	Heraeus (England)
ELISA	Bio test (Germany)
FUJIFILM DRI CHEM NX500i	Fujifilm (Japan)
Furuno CA-180 chemistry analyzer	Furuno (Japan)
Light microscope	Olympus (Japan)
Metal Base Molds	Tanner (Germany)
Oven	Memmert (Germany)
Paraffin embedding system	Leedo (China)
Refrigerator	LG (Korean)
Scanning Electron Microscope (quanta 450)	FEI (U.S.A)
Sensitive electrical Balance	Sartorius (Germany)
Slide Staining Racks	Eisco (U.S.A)
Spectrophotometer	Unico, TM (U.S.A)
Thermoscientific Fischer pathology system	Arcos (united kingdom)
TOSOH AIA360	TOSOH (Japan)
Vortex	GMBH (Germany)
Water bath	Memmert (Germany)

3.1.2 Plastic and Glass Wares

Table (3-2) Plastic and Glass Wares

Equipment	Company (origin)
Cover slipping	Tanner (Germany)
Embedding Cassettes	Simport (China)
Eppendorf Tubes (1.5ml)	AL-Rawan (china)
Gel and Clot Activator Tube	AL-Rawan (China)
Glass slides	MEHE (China).
Medical examination gloves	Cliniva (Malaysia)
Micropipettes	Dragon lap (China)
Plastic container	Meheco (China)
Plastic disposable syringes	Meheco (China)
Veterinary Dissection Box	Indiamart (India)

3.1.3 Chemicals:

The following chemicals were used throughout this study:

Table (3-3) chemicals

Material	Company(Origin)
5,5 -dithiobis-(2-nitrobenzoic acid) (DTNB)	BDH (U.K)
Butan	Sigma (U.K
DPX	Fisher Chemical (UK)
Eosin y solution	Tissue pro (USA)
Ethanol alcohol	Chem lap (BELGIUM)
Ethylene Diamin Tetra Acetic Acid Disodium (EDTA-Na2)	Merck (Germany)
Formalin	BDH company (U.K)
Glutathione stander	BDH (U.K)
HCL	BDH (U.K)
Hematoxylin solution	Tissue pro (USA)

Hydroxyl Butylated Toloune	Merck (U.K)
Ketamine	alfasan (Netherlands)
L-Methionine Powder	NuSci (USA)
Nitric acid	Ecochem (New Zealand)
paraffin	HISTO Line (Italy)
Phosphoric acid	Merck (U.K)
Thiobarbituric Acid (TBA)	Sigma (U.K)
Trichloroacetic acid	BDH (U.K)
Xylazine	Indian Immunologicals (India)
xylene	Emsure (Germany)

3.1.4 The kit and reagent used in present study:

Table (3-4) kit and reagent

kit and reagent	company (Origin)
FUJI DRI-CHEM SLIDE ALP	FUJIFILM (Japan)
FUJI DRI-CHEM SLIDE Na-K-Cl	FUJIFILM (Japan)
IRON FZ reagent	Chema Diagnostica (Italy)
Rabbit Homocysteine ELISA Kit	bioassay technology laboratory (China)
Rabbit Receptor Activator of Nuclear Factor Kappa B Ligand ELISA	bioassay technology laboratory (China)
Rabbit Tumor Necrosis Factor Receptor Superfamily Member 11A ELISA Kit	bioassay technology laboratory (China)
ST AIA-PACK Intact PTH	TOSOH (Japan)

3.2 Methods

3.2.1 Animal and study design

Twenty white males New Zealand rabbits at Six months of age with average weight (3.3±0.2kg) were purchased from the Al-Razi Center that belongs to the Ministry of Science and Technology in Baghdad.

The rabbits were housed in a typically housing with regulated temperature (25±3C°), with maintained on a 12-h:12-h light: dark cycle in addition to the free access to food and water, after 2 weeks of adaptation**,** the male rabbits were randomly divided into the two groups (10/group), the control group and treated group with L- methionine 100mg/kg of Body Weight (BW), daily for twelve weeks**.**

At the end of the experiment, the average weight of control was (3.72±0.11kg), while treated group was (1.7±0.87kg). The two groups were anesthetized using a single dose of Ketamine (50mg/kg) administered slowly through the (lateral marginal vein) with administration of xylazine (0.6ml) Inter Muscular. The autopsy, blood samples collect and bones tissue collect were done in the research laboratory of the College of Veterinary Medicine of Karbala University.

3.2.2 Collection of Blood Samples

After performing anesthesia, ten milliliters of bloods were collected through heart puncture from each subject. The collected bloods was transferred immediately to the gel tube (Serum-separating tube with clot activator) The tubes was left at room temperature for Twenty minutes, and after clotting of blood, serum was separated by centrifugation at 3000 rpm for Twenty minutes. The collected serum was divided into labeled Eppendorf Tubes (1.5ml) then transported to the laboratory in cool box, in the laboratory it was kept at -20°C until analysis.

3.2.3 Collection of bone samples

All anesthetized subjects were killed by cervical dislocation**.** During autopsy process one femur from each subject was separate it from the carcass. Remove the fur and flesh, and preserved in labeled plastic containers containing Formaldehyde 10 percent and sent to the laboratory to complete histological preparation and examination.

3.2.4 Preparation and staining of bone tissue slides

Gross dissection and paraffin block preparation done after one day (twenty four hr) decalcification by twelve percent nitric acid , thereafter automated processing of the sample using Thermoscientific Fischer pathology system /UK, formed by automated processor, paraffin embedding system and automated microtome used to prepare slides after paraffin blocking **(Suvarna *et al*., 2018) .**

Slides are stained by Haematoxylen and Eosin stain through the following steps:-

1. Place the glass slides that hold the paraffin sections in staining racks. Clear the paraffin from the samples in three changes of xylene for two min per change.
2. Hydrate the samples as follows.

i. Transfer the slides through three changes of 100% ethanol for two min. per change.
ii. Transfer to Ninety-five percent ethanol for two min.
iii. Transfer to Seventy percent ethanol for two min.
iv. Rinse the slides in running tap water at room temperature for at least two min.

3. Stain the samples in hematoxylin solution for three min.
4. Place the slides under running tap water at room temperature for at least five min.
5. Stain the samples in working eosin Y solution for two min.
6. Dehydrate the samples as follows.

i. Dip the slides in Ninety-five percent ethanol about twenty times.
ii. Transfer to Ninety-five percent ethanol for two min.
iii. Transfer through two changes of 100% ethanol for two min. per change.

7. Clear the samples in three changes of xylene for two min per change.
8. Place a drop of DPX over the tissue on each slide and add a cover slip. View the slides using ordinary microscope **(Cardiff *et al*., 2014)**

3.2.5 Prepare longitudinal section of bone sample for microscopic examination

1. Place the Bone in diluted formalin solution at a concentration of ten percent for two days to prevent the biological tissues from decay due to autolysis or putrefaction.
2. After two days, remove the muscle and tissue residue from the bone by using:-

a. sharp scalpel
b. Salt. (Rub the bone with salt, which helps remove muscle residue).

3. Separate the head of bone with a little part of shaft from the rest of the bone by using a bone saw.
4. Cut the head of bone longitudinally using a sharp scalpel and hammer.

5. Place the bones in 500 ml of boiling water with two teaspoon of washing powder and gently stirring the mixture for one minute in order to remove the bone marrow from spongy bone.
6. Place the bone on plate then wash it with tap water by using syringe with needle to provide high presser water on spongy bone to remove leftover of bone marrow**.** Repeat the process many times until the spongy bone become clear from the remnants of the marrow.
7. Place the bone closed to heat source like electrical heater to dry. Exam the bone sample using:
   i. Binocular Stereo Microscope (dissecting microscope)
   ii. Scanning Electronic Microscope can directly imaged bone sample without need for special preparations or conductive layer for sample, when use low vacuum mode setup **(Donald, 2003).**

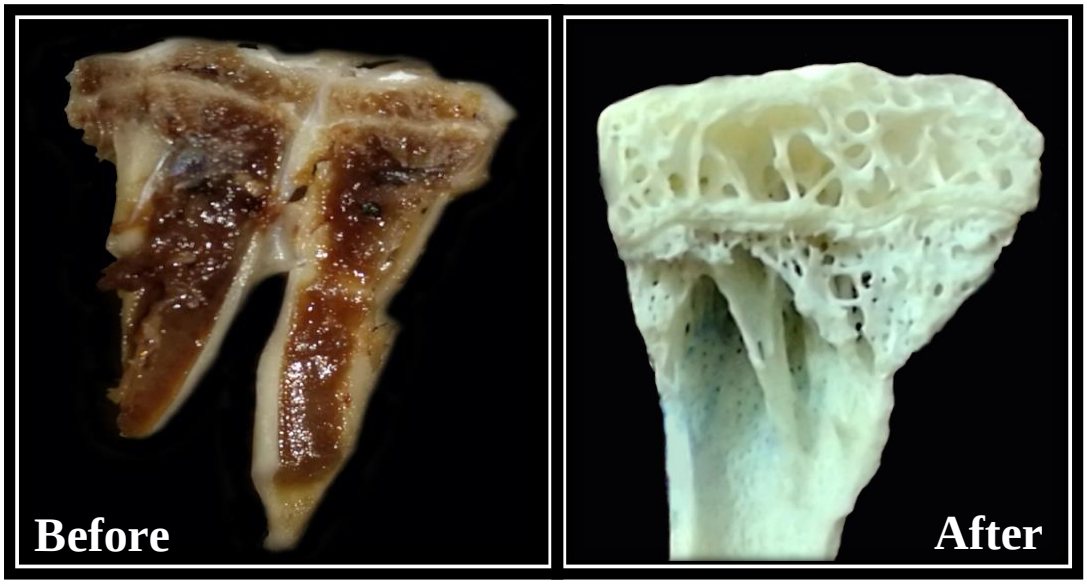

Figure (3-1) longitudinal section of bone sample before and after cleaning.

3.3 Measurement of serum Receptor Activator of Nuclear Factor Kappa b (RANK)

This assay was used to measure the levels of Tumor Necrosis Factor Receptor Superfamily Member 11A (RANK) content in serum of experimental male rabbits by using Rabbit Tumor Necrosis Factor Receptor Superfamily Member 11A ELISA Kit from Bioassay Technology laboratory, as per the manufacturer's instructions. Appendix (1)

3.4 Measurement of serum Receptor Activator of Nuclear Factor Kappa B Ligand (RANK-L)

This assay was used to measure the levels of Receptor Activator of Nuclear Factor Kappa B Ligand (RANK-L) content in serum of experimental male rabbits by using Rabbit Receptor Activator of Nuclear Factor Kappa B Ligand ELISA Kit from Bioassay Technology laboratory, as per the manufacturer's instructions. Appendix (2)

3.5 Measurement of serum Homocysteine (Hcy)

This assay was used to measure the total Homocysteine (Hcy) content in serum of experimental male rabbits by using a Rabbit Homocysteine ELISA Kit from Bioassay Technology laboratory, as per the manufacturer's instructions. Appendix (3)

3.6 Measurement of serum Malondialdehyde (MDA)

The level of Malondialdehyde (MDA) was determined by a modified procedure described by **(Jetawattana, 2005)**. Appendix (4)

3.7 Determination of serum reduced glutathione (GSH)

Reduce glutathione (GSH) determent by using the disulfide chromogenic compound 5,5- dithiobis (2-nitrobenzoic acid) (DTNB) which is readily reduce by sulfhydryl group of GSH to an intensely yellow compound. The absorbance of the reduce chromogen is measured at 412 nm directly proportional to the concentration of GSH **(Burtis and Ashwood, 1999).** Appendix (5)

3.8 Minerals and alkaline phosphatase (ALP) measurement assay

In this assay the serum of each subject was used to masseur Ca^{+2}, Na^{+}, K^{+} levels and ALP by FUJIFILM DRI CHEM NX500i chemistry analyzer device. The FUJIFILM DRI CHEM analyzer is fully automated and can masseur different types of minerals and enzymes at the same time by depending on two types of FUJIFILM DRI CHEM slides; the Colorimetric method slides and the Potentiometric method

slide ; neither type requires the preparation of any reagent and they storage at temperature range between (2 - 8c^{o}).

1. **Potentiometric method slide for Ca^{+2}, Na^{+} and K^{+} measurement assay:** Assays the electrolytes in the specimen. The specimen and an electrolyte reference solution with a fixed concentration of electrolytes are applied to the slide. The concentrations of the electrolytes are measured by the change in potential between two electrodes. One slide contains 3 types of film electrodes (Na^{+}, K^{+}, and Cl^{-}) and all three can be measured at once simultaneously in only one minute.
2. **Colorimetric method slides for alkaline phosphatase (ALP) measurement assay:** The enzymes, general chemical components, and immunological components in the samples are measure by colorimetry. Application of the sample to the slide results in a reaction between the component and the reagent, the formation of a pigment, and measurement of the concentration of chromophore that corresponds to the amount of component. The multilayered film slide begins with dry reagent needed for one measurement and has successive layers of functional materials. In the colorimetry slides, there is the end-point method (general chemistry) and rate method. Load sample either serum or plasma in special tube, Just 10 µl of serum or plasma are used per parameter in case of colorimetric slide. While to measure the electrolytes Na^{+}, K^{+} and cl^{-} on a single slide, a 50 µl specimen is required.

3.9 Determination of serum Iron concentration

The concentration of serum Iron (Fe^{+2}) was obtained by using Furuno CA-180 fully automated chemistry analyzer with special reagent kit that is ready to use, through following User's Manual of device. Appendix (6)

3.10 Determination of serum Parathyroid hormone (PTH)

The levels of parathyroid hormone (PTH) in serum of experimental subjects were determents by Fluorescence Enzyme Immunoassay technique through using (TOSOH-AIA-360 fully Automated Immunoassay Analyzer) through following User's Manual of device. Appendix (7)

3.11 Statistical analysis

Data were presented in mean ± Standard Deviation (SD), the data was analyzed by using "SPSS-25" by measuring independent samples T test after being coded. The comprised of significant P-value in any test were Significant difference $P \leq 0.05$ and Non Significant difference $P > 0.05$**(Kaps & Lamberson, 2017**).

Chapter Four

Results

4.1 Serum total homocysteine (Hcy) concentration

Table (4-1) show the effect of daily oral intubation of methionine (100mg/kg of BW) for twelve weeks, on mean value of serum Hcy concentration (µg/ml) of males rabbits. Where showed highly significant ($p \leq 0.001$) increase in the mean value of Hcy concentration of methionine treated group (33.47±3.25) in comparison with the mean value of control group (16.73±1.29).

Table (4-1) Effect of daily oral intubation of methionine (100 mg/kg BW) for twelve weeks on serum homocysteine (Hcy) concentration (µg/ml) in methionine treated males rabbits.

Parameter / Groups	Hcy (µg/ml)	P-value
Control	16.73±1.29	P≤0.001*
treated	33.47±3.25	

Values are expressed as mean ± SD. N = 10/group, statistically highly significant (*) (P≤0.001) difference between treated and control group.

4.2 Serum total receptor activator of nuclear factor kappa-b-ligand (RANK-L) concentration

Serum RANK-L concentration **(**ng/l) in control and methionine treated group are represent in table (4-2). The results show that daily oral intubation of methionine (100mg/kg BW) for twelve weeks to males rabbits caused highly significant (P≤0.001) increase in the concentration of serum RANK-L in methionine treated group as compared to the control group.

Table (4-2) show the effect of daily oral intubation of methionine (100mg/kg BW) for twelve weeks on serum Receptor Activator of Nuclear factor Kappa-b ligand (RANK-L) concentration (ng/l) in methionine treated males rabbits.

Parameter / Groups	RANK-L (ng/l)	P-value
Control	69.18±18.67	P≤0.001*
treated	123.85±15.44	

Values are expressed as mean ± SD. N = 10/group, statistically highly significant (*) (P≤0.001) difference between treated and control group.

4.3 Serum total receptor activator of nuclear factor kappa-b (RANK) concentration

Table (4-3) showed a highly significant (P≤0.001) increase in serum RANK (ng/ml) concentration of methionine treated group when compared to the mean value of control group.

Table (4-3) effect of daily oral intubation of methionine (100mg/kg BW) for twelve weeks on serum Receptor Activator of Nuclear factor Kappa-b (RANK) concentration (ng/ml) in methionine treated males rabbits.

Parameter / Groups	RANK (ng/ml)	P-value
Control	2.84±0.75	P≤0.001*
treated	5.32±0.66	

Values are expressed as mean ± SD. N = 10/group, statistically highly significant (*) (P≤0.001) difference between treated and control group.

4.4 Serum total parathyroid hormone (PTH) concentration

The data pertain to the serum PTH concentration (Pg/ml) of control group and treated group are descript in table (4-4). Comparing to control, the daily oral intubation of methionine caused highly significant (P≤0.001) increase in the concentration of serum PTH in males rabbits treated with daily methionine intubation (100mg /kg BW), for twelve week.

Table (4-4) effect of daily oral intubation of methionine (100mg/kg BW) for twelve weeks on serum parathyroid hormone (PTH) concentration (Pg/ml) in methionine treated males rabbits.

Parameter / Groups	PTH (Pg/ml)	P-value
Control	31.46±1.18	P≤0.001*
treated	44.78±3.36	

Values are expressed as mean ± SD. N = 10/group, statistically highly significant (*) (P≤0.001) difference between treated and control group.

4.5 Serum total alkaline phosphatase (ALP) concentration

The result of table (4-5) show highly significant (P≤0.001) increase in serum ALP concentration (u/l) of methionine treated group with men value (443.6 ±44.27) in comparison to the control group (252.0±29.08).

Table (4-5) effect of daily oral intubation of methionine (100mg/kg of BW) for twelve weeks on serum alkaline phosphatase (ALP) concentration (u/l) in methionine treated males rabbits.

Parameter / Groups	ALP (u/l)	P-value
Control	252.0 ± 29.08	P≤0.001*
treated	443.6 ±44.27	

Values are expressed as mean ± SD. N = 10/group, statistically highly significant (*) (P≤0.001) difference between treated and control group.

4.6 Serum total Glutathione (GSH) and Malondialdehyde (MDA) concentration

The result of table (4-6) display significant (P≤0.001) decrease in the mean value of serum GSH concentration (µmol/l) of methionine treated group in comparison to the mean value of control group after twelve weeks of daily methionine intubation.

The same table revealed a high significant (P≤0.001) increase in the mean value of serum MDA concentration (µmol/dl) of methionine treated group in comparison to the mean value of control group.

Table (4-6) effect of daily oral intubation of methionine (100mg/kg of BW) for 12 weeks on both serums Glutathione (GSH) concentrations (µmol/l) and serum Malondialdehyde (MDA) concentration (µmol/dl) in methionine treated males rabbits.

Parameter / Groups	GSH (µmol/l)	MDA (µmol/dl)
Control	15.3±0.066	0.41±0.006
treated	8.081±0.20	0.96±0.006
P-value	P≤0.001*	P≤0.001*

Values are expressed as mean ± SD. N = 10/group statistically highly significant (*) (P≤0.001) difference between treated and control group.

4.7 Serum total calcium (Ca^{+2}), sodium (Na^{+}), potassium (K^{+}) and iron (Fe^{+2}) concentrations

Depending on the results clarified in table (4-7) there were highly significant (P≤0.001) increase in the mean value of serum Fe^{+2} concentration (µg/dl) of methionine treated group when compared to the mean value of control group after twelve weeks of daily methionine intubation. Table (4-7) also showed significant (P≤0.001) decrease in the mean values of serum Ca^{+2} (mg/dl) and Na^{+} (mEq/l) concentration of methionine treated group when compared to control group. The same table also showed a significant (P≤0.05) decrease in the mean value of serum

K^+ concentration (mEq/l) of methionine treated group when compared to mean value of control group.

Table (4-7) effect of daily oral intubation of methionine (100mg/kg BW) for twelve weeks on serum iron (Fe^{+2}) concentration (µg/dl), calcium (Ca^{+2}) concentration (mg/dl), sodium (Na^+) concentration (mEq/l) and potassium (K^+) concentration (mEq/l) in methionine treated male rabbits.

Parameter / Groups	Fe (µg/dl)	Ca (mg/dl)	Na (mEq/l)	K (mEq/l)
Control	**92.60±12.8**	**14.18±0.042**	**141.0±0.94**	**4.86 ± 0.29**
treated	**181.50±15.93**	**9.92±1.54**	**127.5±1.18**	**4.28 ± 0.58**
P-value	**P≤0.001***	**P≤0.001***	**P≤0.001***	**P≤0.05**

Values are expressed as mean ± SD. N = 10/group statistically highly significant (*) ($P \leq 0.001$) difference between treated and control group, in Fe^{+2}, Ca^{+2} and Na^+ values. K^+ value showed significant difference ($P \leq 0.05$) between treated and control group.

4.8 Correlation

4.8.1 Correlation between Hcy and biomarkers levels.

The results of correlation and liner regression between Hcy and biomarkers levels of methionine treated rabbits are indicated:

1. The presence of a significant positive correlation (p=0.01) between Hcy and RANK-L levels of methionine treated male rabbits, (r=0.909), (figure 4-1).
2. The presence of a significant negative correlation (p=0.01) between Hcy and Ca^{+2} levels of methionine treated male rabbits, (r=-0.906), (figure 4-2).
3. The presence of a significant positive correlation (p=0.01) between Hcy and Fe^{+2} levels of methionine treated male rabbits, (r=0.916), (figure 4-3).
4. The presence of a significant positive correlation (p=0.01) between Hcy and ALP levels of methionine treated male rabbits, (r=0.799), (figure 4-8).

4.8.2 Correlation between RANK-L and RANK levels.

The results of correlation and liner regression between RANK-L and RANK levels of methionine treated rabbits are indicated:

The presence of a significant positive correlation (p=0.01) between RANK-L and RANK levels of methionine treated male rabbits, (r=0.826), (figure 4-4).

4.8.3 Correlation between PTH with all of Ca^{+2} and RANK-L.

The results of correlation and liner regression between PTH with all of Ca^{+2} and RANK-L in methionine treated male rabbits are indicated:

1. The presence of a significant positive correlation (p=0.01) between PTH and RANK-L levels of methionine treated male rabbits, (r=0.822), (figure 4-5).
2. The presence of a significant negative correlation (p=0.01) between PTH and Ca^{+2} levels of methionine treated male rabbits, (r=-0.811), (figure 4-6).

4.8.4 Correlation between Hcy and GSH levels.

The results of correlation and liner regression between Hcy and GSH levels of methionine treated rabbits are indicated: The presence of a significant negative

correlation (p=0.01) between Hcy and GSH levels of methionine treated rabbits, (r=-0.88), (figure 4-7)

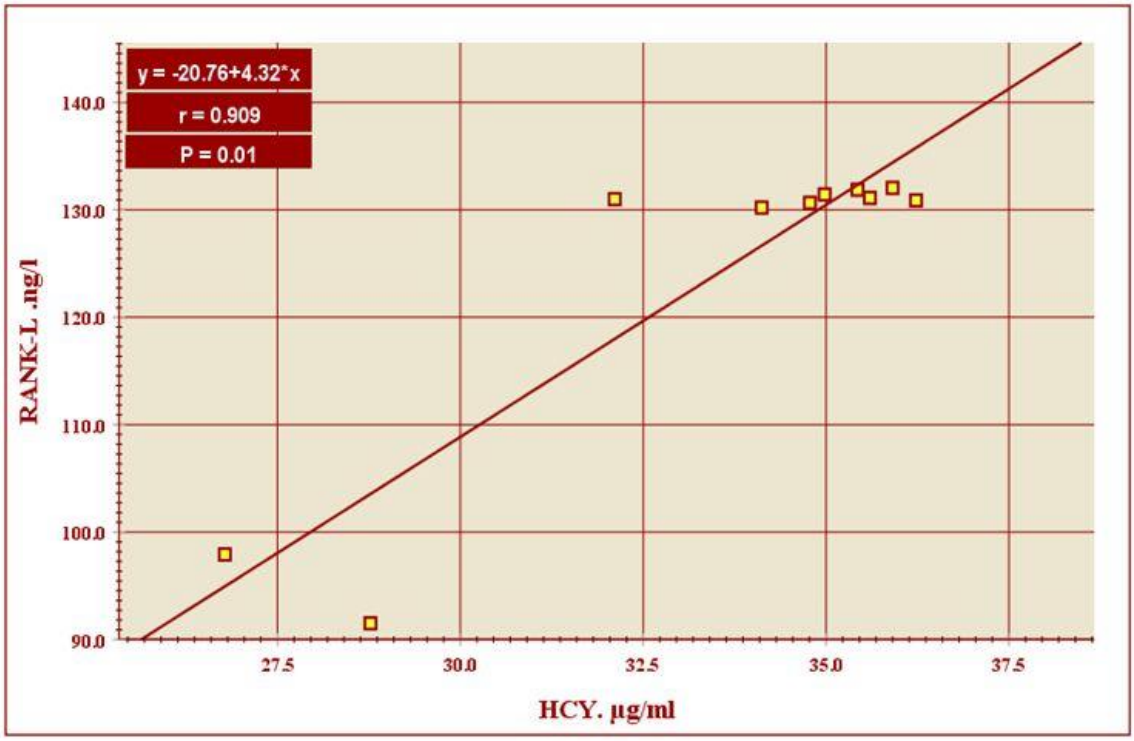

Figure (4-1): the correlation between serum Homocysteine (Hcy) and (RANK-L) in males rabbits treated with L-methionine 100mg/kg BW.

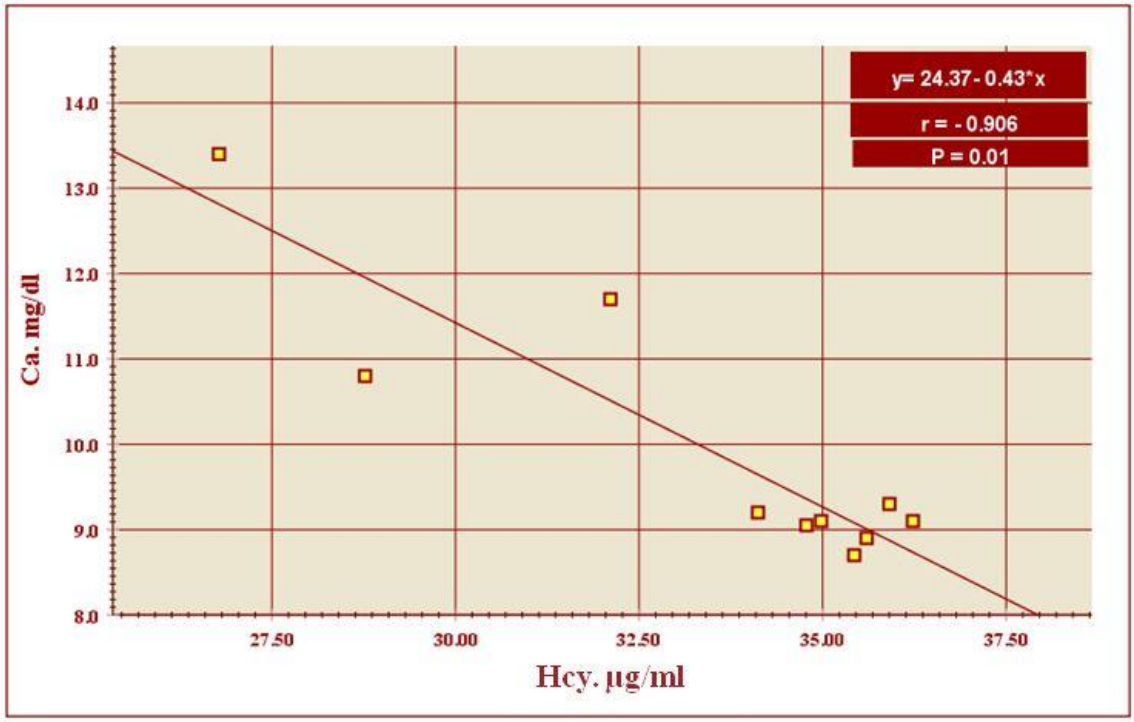

Figure (4-2): the correlation between serum Homocysteine (Hcy) and (Ca) levels in males rabbits treated with L-methionine 100mg/kg BW.

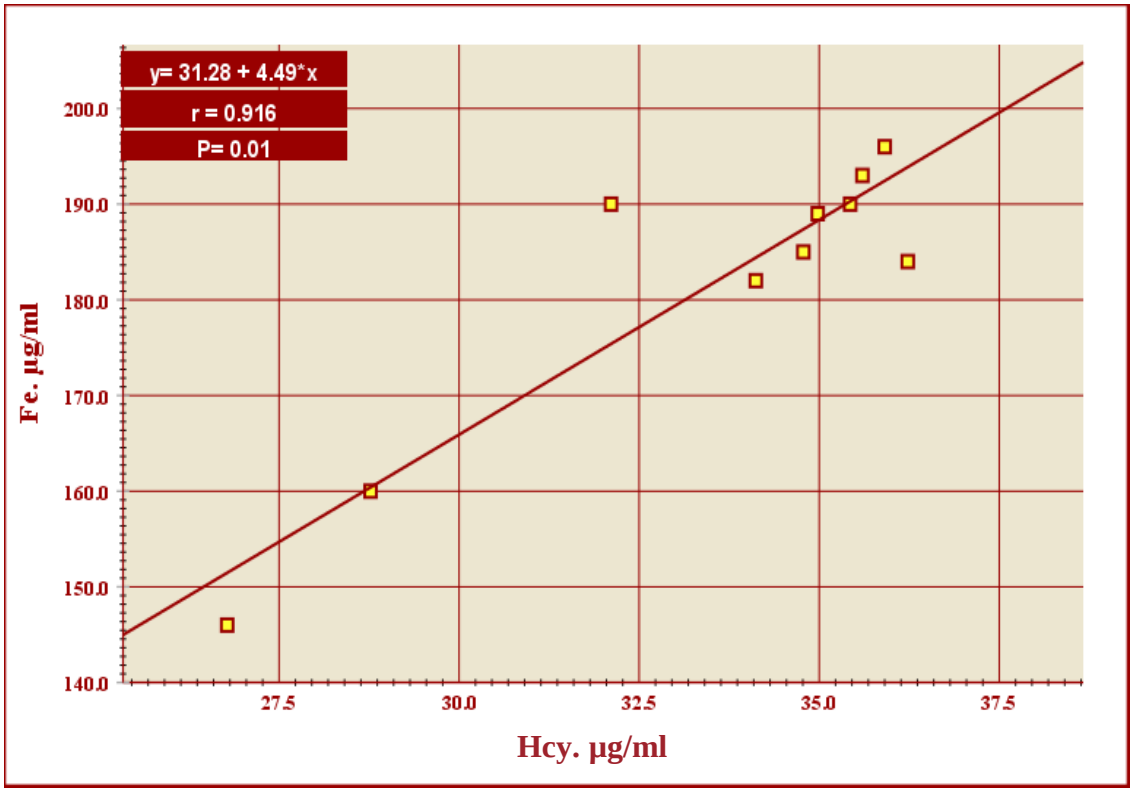

Figure (4-3): the correlation between serum Homocysteine (Hcy) and the (Fe) levels in males rabbits treated with L-methionine 100mg/kg BW.

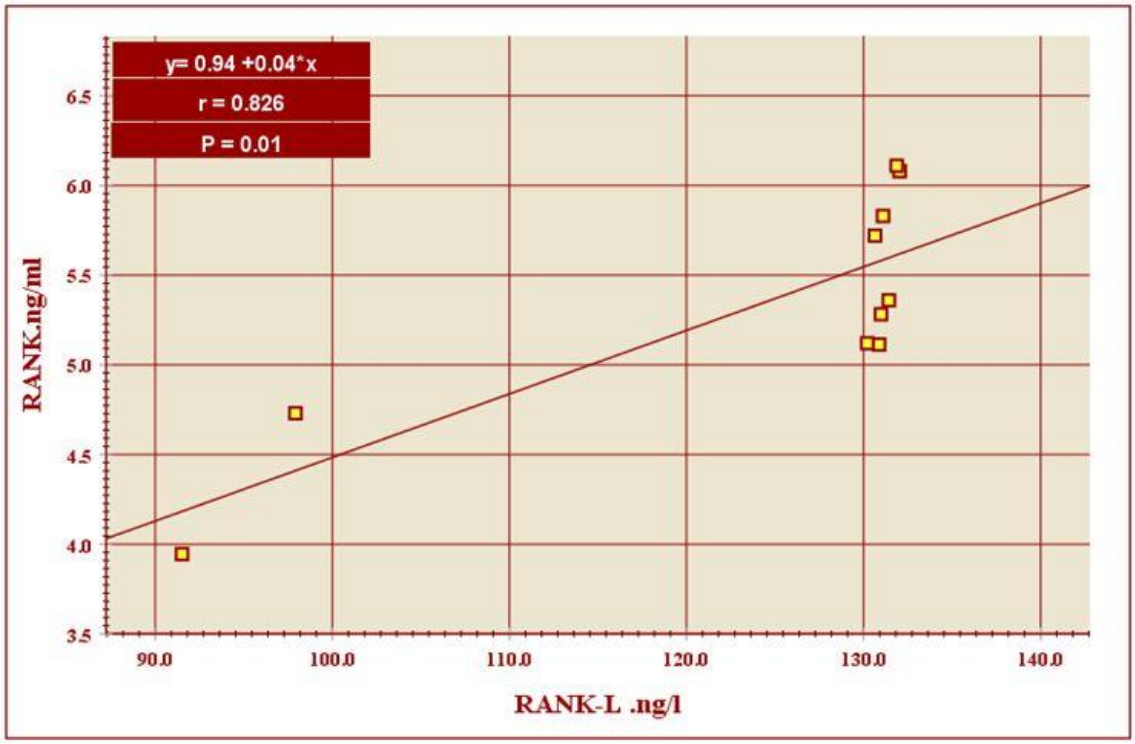

Figure (4-4): the correlation between serum (RANK-L) and (RANK) levels in males rabbits treated with L-methionine 100mg/kg BW

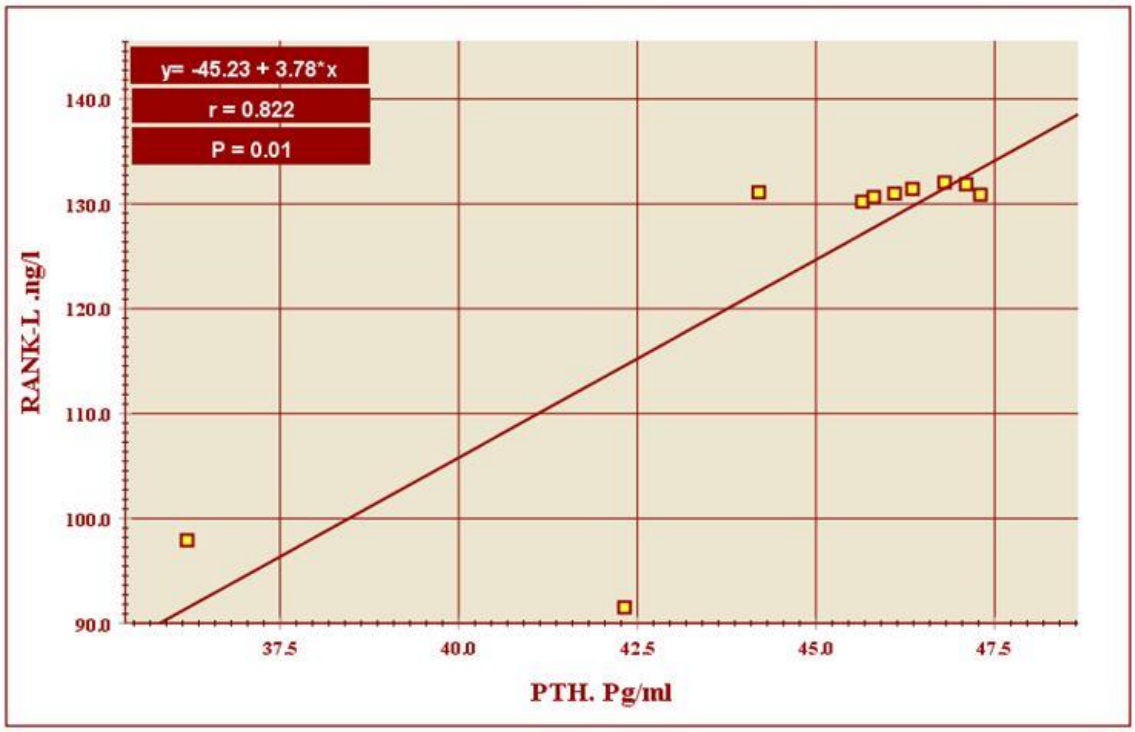

Figure (4-5): the correlation between serum (PTH) and (RANK-L) levels in males rabbits treated with L-methionine 100mg/kg BW.

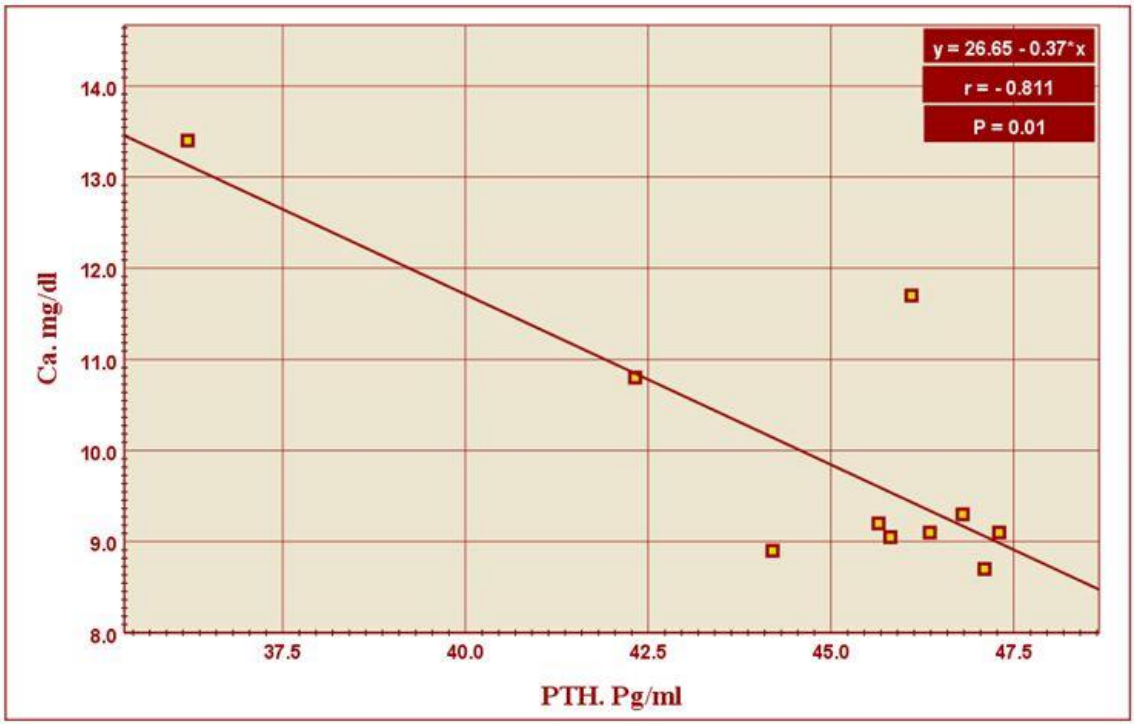

Figure (4-6): the correlation between serum (PTH) and (Ca) levels in males rabbits treated with L-methionine 100mg/kg BW.

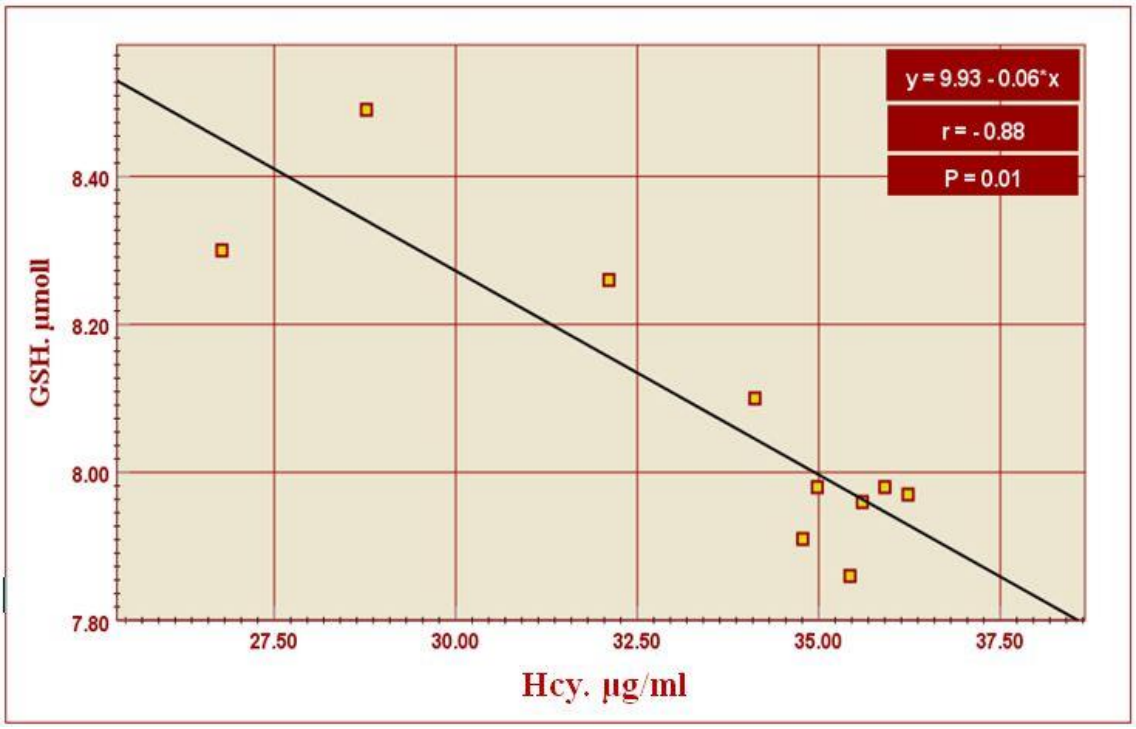

Figure (4-7): the correlation between serum Homocysteine (Hcy) and (GSH) levels in males rabbits treated with L-methionine 100 mg/kg of BW.

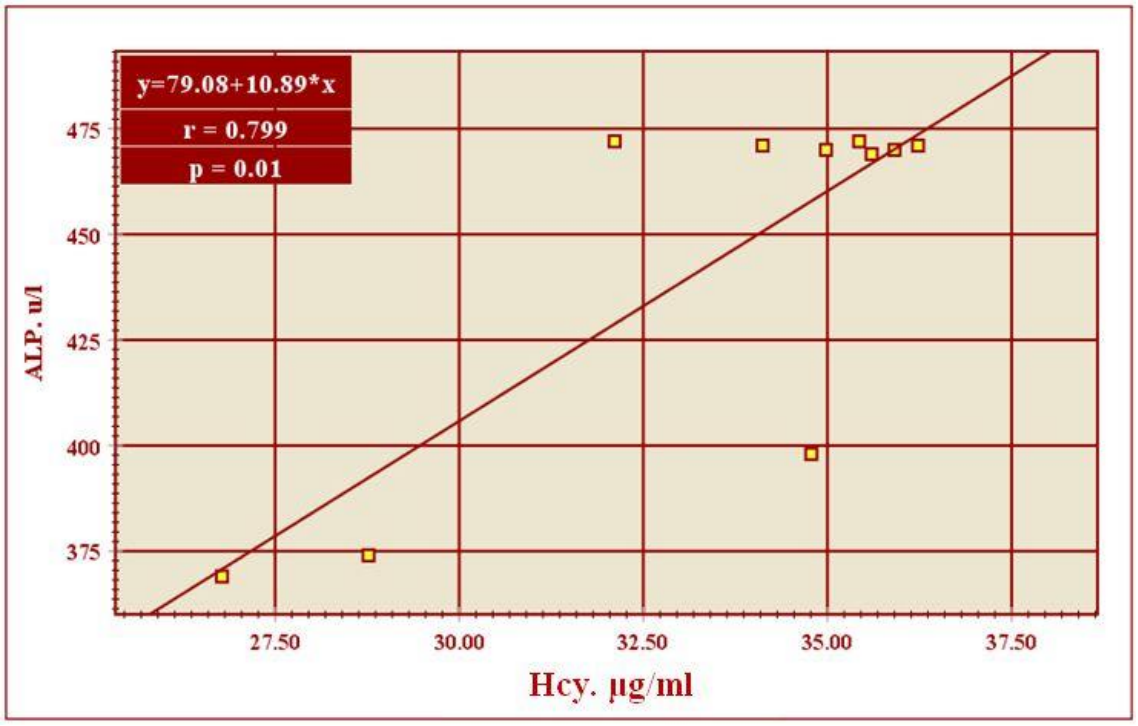

Figure (4-8): the correlation between serum Homocysteine (Hcy) and (ALP) levels in males rabbits treated with L-methionine 100 mg/kg BW.

4.9 Microscopic examination

4.9.1 Examination of longitudinal section of Femur

4.9.1.1 Examination of longitudinal section of femur head

In control group the longitudinal examination of femur head show; normal trabecular bone with small spaces between them which what make bone structure more solid and coherent **figure 4-9.** In methionine treated group the longitudinal examination of femur head showed; thin and corrosive trabecular bone, with large spaces that make bone fragile and easily to breakable **figure 4-10**.

4.9.1.2 Examination of longitudinal section of distal end of femur bone (The epicondyle region)

In control group the longitudinal examination of epicondyle region showed; normal and strongly interconnected bone plates with a few spaces between them which what make bone more solid and strong **figure 4-11.** In methionine treated group the longitudinal examination of distal part showed; the weaker rod-like structure bone plates, increase in numbers of porous and spaces with lost in bone density **figure 4-12**.

4.9.2 Histological examination

4.9.2.1 Histological section of femur head of rabbits (control group)

Microscopic examination of histological sections of femur head of control group showed normal bone trabecular, normal thickness, normal density with no surface resorption pits **figure 4-13**.

4.9.2.2 Histological section of femur head of rabbits treated with methionine

Microscopic examination of histological section of femur head for rabbits treated with methionine 100mg/kg BW daily for 12 weeks, showed very thin and disconnected bone trabeculae **figure 4-14.**

Figure 4-15 & 4-16 Clarify the histological section of femur head under light microscope of rabbits treated with methionine, showed bone trabecular surface with increase in osteoclasts percentage and resorption pits.

4.9.3 Electron microscopic examination

The electronic microscope examination of control group for longitudinal section of femur head shows: normal trabecular bone and strong interconnected plates with small spaces between them that make bone solid and strong **figure (4-17).**

While in methionine treated group the longitudinal examination of femur head showed: thin trabecular with weaker rod like structure and some of rods are completely disconnected that make bone fragile and easily breakable (osteoporotic bone) **figure (4-18).**

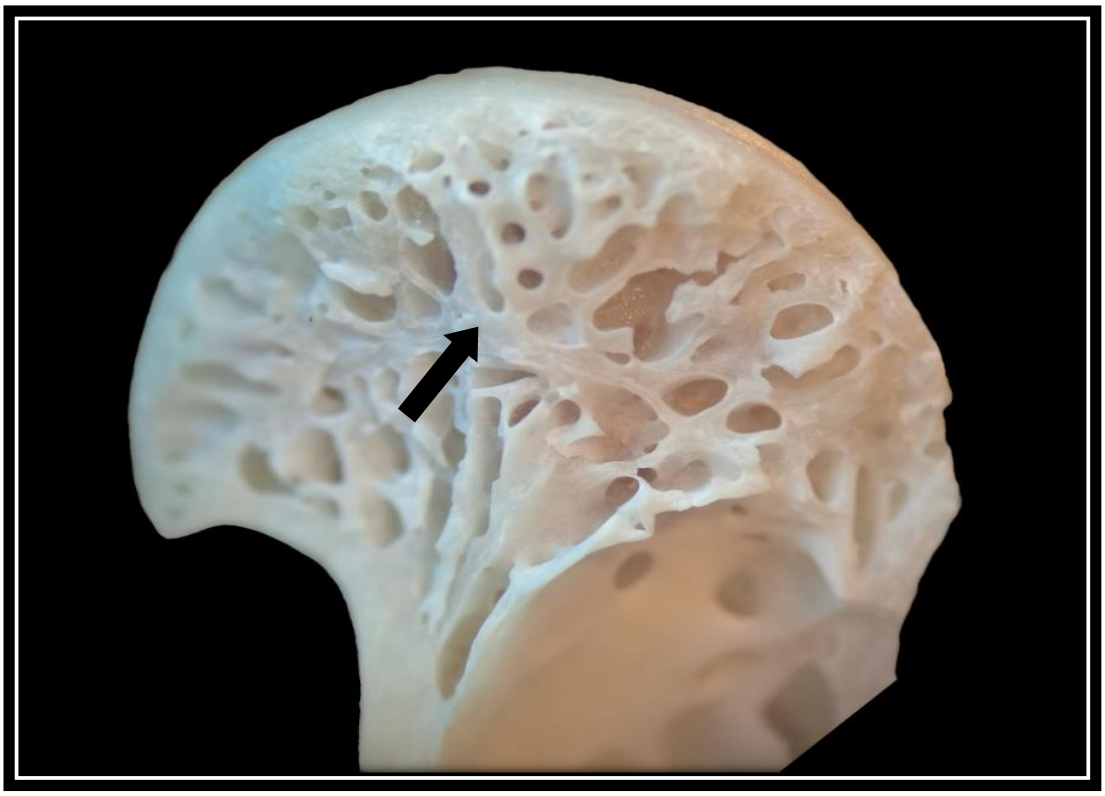

Figure (4-9) longitudinal section of femur head under stereo microscope of control males rabbits note: normal and intensive trabecular bone (arrow) with small spaces between them which give bone solid and strong structure (20X).

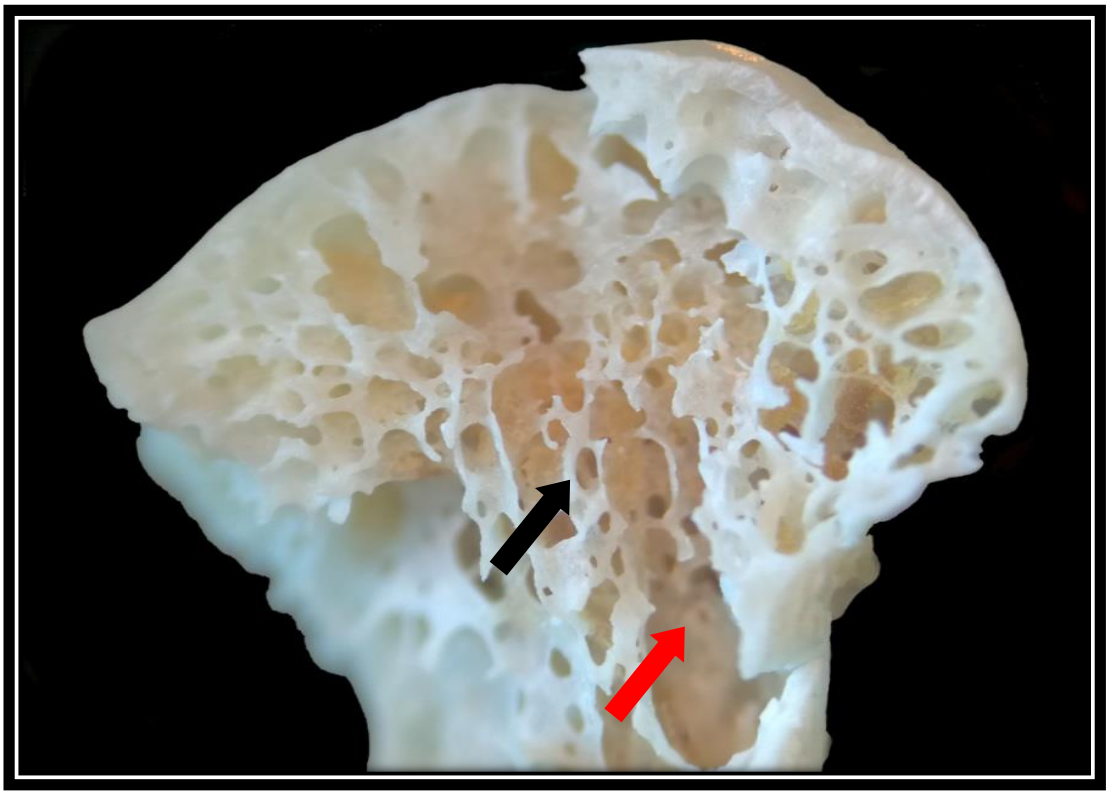

Figure (4-10) longitudinal section of femur head under stereo microscope of males rabbits treated with 100mg/kg BW methionine for twelve week note: thin and corrosive trabecular (black arrow) with large space (red arrow) that make bone fragile and easily breakable (20X).

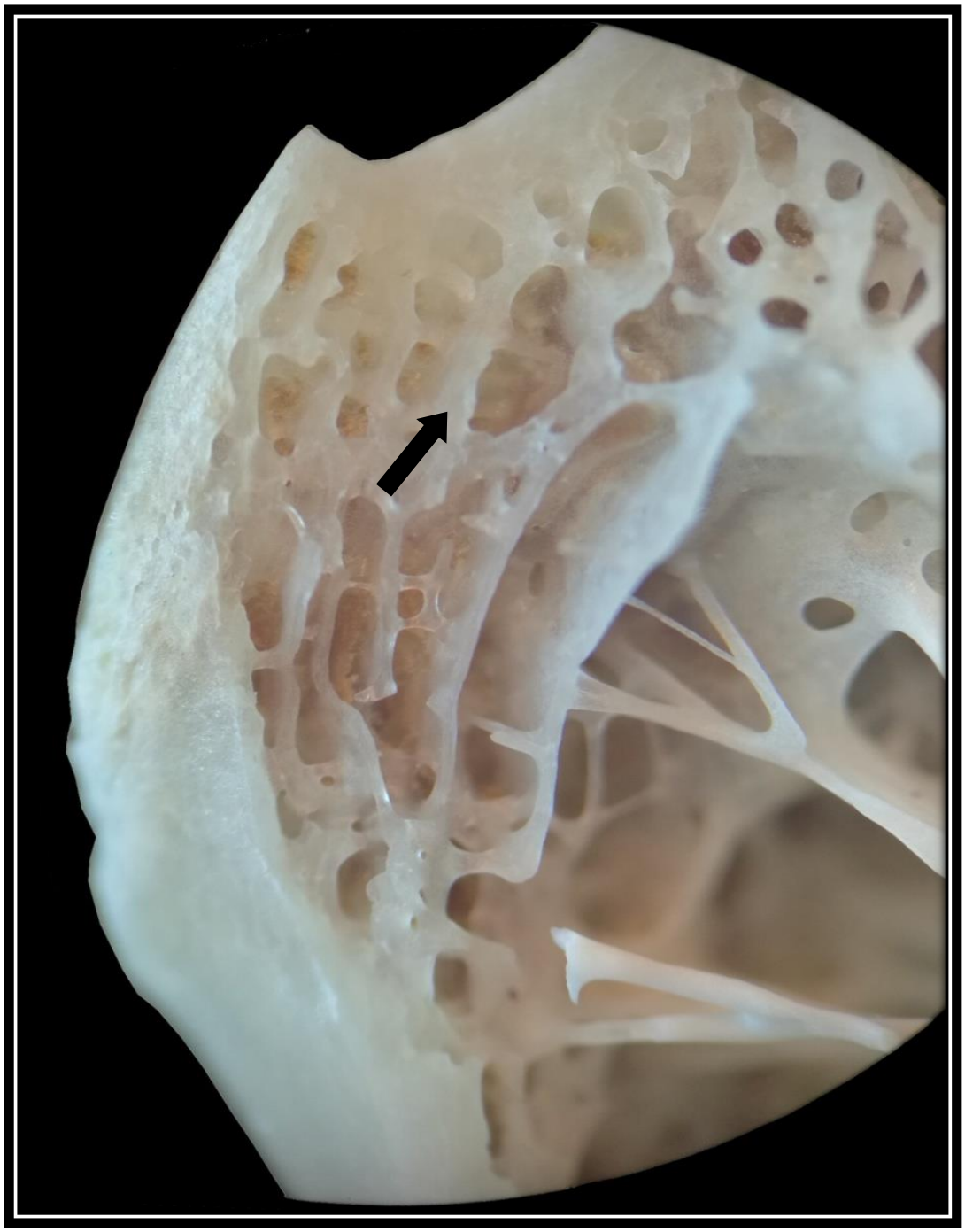

Figure (4-11) longitudinal section of distal part of femur bone (epicondyle region) under Stereo Microscope of control males rabbits note: Strongly interconnected bone plates (arrow) with a few spaces between them that make bone solid and tough (20X).

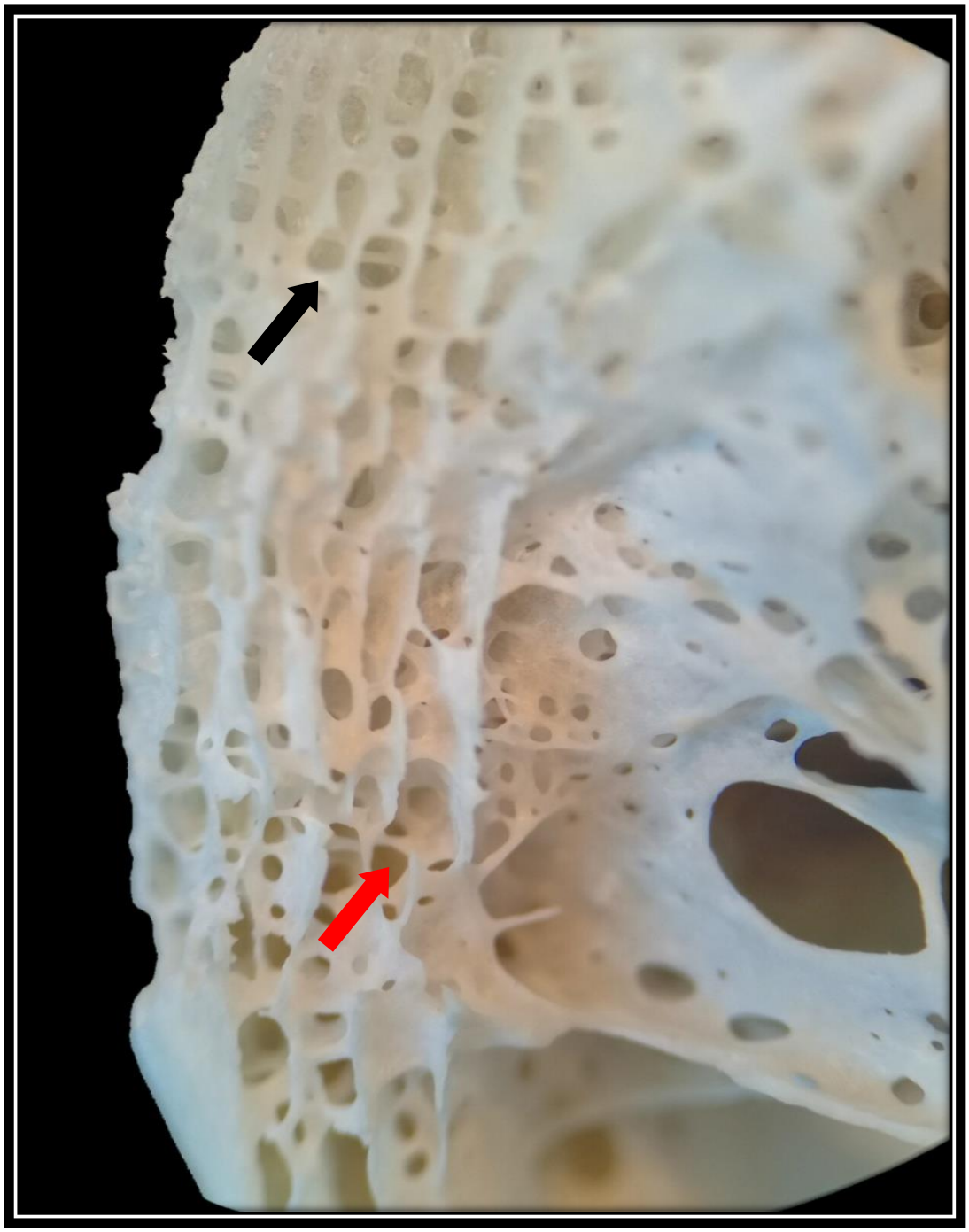

Figure (4-12) longitudinal section of distal part of femur bone (epicondyle region) under Stereo Microscope of males rabbits treated with 100mg/kg BW methionine for twelve weeks note: thin trabecular with weaker rod like structure and some of rods are completely disconnected (black arrow), increase in numbers of porous and spaces (red arrow) with lost in bone density (20X).

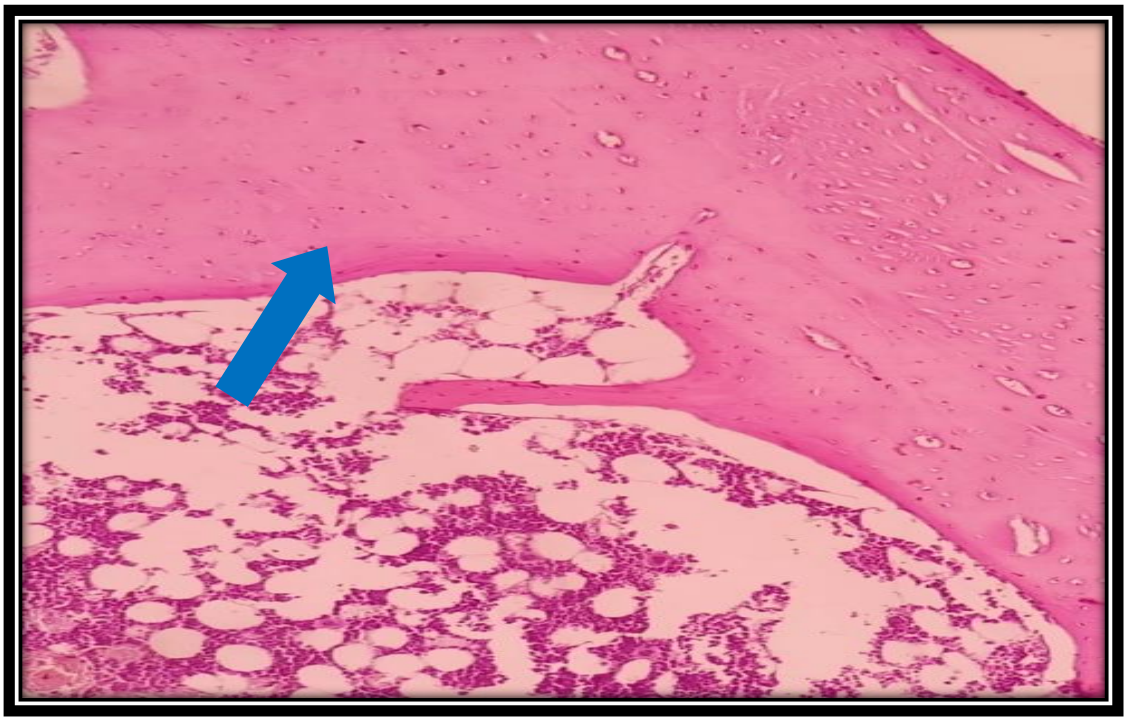

Figure (4-13) Histological section of femur bone (head) under light microscope of control male rabbit note: normal bone trabecular (arrow), normal thickness, normal density and with no surface resorption pits (H&E X200).

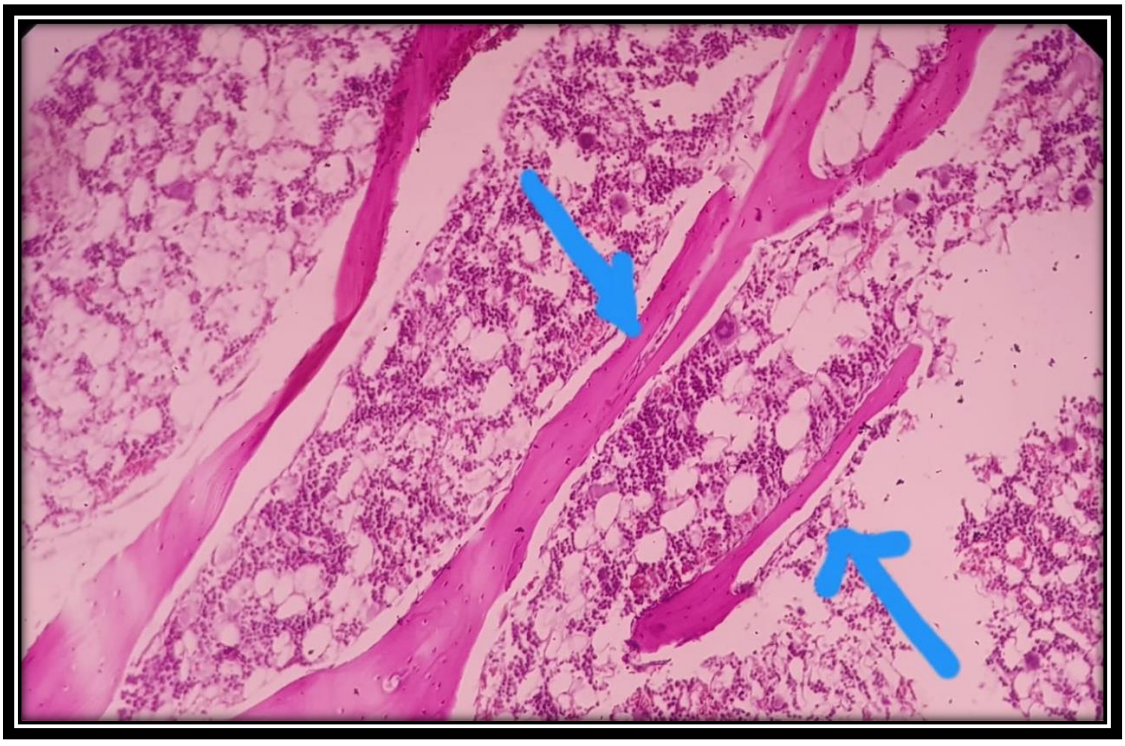

Figure (4-14) Histological section of femur bone (head) under light microscope of male rabbit treated with methionine 100mg/kg B.W note: very thin and disconnected bone trabeculae (Arrow) (H&E X100).

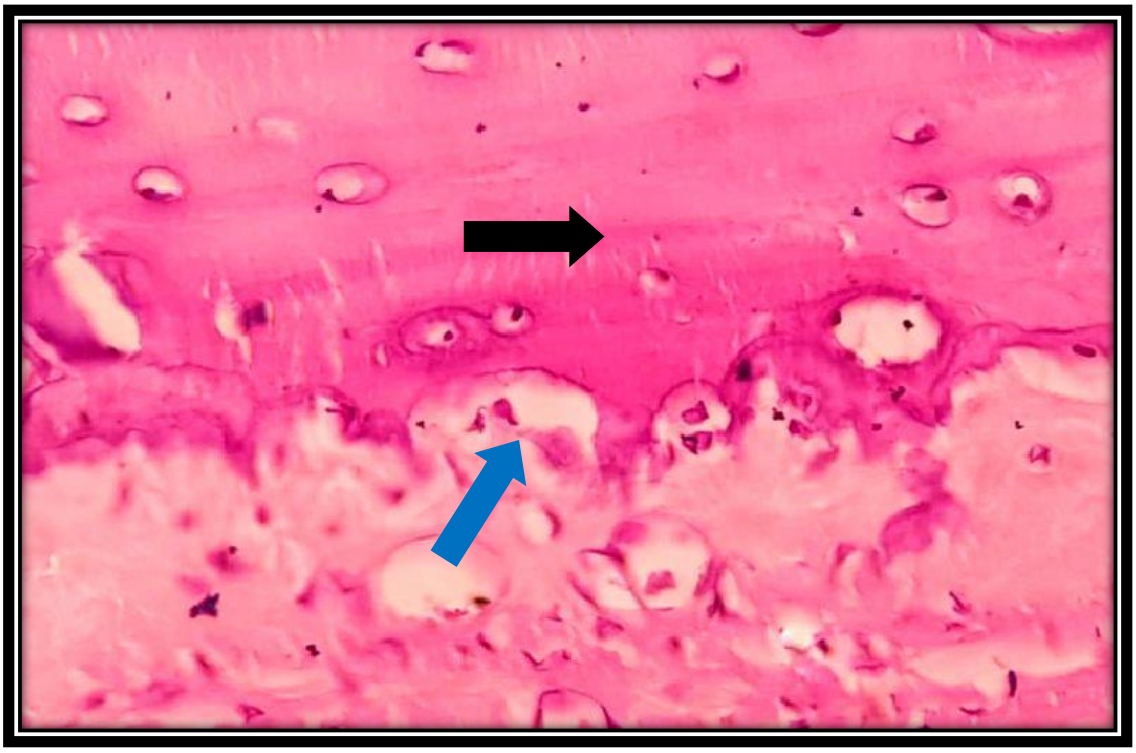

Figure (4-15) Histological section of femur bone (head) under light microscope of male rabbit treated with methionine 100mg/kg B.W note: bone trabeculae (black arrow) with increase in resorption pits (blue arrows) (H&E X400).

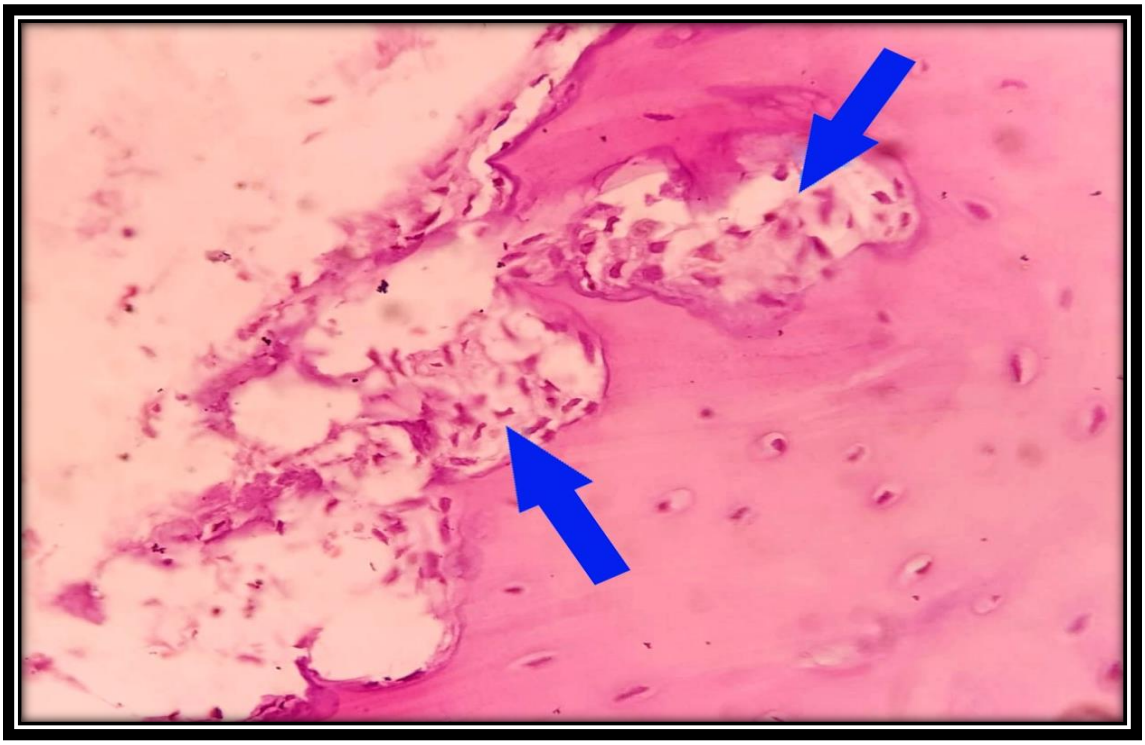

Figure (4-16) Histological section of femur bone (head) under light microscope of animal treated with methionine 100mg/kg B.W note: resorption pits with increase in osteoclasts size, percentage and activity. (Arrow) (H&E X400).

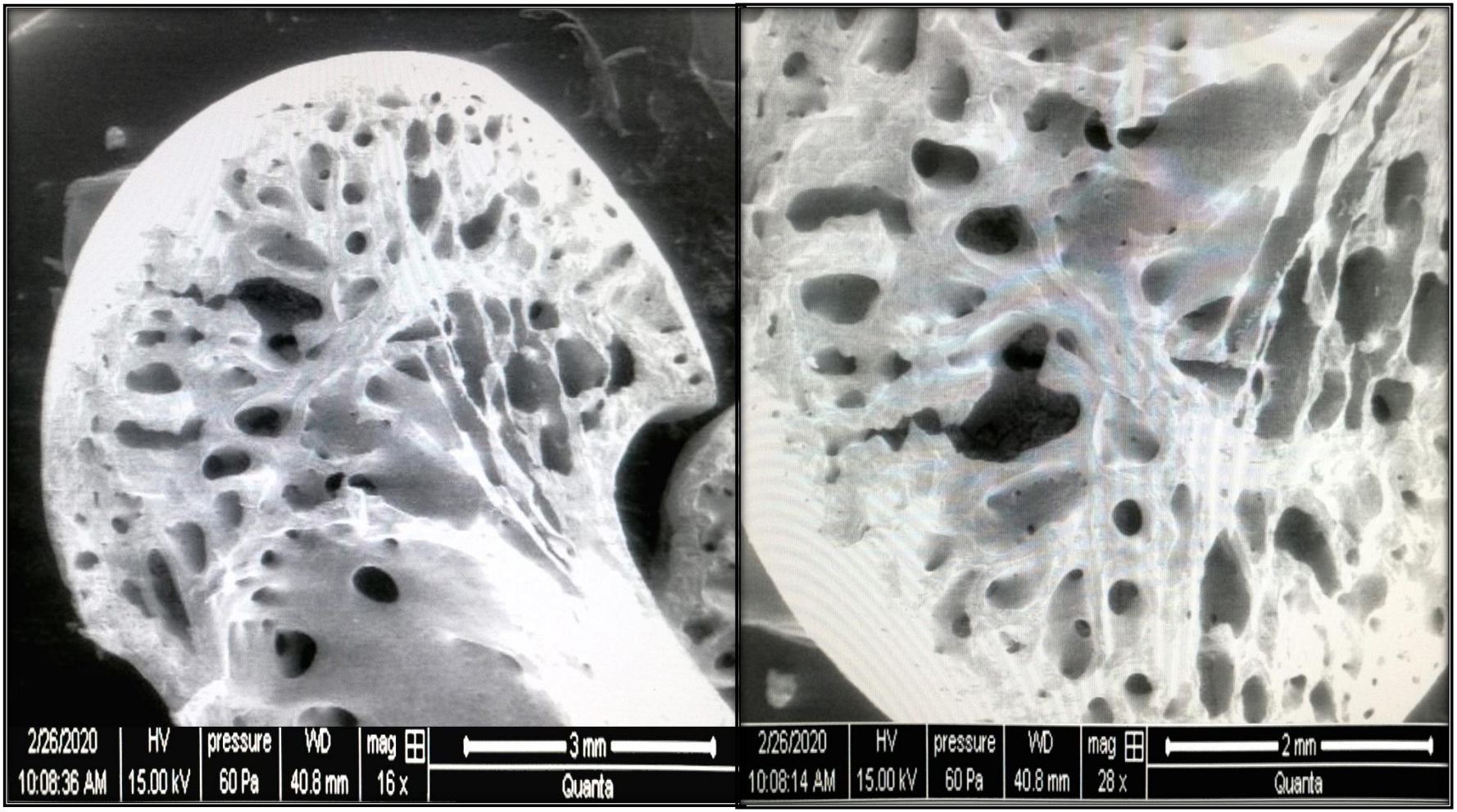

Figure (4-17) longitudinal section of femur head in different magnifications obtained by scanning electron microscope in low power mode of control animal note: normal trabecular bone with small spaces between them which make bone solid and strong (normal bone).

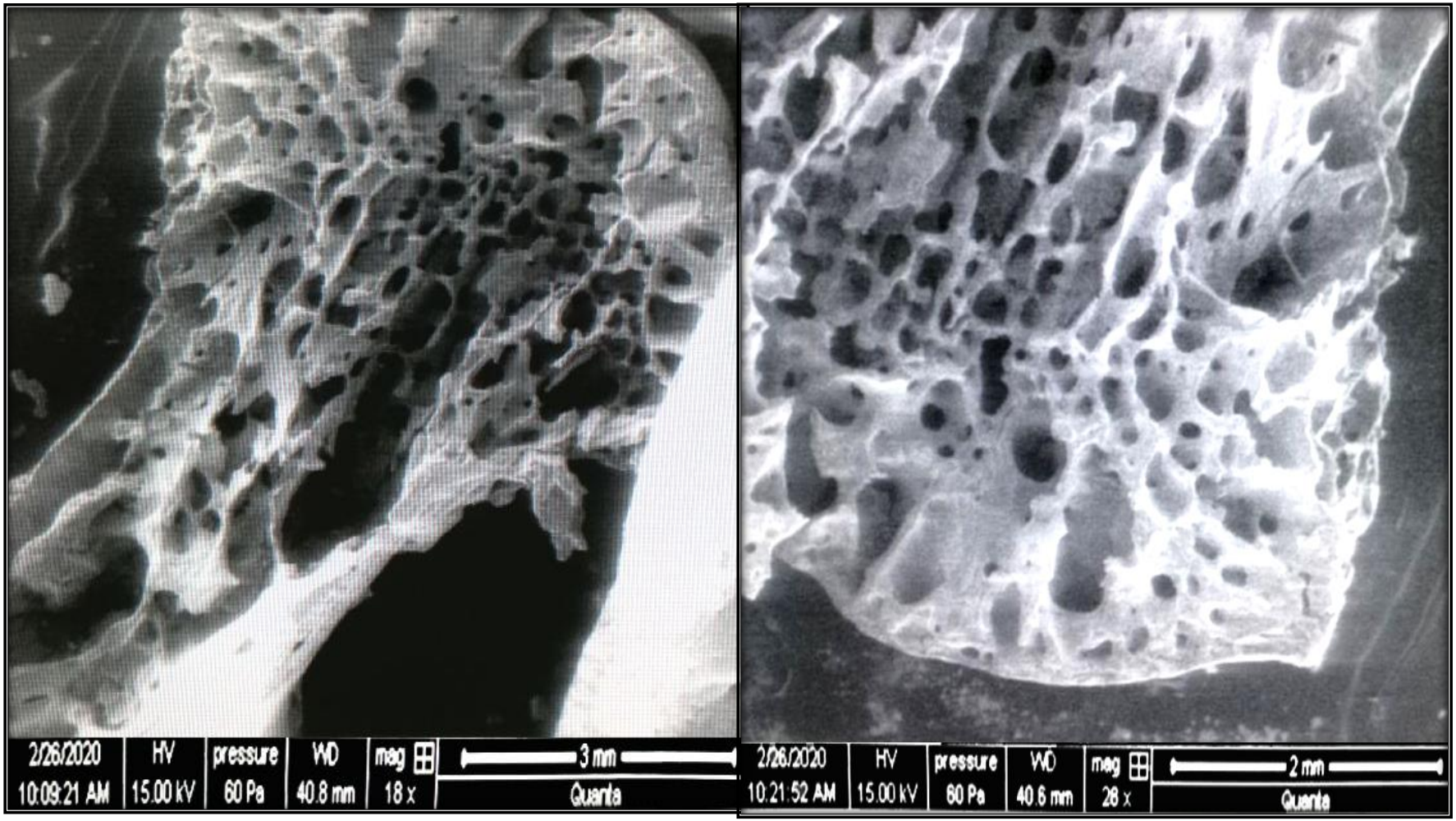

Figure (4-18) longitudinal section of femur head in different magnifications obtained by scanning electron microscope in low power mode of male rabbit treated with 100mg/kg BW methionine for 12 week note: thin and corrosive trabecular with large space that make bone fragile and easily breakable (osteoporotic bone).

Chapter Five
Discussion

5.1 Hyperhomocystiminma (HHcy) induce by methionine overload

The results of this study show that daily administration of methionine (100mg/kg of BW) caused a significant increase in the mean value of serum Hcy when compared to the control group, table (4-1). It was found that one of the classical ways to study the effects of HHcy is to induce this condition by means of an oral methionine load test (100mg/kg BW), which can be applied to both animals and humans **(Ventura *et al*., 2000).**

The results of this study agreed with (**Salman**, **2014; Al-kenany *et al*., 2019**), where they induced HHyc in experimental rabbits by daily intubation of methionine 100mg/kg BW. Also its agreement with (**Hrnčić *et al*.**, **2014; Meng *et al*.,2015; Hemanth Kumar *et al*., 2017; Milovanovic *et al*., 2017; Kim *et al*., 2018)** Animals (mice , rats) fed with methionine enriched diet developed HHcy and had significantly higher serum total Hcy concentrations comparing to control group.

This high level of serum Hcy in methionine-treated group may attribute to the depletion of vits B group that responsible for Hcy metabolism **(Kałużna-Czaplińska *et al*., 2011),** thus, the high level of Hcy (HHcy) may reflecting the deficiency of one or more of the three vits (folic acid, vit B6, B12) that involved in the process of methionine metabolism (**Jordao *et al*., 2009),** and this corresponds to a studies conducted by **(Yamamoto *et al*., 2012; Barroso *et al*., 2017)** where they attributed this high level of serum Hcy to vits B (folate, B12 and B6) deficiency, and this deficiency in turn lead to disturbance in remethylation and transsulfuration pathway, by preventing normal conversion of Hcy to methionine and conversion of Hcy to cysteine respectively, and farther to GSH. Thus, a deficiency in one or more of these three vitamins B group may lead to cause disturbance in methionine metabolism and hence the high level of Hcy (HHcy).

Another reason for this increase in serum Hcy level may attributed to the continuous intake of excess L- methionine, which may caused high generation of ROS that lead to oxidative stress and damage in liver and kidney tissues and thus, derange of Hcy metabolism and their excretion **(Hemanth Kumar *et al*., 2017)**. Hcy has highly reactive thiol group and can undergo rapid autoxidation in the presence of oxygen and metal ions (copper and iron) generating potent ROS including superoxide anion, H_2O_2 and hydroxyl radical **(Long& Nie, 2016)**, where a studies by

(Kim *et al*., 2015; Fernández-Rodríguez *et al*., 2016; Barroso *et al*., 2017) they found that methionine overload contributed in liver damage and subsequently an increase in the level of serum Hcy, through augments of oxidative stress, DNA damage and increasing in liver cells apoptosis and thus, disrupt of Hcy metabolism due to impairment of remethylation and transsulfuration pathways. It was suggested that 4-week exposure to high Hcy level (HHcy) was enough to cause liver cells injury and damage, where remethylation mainly occurs **(Pacana *et al*., 2015)**.

The kidney also plays an important role in metabolism and clearance of amino acids, by presence of specific Hcy uptake mechanisms and Hcy metabolizing enzymes in the kidney, suggests that the kidney has a role in Hcy metabolism **(Ostrakhovitch & Tabibzadeh, 2015; El-Deeb *et al*., 2017**), and this is represented by the unique role in the removal of SAH from the circulation (**Long** & **Nie, 2016).** A study by (**Levi *et al*., 2014)** suggests that moderately elevated serum Hcy level is a significant risk factor for induce kidney damage. The **(Long** & **Nie, 2016)** find that Hcy accelerates the progression of kidney damage by causing inflammation, which in turn reflects on kidney functions in metabolize and excretion of Hcy.

Our study conclude from what mentioned above that continues intake of excess L- methionine caused a high level of serum Hcy either as a result of the depletion of the vitamins B group or as a result of tissues damage of liver or kidney or both together and, thus, caused derange in the process of Hcy metabolizing and their excretion outside the body which in turn lead to high Hcy level (HHcy).

Finally our results were disagreement with **(Troen *et al*, 2003)** where found that mice with methionine rich diets had normal Hcy level, and this was attributed to the adequate levels of vits B group that necessary for methionine metabolism and prevents HHcy status.

5.2 The effect of HHcy that induce by methionine overload on the serum RANK biomarker

The results of our study showed that daily intubation of methionine for 12 weeks caused high significant increase in serum RANK of methionine treated group when it's compared to the control group (table 4-3).

This raise in serum RANK may be attributed to the excess production of ROS by Hcy after methionine overload, where several previous studies have indicated that ROS are important for differentiation process of osteoclasts through stimulations of RANK production, where any increase of ROS level may lead to bone disorder and destruction (**Callaway**& **Jiang, 2015; Collins *et al*., 2018**; **Agidigbi & Kim, 2019; Schröder, 2019).**

In a study conducted by (**Nakanishi *et al*., 2013)** where they showed up the role of M-CSF in differentiation of hematopoietic precursor cells into osteoclasts by increase the intracellular level of ROS through, NADPH oxidase and induced RANK production.

On other hand the increase in serum Hcy level also lead to increases the levels of ROS through activation of NADPH oxidase, where several studies have suggested that NADPH oxidase enzymes are very important sources of ROS **(Bevilacqua *et al*., 2012; Chen *et al*., 2013; Sun *et al*., 2016; Schröder, 2019).** It was found that Hcy stimulates phosphorylation of NADPH oxidase subunits that involved p47phox; p67phox in addition to up regulation of NADPH oxidase 2 subunits and this stimulation in turn will raise the ROS levels **(Feng *et al*., 2018; Zhu *et al*., 2018; Korkmaz *et al*., 2019).**

Thus, the role of Hcy is nearly similar to the role of M-CSF in ROS production but with more harmful effect, because the high levels of Hcy will contribute in elevation of ROS production **(Chen *et al*., 2017; Zhong *et al*., 2017)**, and this subsequently lead to increases in RANK expression and production, thus enhancing osteoclast differentiation and activation by RANK-L (**Oh & Lee, 2017)**, this in turn negatively reflected on bone health **(Ono *et al*., 2020).**

Also this raise in serum RANK may be attributed to the role of PGE2 that mediated by Hcy. Where several studies have suggested that one of the RANK, RANK-L stimulation factors is PGE2 **(Hegde *et al*., 2016; Park *et al*., 2017),** several previous studies showed that Hcy promote inflammation by activating p38 MAPK signaling pathway **(Poddar & Paul, 2013; Liu *et al*., 2019; Rajagopal *et al*., 2019)**. The p38 MAPK is an upstream regulator of phospholipase A2 (PLA2) and can activate PLA2 **(Kim *et al*.,2016),** upon the activation of PLA2, arachidonic acid will be released from the membrane phospholipids, the arachidonic acid can be further

converted into prostaglandins by Cyclooxygenases 2 (COX2) **(Stipanuk & Caudill, 2018),** and according to a study conducted by **Liu *et al*., 2005** they found that COX2 and PGE2 stimulate osteoclastogenesis through inhibition of OPG secretion, stimulation of RANK-L production by osteoblasts and increase of RANK production by osteoclasts, PGE2 also stimulated IL-6 production.

The IL-6 act as osteoclast differentiation modulators and encourages osteoclastogenesis and can cause excessive osteoclastic activity (**Wu *et al*., 2017; Brunetti *et al*.,2020)**, the IL-6 is tied to excessive promotion of RANK-L, makes preosteoclasts more sensitive to RANK-L stimulation, up regulation of RANK expression and inhibition of OPG, the IL-6 has been linked to a numerous of bone disease including osteoporosis, rheumatoid arthritis, and bone-metastatic cancers **(Hashizume *et al*., 2011; Harmer *et al*., 2019).**

Our results also showed present of a significant (p=0.01) positive correlation (r=0.826) between RANK-L and RANK concentration in serum (figure 4-4), and this finding agree with (**Zhang *et al*., 2018).** These high levels of RANK, RANK-L that is positively correlated would cause signaling pathway which is essential for osteoclastogenesis. In brief, the binding of RANK-L (produce by osteoblast) to RANK, located on the surface of osteoclast precursors, recruits the cytoplasmic adapter protein TRAF6, leading to NF-κB activation and translocation to the nucleus; in the nucleus, the translocated NF-κB increases the expression of c-Fos; the interaction of c-Fos with NFATc1 leads to increased expression of various osteoclastogenic genes; consequently, osteoclast formation is increased, thereby resulting in increased bone resorption (**Li *et al*., 2010)**. The positive correlation between serum RANK and RANK-L and subsequently the signaling pathway that resulting from the reaction between them, explain the result of histological examination of methionine treated males rabbits that showed clearly increase in the numbers and the activities of osteoclasts. This high number in osteoclasts strongly contributed in occurrence of osteoporosis **(Nagy & Penninger, 2015; Zhang *et al*., 2107).**

Through these evidences, we conclude that Hcy may contributed in raising the level of serum RANK either by ROS generation or stimulating the production of

PGE2 which in turn stimulate IL-6 production, where the both PGE2 and IL-6 are considered as stimulating factor for production both of RANK and RANK-L.

5.3 The effect of HHcy that induce by methionine overload on serum RANK-L biomarker

The result of our study shows that daily methionine intubation to adult males rabbits for 12 weeks caused highly significant ($P \leq 0.001$) increase of serum RANK-L as compared to control group, table (4-2).

Our results are consistent with (**Vijayan & Gupta, 2018)** their interpretation to high RANK-L levels was attributed to the oxidative stress induced by Hcy that deranges insulin-sensitive- Forkhead Box O1(FOXO1) due to phosphorylation of protein phosphatase 2A (PP2A) was the key event that inhibited OPG synthesis in Hcy-treated osteoblast cultures. At the same time Hcy stimulate MAPK signaling cascades and increase RANK-L synthesis in osteoblast cultures through c-Jun/JNK MAPK signaling mechanisms independent of FOXO1.

The HHcy following high methionine diet caused lower FOXO1 and OPG expression and increased synthesis of proresorptive and inflammatory cytokines such as RANK-L, M-CSF, IL-1α, IL-1β, IFN-γ, IL-17, and TNF-α, these results emphasize that HHcy alters the redox regulatory mechanism in the osteoblast by activating PP2A and deranging FOXO1 and stimulating MAPK signaling cascades, eventually shifting the OPG: RANK-L ratio toward increased osteoclast activity and decreased bone quality (**Vijayan *et al.*, 2013)**.

Our results agrees with **(Behera *et al.*, 2018)** where that RANK-L expression was significantly increased in HHcy mice, they find that 3 micromole concentration of Hcy significantly reduced the OPG level and increased the RANK-L level in bone marrow mesenchymal stem cells, they also found that HHcy induced RANK-L synthesis via oxidative stress mediated c-Jun/JNK signaling through phosphorylation of JNK (T183/Y185) as compared to the control group. In the same study it was confirm the role of oxidative stress that induce by HHcy in increasing the levels of RANK-L by administration N-acetyl cysteine (NAC) as anti oxidant drug they found that there were significant decreases in the RANK-L levels in HHcy+NAC group as compared to HHcy group (**Behera *et al.*, 2018**).

Our results also showed strong positive correlation (r= 0.909, p= 0.01) between high serum Hcy levels and RANK-L level (figure 4-1).

In a study conducted by **Handono *et al.*, 2014** their results appeared to be consistent with our results by present positive correlation between Hcy and RANK-L, they attributed the high in Hcy levels (HHcy) to decrease in coenzymes such as folic acid, vitamin B6, and vitamin B12, these coenzymes (folate, B12, B6) plays a major role in metabolism of methionine and reducing the high levels of Hcy which is considered a strong stimulation of osteoclasts, therefore, a deficiency in vits B group cause HHcy seems to have a potential role in the development of osteoporosis **(Herrmann *et al*., 2005).**

The Handono *et al* also found that auto-oxidation of Hcy results in increased production of intracellular ROS and stimulates p38 MAPK activation which influence the differentiation of osteoclast precursor cells, through ROS-RANK-L pathway and activation of RANK.

The results of our study also showed a high elevation of serum PTH in methionine-treated group. Where PTH has a fundamental role in increasing the RANK-L levels **(Silva & Bilezikian, 2015),** where Figure (4-5) shown positive correlation (r=0.822, p= 0.01) between serum PTH levels and serum RANK-L concentration, this result was compatible with (**Jilka *et al.*, 2010)** A sustained rise in PTH increases the local concentration of RANK-L and thereby the development of new osteoclasts. And also compatible with **(Fu *et al.*, 2002; Teti, 2013; Silva & Bilezikian, 2015)** the PTH stimulates osteoclast formation by binding to its receptor on osteoblast, osteocytes cells and stimulating the production of RANK-L, and inhibiting the expression of OPG, and increasing both osteoclast recruitment and osteoclast activity, and thereby stimulating bone resorption.

Regarding the results of our study about RANK-L and PTH were incompatible with study conducted by (**Nakchbandi *et al.*, 2008)** about the role of RANK-L/OPG cytokine system in Primary HHcy, Where they found, the patients with mild primary hyperparathyroidism, have elevated RANK-L level which positively correlated with bone loss, but neither RANKL nor OPG correlated with PTH**,** they suggests that skeletal responsiveness to PTH may differ in this disease.

5.4 The effect of HHcy that induce by methionine overload on serum Ca

The current study reveals lower in the mean value of serum Ca^{+2} level of methionine treated male rabbits compared with control group, as shown in table (4-7). The results of our study was agreement with (**Zemel *et al*., 1981; Wang & Zhao, 1998)** through their research about the relationships between dietary protein, sulfur containing-amino acid (methionine and cystine) intakes and urinary Ca^{+2} excretion were examined both in animals and in young men, the results showed that Ca^{+2} and sulfate excretion were significantly higher to the double when compared with basal diet group. Thus, the reason for this decrease in serum Ca^{+2} level may attribute to it's highly excretion by kidney with urine**.**

According to a study conducted by National Aeronautics and Space Administration (NASA) in 2013, they found excessive amounts of sulfur-containing amino acids (methionine and cysteine) can metabolize to create a slightly acidic environment in the body, and the basic components such as Ca^{+2}, K^{+} salts can help neutralize these acid loads, and because bone itself is a large reservoir of Ca^{+2} containing base, an increase in acidity can affect Ca^{+2} metabolism and lead to the resorption (or loss) of bone mineral and increased urinary Ca^{+2} excretion when there is not enough base in the diet, and this excretion in Ca^{+2} contributes in renal stone formation and bone disorder diseases (**Zwart *et al*., 2013)**.

Our results also show significant ($p=0.01$) negative correlation ($r=^{-}0.906$) between Hcy concentration and serum Ca^{+2} level, figure (4-2). This strong negative correlation can explained via number of mechanisms that show the direct and indirect effect of Hcy on lowering the Ca^{+2} level of serum; one of these mechanisms may attributed to the role of ROS that formed by Hcy, where Hcy is consider one of the most potent oxidant agents in the living cells (**Cristiana *et al*., 2012)**.

It was found that ROS stimulate ion channels, such as plasma membrane Ca^{+2}, K^{+} channels, leading to changes in cation concentration, where cytosolic Ca^{+2} level can be increased by ROS in various cell types, including epithelium cell, through the influx of extracellular Ca^{+2} (**Novo & Parola, 2008; Kiselyov & Muallem, 2016).** Thus, this mechanism might have role in lowering of serum Ca^{+2} levels.

Another mechanism was clarifies the lowered of Ca^{+2} level due to induced kidney disease by Hcy (**Blaine *et al*., 2015).** A study by **(Long& Nie, 2016)** found that HHcy contribute in kidney damage through several of cellular and molecular mechanisms that involved oxidative stress, inflammation, DNA hypomethylation and endoplasmic reticulum stress that lead to; glomerular injury, endothelial and mesangial cell dysfunction, mesangial apoptosis via ROS generation and p38-MAPK activation, and finally injury of the proximal tubular cells (PTCs) due to endoplasmic reticulum stress. Where the PTCs have important role of Ca^{+2} absorption in addition to express 1-α-hydroxylase enzyme which have important role in activation of vit D (**Felsenfeld *et al*., 2013; Blaine *et al*., 2015).**

It was found that moderately elevated serum Hcy level is a significant pathogenic factor for the development of chronic kidney disease (CKD) in the general population **(Levi *et al*., 2014).** In patients with CKD had marked disruption in bone and mineral metabolism resulting in a complex disorder that has been termed CKD-mineral bone disorder (CKD-MBD), Perturbations begin in the earliest stages of the CKD and worsen with progressive kidney disease **(Isakova *et al*., 2011).** The biochemical alterations of CKD-MBD include elevated PTH, decreased (1,25-dihydroxyvitamin D 1,25D important for intestinal absorption of Ca^{+2} in addition to elevated of serum phosphate, and decreased serum Ca^{+2} **(Perwad *et al* 2007; Moorthi & Moe, 2011)**.

The Hcy have another mechanism by which the level of serum Ca^{+2} can be reduced through stimulation of N-methyl-D-aspartate receptors (NMDA receptors), the NMDA receptor: is glutamate-gated cation channels with high Ca^{+2} permeability, where found throughout the mammalian central nervous system (CNS), neuromuscular junction of skeletal muscles **(Blanke & VanDongen., 2008; Malomouzh *et al*., 2011; Rajani *et al*., 2020),** and express on vascular cells, (**Dumas *et al*., 2018).**

The NMDA-receptor activated when glutamate and glycine (or D-serine) bind to it, and when activated it allows to positively charged ions such as Ca^{+2} to flow to inside cells (**Paoletti *et al*., 2013).** It was found that Hcy also binds to NMDA-receptor lead to increase of the intracellular level of Ca^{+2} **(Moshal *et al*., 2006).**

The Hcy induces excitotoxic effects in cells expressing glutamate receptors of the NMDA class, these receptors were found not only in neurons but also in immune-competent cells, neutrophils, red blood cells, cardiomyocytes, and osteoblasts, it was found that Hcy-NMDA receptor mediated Ca^{+2} influx results in a rapid and relatively sustained ERK/MAPK phosphorylation, the activation of the ERK/MAPK- signaling pathway plays a critical role in mediating Hcy dependent cell damage and death **(Poddar & Paul, 2009; Ganapathy *et al.*, 2011; Boldyrev *et al.*, 2013)**.

The NMDA receptor: also found in bone forming osteocytes, osteoblast **(Itzstein *et al.*, 2001; Brakspear& Mason, 2012)**. It was found that Hcy-mediated bone remodeling via oxidative stress, the increased Hcy level elevates intracellular Ca^{+2} by agonizing NMDA-receptor, this in turn increases activation of Ca dependent calpain-I, which disrupts the mitochondrial membrane potential **(Itzstein *et al.*, 2001; Behera *et al.*, 2017**), thus, increasing ROS which further activates MMPs, the MMPs are a series of enzymes involved in matrix degradation **(Vacek *et al.*, 2013)**.

Also increased in Hcy levels induces apoptosis via ROS-mediated oxidative pathways which further switches off osteoblast activity and mineralization, by up regulating RANK-L expression, leading to osteoporosis by reducing bone formation **(Tyagi *et al.*, 2011; Behera *et al.*, 2017)**. Our finding provides evidence about the strong effect of Hcy on reducing the level of serum Ca^{+2}, which may be attributed to one or all of the mechanisms that mentioned above.

Our result was disagreement with a study conducted by (**Gopinath & Abdul, 2018)** about biochemical bone markers (ALP, Ca^{+2} and phosphorous) in evaluation of osteoporosis in post-menopausal women; where they found, Serum Hcy levels were significantly higher in osteoporotic women, with no statistical difference was seen in biochemical bone markers like Ca^{+2}, Phosphorus and ALP.

5.5 The effect of HHcy that induce by methionine overload on the serum PTH

Our results previously shown a significant decrease in the level of serum $Ca^{+2}$of methionine treated group. It is already known that is a low level of serum Ca^{+2}will stimulate the parathyroid gland to produce PTH (**Lofrese *et al.*, 2019; Khan & Sharma, 2020)**. Where the results of present study, showed a significant ($p \leq 0.001$)

increase in serum PTH concentration of methionine treated group as compared to the control group, table (4-4). In addition to a significant (p=0.01) negative correlation (r= ¯0.811) between PTH concentration and Ca^{+2}level in serum, figure (4-6).

Our results agree with (**Arora *et al.*, 2018)** where their study indicated a strong negative correlation ($P \leq 0.001$; $r = -0.805$) between PTH concentration and Ca^{+2} level in serum**.** Our results also agree with **(Kohrt *et al.*, 2018)** where they found elevated in PTH levels with low of serum Ca^{+2} levels, and this rise in PTH level contributed in bone resorption.

It was found that low of serum Ca^{+2} level is sensed by the Ca-sensing receptors on the parathyroid gland, which stimulates PTH synthesis and secretion, the PTH in turn exerts several effects to raise serum Ca^{+2} level: through (1) stimulates bone osteoclast activity to increase bone resorption and Ca^{+2} release, (2) acts on the kidney proximal tubules to increase renal reabsorption of Ca^{+2}, and (3) increases the renal enzyme, 1-α-hydroxylase, responsible for the conversion of 25D to the most active form of 1,25D, the main role of 1,25D is to increase intestinal Ca^{+2}absorption **(Fleet & Peacock, 2014; Gallant & Spiegel, 2017),** thus, the PTH is essential for the maintenance of Ca^{+2} homeostasis **(Silva & Bilezikian, 2015)**.

our results also show a significant positive correlation between serum PTH concentration and serum RANK-L level, figure (4-5), and this agree with **(Ben-awadh *et al.*, 2014)** the PTH considers a potent stimulator of RANK-L expression.

It was found that high PTH level consider a leading cause of the bone loss disease osteoporosis (**Malluche *et al.*, 2015; Yeh *et al.*, 2016),** because one of the main mechanisms that PTH uses to control bone turnover is stimulation of RANK-L production **(Xiong & O'Brien, 2012; Hatch, 2020)**. The PTH stimulates osteoclast formation by binding to its receptor on stromal/osteoblastic cells and osteocytes, stimulating the production of RANK-L, and inhibiting the expression of OPG, where the PTH potently suppressed of OPG expression **(Silva & Bilezikian, 2015).** So the high PTH contributes in bone loss by increase RANK-L production and inhibits OPG production.

5.6 The effect of HHcy that induce by methionine overload on ALP

The results of the study show that daily administration of methionine for 12 weeks caused a significant increase in the mean value of serum ALP as compared to the control group table (4-5). The function of ALP is not well defined and it is unclear why ALP is elevated **(Kirkham & Cartmell, 2007)**.

The ALP is a ubiquitous enzyme present in all tissues but is mainly concentrated in the liver and bone that considers primary sources of serum ALP and the damage of these tissues causes release of ALP into the bloodstream **(Krishnamurthy *et al*., 2011; Sharma *et al*., 2014; Kim *et al*., 2017).** So the elevated in ALP concentrations are attributed to the two groups of conditions which are; hepatobiliary and bone diseases, the hepatobiliary that causes elevated in ALP concentrations include; tumors, biliary obstruction and liver cirrhosis **(Roudsari & Mahjoub, 2012; Ramasamy, 2020).**

The result of the present study was consistent with a study conducted by (**Al-Shammry & Al-Okaily, 2009)** about studying the effects of methionine overload, where they found that administration of methionine (100mg/kg BW) for 42 days, caused a significant increase in serum ALP, alanine aminotransferase (ALT) and aspartate amino transferase (AST) levels, indicating the occurrence of liver injury. The activity of ALP is a standard indicator of liver status, and a significantly increased level indicates liver damage **(Saeid *et al*., 2014; Das *et al*., 2018).** The histological examination of liver in **Al-Shammry & Al-Okaily, 2009** study supported this speculation in which hepatic damage were observed following methionine overload. An increase in ALP may be taken as an index of hepatic paranchymal damage and hepatocytic necrosis **(Sherwin, 1989).** Such positive correlation between HHcy concentration and ALP activity was documented (**Lambert *et al*., 1997)**.

the ALP is abundant in osteoblasts and is considered to play a role in the mineralization of newly formed bone, the elevated serum ALP levels have been detected in diseases characterized by increased bone resorption as well such as; Paget's disease, hyperparathyroidism, metastatic bone disease, a recent fracture (**Kubo *et al*., 2012**; **Verma & Gorard, 2012)** and osteoporosis (**Halleen *et al*., 1996)**, in addition to rickets and osteomalacia **(Chinoy *et al*., 2011; Sahay & Sahay, 2012).**

As we mentioned earlier our results showed lower in serum Ca^{+2} levels with elevated in serum PTH of methionine treated group, where High serum PTH levels are associated with elevated of serum ALP levels **(Einollahi *et al*., 2014; Román-Flores *et al*., 2015).** So this increase in serum ALP may also attributed to increased in bone resorption due to an increase in sensitivity of bone to PTH **(Gol *et al*., 2006**), as a result of decreased plasma Ca^{+2} level **(Rudin *et al*., 2019; Sharma *et al*., 2019)**. These agree with (**Sharma *et al*., 2014)** the diseases that affect blood Ca^{+2} level (hyperparathyroidism) were associated with elevated ALP.

The administration of methionine leads to increase in the acidic condition of the body and may also elevate cortisol concentration leading to severe chronic bone loss and subsequently elevation of ALP levels **(Maurer, 2003)**. **Elliott *et al*., 2007** found that Hcy positively correlated with serum ALP, and this consistence with our finding by presences significant ($p \leq 0.01$) positive correlation ($r=0.799$) between serum Hcy and ALP levels as shown in Figure (4-8).Thus, the high level of Hcy after methionine overload may leads to increase the level of serum ALP either due to liver damage, bone damage, or both together.

5.7 The effect of HHcy that induce by methionine overload on the serum iron (Fe)

The Results of the current study showed an increase in the mean value of serum iron of methionine-treated group, table (4-7). Our result is consistent with **(Micovic *et al*., 2016)** they found the groups that treated for 22 days with methionine, methionine + L-cysteine, showed increase in blood iron level, while the group that treated with methionine + NAC (antioxidant drug) showed significantly lower in iron level, they attributed this high in iron level to mitochondrial dysfunction, according to (**Lee *et al*., 2015),** they noticed that increased values of iron in blood were related to mitochondrial dysfunction, the disturbances in mitochondrial function caused excess iron deposition and unbalanced expression of iron metabolism-related proteins such as ferritin and ferroportin.

Our result also showed a significant ($p=0.01$) positive correlation ($r=0.916$) between serum Hcy and serum iron level of methionine treated group, figure (4-3). This result consistent with **(Baggott & Tamura, 2015)** they reported a significant positive

correlation between plasma Hcy and serum iron concentrations or iron deposits in arterial.

This elevation of iron level may attributed to the liver damage due to expos to excess L-methionine and this damage may reflected on production of necessary enzymes for iron metabolites. Where the liver produces the majority of proteins involved in iron metabolism, including hepcidin and transferrin **(Anderson & Shah, 2013; Rishi & Subramaniam, 2017).** The excessive dietary of methionine is hepatotoxic **(Mori & Hirayama, 2000).** Where a study by **(Klavins *et al.* 1963)** found excess intake of L-methionine by rats caused tissues damage including; liver enlargement, fatty liver and erythrocyte membrane damage**.**

It was found that L-methionine supplementation increased hepatic lipid peroxidation and iron levels **(Lynch** & **Strain, 1989)**, and also a study by **(Woo *et al*., 2006)** found a significant increase in the serum AST and ALT of hyperhomocysteinemic rats after 4 weeks of high methionine diet which indicating the occurrence of liver injury, and this finding suggested that 4-week exposure to HHcy was able to cause liver injury.

The HHcy status associated with increased superoxide anion and peroxynitrite formation in the liver, where peroxynitrite considered a potent oxidant agent that can cause liver tissue damage, as a consequence, the level of lipid peroxides was significantly elevated in livers of hyperhomocysteinemic animals **(Estévez & Jordán, 2002; James *et al*., 2003)**. In our study the degree of lipid peroxidation was examined by measuring the level of MDA, an indicator of lipid peroxidation.

The liver plays a major role in iron homeostasis; thus, the patients that suffer from liver diseases, the iron regulation may be disturbed**,** liver diseases decrease the synthetic functions of the liver, including the production of hepcidin, a key protein in iron metabolism (**Milic *et al*., 2016; Gkamprela *et al*., 2017).** The level of iron in the bloodstream serves as a stimulus for hepcidin synthesis (**Meynard *et al*., 2014)**. Hepcidin as the 25-amino-acid peptide is mainly synthesized in the liver **(Campostrini *et al*., 2012).** It is the essential hormone in regulating iron homeostasis and hepatic secretion of hepcidin is associated with response to iron overload **(Ganz & Nemeth, 2012; Koenig *et al*., 2014).**

At the molecular level, hepcidin mechanism in iron homeostasis is related to inhibition of iron efflux from enterocytes, macrophages and hepatocytes into the blood plasma by inducing internalization and degradation of the iron exporter ferroportin in these cells (**Lee *et al*., 2010; Sam *et al*., 2013).** Therefore, the interaction between ferroportin and hepcidin controls systemic iron homeostasis (**Sangkhae & Nemeth, 2017).** Thus, the low levels of hepcidin due to liver disease will result in iron overload, and this leads to iron deposits in the liver and higher levels of non-transferrin-bound iron in the bloodstream **(Milic *et al*., 2016)**. So the deficiency of hepcidin leads to increased dietary iron absorption with uncontrolled release of iron to the plasma, leading to iron overload **(Katsarou & Pantopoulos, 2018; Camaschella *et al*., 2019).**

The excessive dietary iron uptake may cause iron deposition in many vital organs, including the liver, heart, skin, and especially pancreas **(Barton *et al*., 2010; Porter & Rawla, 2019).** It has been reported that excess iron in cells stimulates hydroxyl radical formation and exerts potent toxic effects that can cause damage to membranes and DNA of these organs, and this damage will be accompanied by increased the levels of lipid peroxidation **(Anderson & Shah, 2013; Ivanov *et al*., 2013).**

From what mention above the excess methionine intake causes oxidative stress in the liver and may subsequently affect on iron regulation **(Lan & Jiang, 1997).** Also oxidative stress play role in inhibition of hepcidin promoter activity and transcription in the liver **(Kotze *et al*., 2009)**, thus, low hepcidin production with elevation of serum iron level **(Arezes & Nemeth, 2015).**

5.8 The effect of HHcy that induce by methionine overload on the serum of GSH and MDA

Our results showed a significant ($p \leq 0.001$) decrease in the mean value of serum GSH of methionine treated males rabbits as compared to control group, table (4-6). With present of a significant ($p=0.01$) negative correlation ($r=-0.88$) between GSH concentration and serum Hcy level, figure (4-7). The results of the present study were consistent with **(Waly *et al*., 2011)** the low serum level of GSH; is a metabolic condition that involves high level of Hcy and considered a significant sign of oxidative stress.

The GSH is one of the most important and potent antioxidants **(Aquilano *et al.*, 2014; Pizzorno, 2014),** and decrease of the antioxidant GSH is an important indicator of the oxidative stress **(Rose *et al.*, 2012)**. The GSH consumed in the process of scavenging superoxide and superoxide-derived ROS, and the accelerated of superoxide production is the primary cause of GSH depletion **(Won *et al.*, 2015)**

The HHcy associated with potent production of ROS, where the autoxidation of Hcy metabolites leads to the accumulation of strong oxidizing agent such as superoxide anion radical, H_2O_2 and hydroxyl radical **(Boldyrev *et al.*, 2013; Fu, 2018; Esse *et al.*, 2019)**. The greater the rate of H_2O_2 generation the greater the effect 50% GSH depletion had on enhancing H_2O_2 production (**Han *et al.*, 2003)**. Where a study by **Chern *et al.*, 2001** and **Balestrino, 2012** they found Hcy-mediated overproduction of H_2O_2. Even in low Hcy concentration, H_2O_2 yielding, promoting ROS generation in both extra and intracellular compartments **(Cristiana *et al.*, 2012)**.

The GSH is easily oxidized by free radicals and other ROS such as H_2O_2, and hydroxyl radical to form glutathione disulfide (GSSG) by the catalysis of GSH peroxidase (GSH-Px), and then by the catalysis of glutathione reductase; GSSG is reduced to GSH **(Agar *et al.*, 2000; Bin et al., 2017).** The high production of oxidative stress can overcome the ability of the cell to reduce GSSG to GSH leading to accumulation of GSSG (**Minich** & **Brown *et al.*, 2019).**

The GSSG has intrinsic cytotoxicity, and consequently cells export GSSG when formation exceeds capacity for recycling to GSH **(Váradi & Sarkadi, 2003),** thus, GSH may lose in the form of exported GSSG **(Berman *et al.*, 2011; Samuni *et al.*, 2013).** In addition to that the Hcy decreases the tissue levels of antioxidant vitamins A, C and E, and also lowering the activity of antioxidant enzymes, thereby reducing the antioxidant reserves such as GSH (**Huang *et al.*, 2002; Khuzakhmetova *et al.*, 2019).**

Another reason could be behind this low level of GSH, which is the impairment of transsulfuration pathway, due to depletion of vit B6 after methionine overload. The GSH; is the final product of Hcy metabolism in the transsulfuration pathway, and the major determinants of GSH synthesis are the availability of cysteine, so the GSH that is lost in the form of exported GSSG must be replaced by *de novo*

synthesis from cysteine, it was concluded that a vit B6 deficiency may contribute to impaired transsulfuration pathway, and this leads to HHcy and reduced GSH synthesis **(Berman *et al.*, 2011; Samuni *et al.*, 2013; Cacciapuoti, 2019).** two enzymes based on vitamin B6, CBS and CSE, which ultimately developed antioxidant GSH (**Kharbanda**, **2009**). By driving Hcy to cysteine and further into GSH **(Liu *et al.*, 2013; Dalto& Matte, 2017)**.

Thus, the cysteine that synthesized by the transsulfuration pathway is an important contributor to GSH synthesis, and the decrease in vit B6 level would significantly reduce of cysteine concentrations and this reflect on GSH level, it would then be reasonable to hypothesize that lower vit B6 status might be associated with HHcy, oxidative stress, and an impaired GSH-dependent antioxidant defense system (**Hsu *et al.*, 2015)**.

The administration of methionine overload to experimental animals is generally used to produce HHcy to investigate the effects of ROS that induced by Hcy on the body fluids and tissues of animals **(Mahfouz & Kummerow, 2004; Hidiroglou *et al.*, 2004; Micovic *et al.*, 2016)**. The indicators of oxidative stress include a decrease in both of GSH concentrations and the activities of antioxidant enzymes, and an increase in MDA levels (**Subbaiah *et al.*, 2011).**

Our results revealed a presence of a significant raise in the serum MDA of methionine treated males rabbits, as shown in table (4-6). And this finding agree with **(Mansour *et al.*, 2011; Anita *et al.*, 2012; Liu *et al.*, 2013; Çelik *et al.*, 2017; Huo *et al.*, 2018)** the exposure to Hcy that induced by high methionine diet leads to oxidative stress reflected on increased MDA and decreased antioxidant GSH levels**.**

The auto oxidation of Hcy enhances lipid peroxidation through production of free radicals, an elevation of free radicals generation caused a gradual cell injury and liberation of lipoxygenase enzyme which oxidized unsaturated fatty acids and subsequent production of MDA, the suppression of endogenous scavenging system including GSH resulting in oxidative stress (**Negre-Salvayre *et al.*, 2010; Al-Okaily *et al.*, 2015).**

These outputs are in agreement with several laboratory studies that alterations in the oxidant/antioxidant status of different tissue due to an elevation in lipid

peroxidation after exposure to experimental oxidative stress **(AL-Bazii, 2009; Xie *et al.*, 2015).** So this raise in serum MDA levels of current study may attribute to the tissues injury by free radicals that induced by HHcy after methionine over load**.**

5.9 The effect of HHcy that induce by methionine overload on serum Na and K

Our result showed significant decrease in the mean value of serum K^+ after 12 weeks of methionine administration, table (4-7). The result of this study was agree with **(Micovic *et al.*, 2016)** where, they found that after 3-weeks of experiment, a significant decrease in serum K^+ in methionine treated group and methionine + L cysteine group, but with no difference was found between these experimental groups. They attributed this result to the high production of ROS due to HHcy; the high production of ROS make homeostasis is disturbed, leading to oxidative stress, which has a key role in the development of liver and other chronic diseases **(Song & Rosenfeld, 2004; Costa *et al.*, 2013).**

Oxidative stress generates liver damage by causing changes in lipids, proteins and DNA molecules and even more importantly, by modifying pathways that control normal biological functions. Moreover, the systemic oxidative stress that develops after liver disease may also lead to damage in other organs, such as the brain and kidneys. Consequently, homeostasis of the whole organs was monitored through biochemical parameters that are primarily associated with the biological processes in the liver, kidneys, pancreas and bowels **(McCully, 2015).**

It was found that low K^+ level associated with; hypertension diseases, stroke, cardiovascular diseases and osteoporosis **(Castro & Raij, 2013; Weaver, 2013).**

The alkaline K^+ salts protect against bone resorption for pH homeostasis, where, Homeostatic mechanisms keep systemic pH tightly controlled at between 7.35 and 7.45**,** the excessive amount of sulfur-containing amino acids (methionine and cysteine) or high in meats and cereal grains with low in fruits and vegetables intake "Western diets" will creates slightly acidic environment in the body, an increase in the acidity with low base in the diet such as Ca^{+2}, K^+ can affect on Ca^{+2} metabolism and lead to the resorption of bone and increased urinary Ca^{+2} excretion **(Weaver, 2013),** because in acidic environment of body, with low base in diet, the OGR7

receptor on osteoblasts sense acid levels, which induces intracellular Ca^{+2} release to mediate RANK-L expression and this in turn make osteoclasts activated, therefore bone resorption **(Dawson-Hughes, 2011).**

The alkaline K^+ salts produced from metabolizing fruits and vegetables or K^+ supplements such as (K^+ bicarbonate or citrate), the K^+ intake has been associated with reduced urinary Ca^{+2} excretion in healthy kidney whether given as citrate or bicarbonate salts and improves Ca^{+2} balance **(Palmer *et al.*, 2020).**

Our results also showed a significant decrease in the mean value of serum Na^+ of methionine treated group, table (4-7). It was found that majority (70%) of Na^+ and water reabsorption under normal conditions occurs in the PTCs of kidney (**Biff *et al.*, 2020)**. So any disturbance or damage in this part may negatively reflect on Na^+ homeostasis and other ions and nutrient (**Earley *et al.*, 1966)**.

The kidney require large quantity of ATP for active transport **(Hall, 2010),** that take place in the proximal tubule, the loop of Henle, the distal tubule and the collecting duct, where the most active transport processes occurs in PTCs, because the PCTs reabsorb about 80% of the filtrate that passes through the glomerulus, including glucose, ions, and nutrients, for that reason the PTCs contain more mitochondria than any other structure in the kidney, to produce ATP that is critical for Na+/K+ ATPase pumps to maintains a low intracellular level of Na^{+2}, which help in regulation of Na^+ reabsorption in this part **(Yan *et al.*, 2013; Bhargava & Schnellmann, 2017).**

Many of previous studies have shown that Hcy causes severe mitochondrial injury with disruption of mitochondrial functions through regulation of oxidative stress **(Bhattacharjee & Borah, 2016; Chen *et al.*, 2017)**. Hcy also associated with overproduction of H_2O_2 **(Chern *et al.*, 2001)**. Where, the H_2O_2 inhibits Na^+/K^+ATPase pumps in PTCs **(Gonzalez-Vicente & Garvin, 2017)**, and this in turn leads to loss of Na^+ with urine (**Liu *et al.*, 2017)**.

In addition to that, H_2O_2 act synergistically with Hcy to cause mitochondrial damage **(Austin *et al.*, 1998).** It is well known that mitochondrial damage can result in the overproduction of ROS and reactive nitrogen species (RNS), which upon accumulation can cause oxidative and nitrosative damage to the lipids, proteins and

DNA, that associated with the pathophysiology of the most kidney diseases **(Radak *et al*., 2011; Ishimoto *et al*., 2018).**

The PTCs have a requirement for ATP for the active reabsorption of filtered nutrients and ions. Thus, the damage of PCTs mitochondria can have two consequences, first; increased production of ROS thereby resulting in increased oxidant stress and what make matters worse that PTCs cannot synthesize GSH and depending on circulating GSH as antioxidant protection, where our result also showed decrease in GSH levels of methionine treated group, so the decrease of cellular antioxidants can lead to the accumulation of endogenous ROS that activates signaling pathways leading to the death of renal tubular cells, and the second consequences is; PTCs dysfunction resulting in Fanconi Syndrome **(Visarius *et al*., 1996; Murphy, 2009; Gyurászová *et al*., 2020).**

The Fanconi syndrome; is a condition that could be an inherited or acquired characterized by defect of PTCs leading to malabsorption of various electrolytes such as Na^+, K^+, Ca^{+2} and other substances **(Keefe & Bokhari., 2018; Hechanova, 2019)**. And this may explain the reason of this decrease in serum Na^+ and K^+ levels.

It was found that restricting intake of sulphur amino acids, such as methionine and cysteine will reduces mitochondrial oxidative stress in the kidneys, liver, brain and heart **(Sanchez-Roman *et al*., 2012; Hine *et al*., 2015),** and this restriction of sulphur amino acids will protect the kidney from acute and chronic injury, and also protect the other organs **(Wang *et al*., 2018; Späth *et al*., 2020)**.

The auto-oxidation of Hcy is associated with generating of potent ROS including superoxide anion, H_2O_2 and hydroxyl radical **(Long& Nie, 2016).** That causes oxidative damage not only to the tissues of the kidneys, liver heart and brain **(Di Meo *et al*., 2016),** but also may extend to other parts like the adrenaline glands.

Where a study by **(Prasad *et al*., 2014)** found that oxidative stress may cause Adrenal insufficiency, although that adrenal cortex, in particular, is well supplied with both enzymatic (GSH-Px) and nonenzymatic (vitamins A, C and E) antioxidants. In addition to ability of Hcy to generate ROS, the Hcy also has ability to lowering the tissue levels of antioxidant vitamins A, C and E, and also lowering

the activity of antioxidant enzymes GSH-Px, thereby reducing in the antioxidant stored **(Handy *et al.*, 2005; Khuzakhmetova *et al.*, 2019).**

Thus, unbalance between the overproduction of ROS and the exhausted antioxidant defense system resulting in oxidative stress that may cause Adrenal insufficiency **(Liguori *et al.*, 2018)**. Where, adrenal insufficiency: is a condition in which the adrenal glands do not produce adequate amounts of steroid hormones, primarily cortisol and also impaired production of aldosterone, which regulates Na^+ conservation, K^+ secretion, and water retention **(Corrigan, 2007).** Where, a study by **(Izumi *et al.*, 2015)** found that low level of Na^+ (Hyponatremia) results from both aldosterone and cortisol insufficiency.

The low of Na^+ or high in K^+ level is associated with activation of the renin-angiotensin-aldosterone system (RAAS) **(Srinivasa *et al.*, 2016; Fountain & Lappin, 2017; Beusekamp *et al.*, 2018)**. The ROS also promote or mediate hypertension initiated by many processes, such as activation of the RAAS (**Welch *et al.*, 2003)**.

The activation of the RAAS causes a widespread increase in ROS in the kidney via NADPH oxidase, mitochondrial dysfunction, decreased NO availability, and decreased antioxidant enzymes; this is implicated in Na^+ retention, K^+ secretion, vasoconstriction, and hypertension **(Palm *et al.*, 2010; Bayorh *et al.*, 2011; Pessôa *et al.*, 2013; Araujo & Wilcox, 2014),** but our results showed low levels of serum Na^+ and this may indicate either a failure of the RAAS associated with renal tubule damage and this failure in RAAS occur as a result of damage to one or all of the following organs, which includes the liver, kidneys, adrenal gland due to oxidative stress or the RAAS its works properly but there may be severe damage to the renal tubes that may associate with loss of electrolytes and substance.

It was found that hyponatremia may also have a direct adverse impact on integrity and metabolism of bone leading to a higher rate of osteoporosis, because up to one third of body Na^+ is stored in bone **(Afshinnia *et al.*, 2015; Upala & Sanguankeo**, **2016**; **Hew-Butler *et al.*, 2019).** It is hypothesized that in hyponatremia, increased bone resorption occurs in an attempt to preserve the homeostasis of Na^+ at the expense of bone structural integrity. Increased osteoclast activity and number in parallel with decreased serum level of osteocalcin (a marker

of bone formation), reduced intracellular Ca^{+2} (**Verbalis *et al*., 2010; Barsony *et al*., 2011; Afshinnia *et al*., 2015),** alteration in metabolism of vitamin D and sterol-containing hormones, and alteration in oxidative stress **(Basu *et al*., 2001; Yılmaz& Eren, 2009)** are viewed as possible mechanistic links between hyponatremia and osteoporosis.

Increased osteoclastic activity may be mediated by increased expression level of vasopressin receptors AVPr1a-alpha and AVPr2-alpha triggering Erk (kinase) signaling pathway in favor of suppressing bone formation and stimulating bone resorption (**Tamma *et al*., 2013)**. Another mechanism for activation of osteoclasts could be via voltage-gated Na^{+} channels which are highly expressed in bone and are sensitive to changes in extracellular tonicity and, therefore, may mediate the activation of osteoclasts in chronic hyponatremia **(Verbalis *et al*., 2010; Carlos Ayus *et al*., 2012; Hoorn *et al*., 2012).**

We disagreement with **(Micovic *et al*., 2016)** where, they found that Na^{+} levels in the blood were significantly higher in methionine treated group and methionine + L cysteine group after 3 weeks of experiment, they attributed this result to oxidative stress due to high ROS production that leads to damage in liver and other organs such as the brain and kidneys, Consequently, homeostasis in these organs will disturbance.

5.10 The effect of HHcy that induce by methionine overload on histological section

The microscopic examination of longitudinal and histological sections of femur head for control group shows normal bone trabecular with normal thickness and density as shown in **figures 4-9**, **4-11** and **4-17.** In addition to very few osteoclasts with no surface resorption pits as shown in **figure 4-13**.

The microscopic examination of longitudinal and histological sections of femur head for methionine treated group shows thin trabecular with weaker rod like structure and some of rods are completely disconnected with large spaces between them as shown in **figures 4-10, 4-12**, **4-14** and **4-18**. Increase in osteoclasts percentage and resorption pits on surface of trabecular bone as shown in **figures 4-15 & 4-16**.

These changes in bone tissues of methionine treated group attributed to increase in osteoclasts number and activity due to increase in RANK and RANK-L levels after methionine overload and occurrence of HHcy, The RANK-L binds to its receptor RANK on hematopoietic precursors of osteoclasts and stimulates their differentiation and survival and thereby stimulating bone resorption (**Liu &Liu, 2015).**

In brief, the binding of RANK-L (produce by osteoblast) to RANK, located on the surface of osteoclast precursors, recruits the cytoplasmic adapter protein TRAF6, leading to NF-κB activation and translocation to the nucleus; in the nucleus, the translocated NF-κB increases the expression of c-Fos; the interaction of c-Fos with NFATc1 leads to increased expression of various osteoclastogenic genes; consequently, osteoclast formation is increased, thereby resulting in increased bone resorption (**Li *et al.*, 2010)**. **)** and this corresponds to what we found in the histological examination, which showed a significant increase in the numbers and activities of osteoclasts, with occurrence of sever osteoporosis.

Chapter Six

Conclusions

&

Recommendations

6.1 Conclusions

From the results and discussion obtained from this study, we can conclude that:

A. Oral intubation of (100mg/kg BW) of methionine for twelve weeks caused a case of oxidative stress with a significant elevation in the serum Hcy (HHcy), RANK, RANK-L, PTH, ALP, Fe^{+2} and MDA, and a significant reduce in the serum of Ca^{+2}, Na^{+}, K^{+} and GSH.

B. The elevation in the serum RANK and RANK-L activate signaling pathway caused increase in the number and activity of osteoclast, which appears clearly in examining the tissue sections of the femur in hyperhomocysteinemic males rabbits, and this increase in the number and activity of osteoclasts cause erosion and necrosis in trabecular structure of femur bone.

C. We conclude that HHcy considered a risk factor for osteoporosis.

6.2 Recommendation

The present study recommended the following:

1. Further biochemical investigations can be carried out to elucidate the mechanism of HHcy on osteoclasts activity.
2. Measurement of tissue oxidative stress, free radicals and antioxidants biomarkers after methionine overload.
3. Histological examination to the tissues of different organs of animals treated with methionine overload, to find its effect on these organs.
4. Electronic microscopic study (Transmission and scanning electronic microscope) of different organs subjected to methionine overload.
5. Responsible health authorities such as WHO and Centers for Disease Control and Prevention (CDC) should consider HHcy as a risk factor for osteoporosis. And emphasizing the role of vitamin B group in reducing the high level of Hcy, as it plays an important role in methionine metabolism

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Appendix

Appendix (1)

Measurement of serum Receptor Activator of Nuclear Factor Kappa B (RANK)

Kit contain:-

compound	Quantity
Biotinylated Rabbit TNFRSF11A/RANK Antibody	1ml x1
Plate Sealer	2 pics
Pre-coated Elisa plate	12*8 well strips x1
Standard Diluent	3ml x1
Standard solution (24ng/ml)	0.5ml x1
Stop Solution	6ml x1
Streptavidin-HRP	6ml x1
Substrate Solution A	6ml x1
Substrate Solution B	6ml x1
User Instruction	1
Wash Buffer Concentrate (25x)	20ml x1
Zipper bag	1 pic

Material Required But Not Supplied:

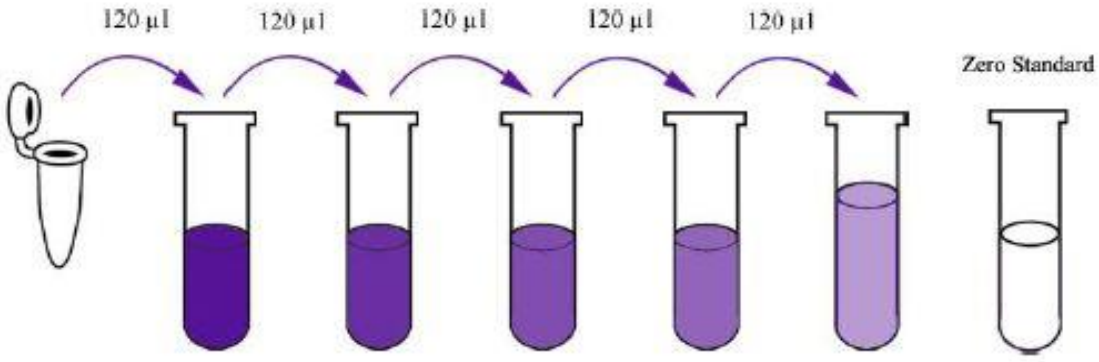
- 37°C±0.5°C incubator
- Absorbent paper
- Precision pipettes and disposable pipette tips
- Clean tubes
- Deionized or distilled water
- Microplate reader with 450 ± 10nm wavelength filter

Reagent Preparation

All reagents should be brought to room temperature before use. **Standard** Reconstitute the 120µl of the standard (24ng/ml) with 120µl of standard diluent to generate a 12ng/ml standard stock solution. Allow the standard to sit for 15 mins

with gentle agitation prior to making dilutions. Prepare duplicate standard points by serially diluting the standard stock solution (12ng/ml) 1:2 with standard diluent to produce 6ng/ml, 3ng/ml, 1.5 ng/ml and 0.75 ng/ml solutions. Standard diluent serves as the zero standards (0. ng/ml). Any remaining solution should be frozen at -20°C and used within one month. Dilutions of standard solutions suggested are as follows:

12ng/ml	Standard No.5	120µl Original Standard + 120µl Standard Diluent
6ng/ml	Standard No.4	120µl Standard No.5 + 120µl Standard Diluent
3ng/ml	Standard No.3	120µl Standard No.4 + 120µl Standard Diluent
1.5ng/ml	Standard No.2	120µl Standard No.3 + 120µl Standard Diluent
0.75ng/ml	Standard No.1	120µl Standard No.2 + 120µl Standard Diluent



Standard Concentration	Standard No.5	Standard No.4	Standard No.3	Standard No.2	Standard No.1
24ng/ml	12ng/ml	6ng/ml	3ng/ml	1.5ng/ml	0.75ng/ml

Wash Buffer

Dilute 20ml of Wash Buffer Concentrate 25x into deionized or distilled water to yield 500 ml of 1x Wash Buffer. If crystals have formed in the concentrate, mix gently until the crystals have completely dissolved.

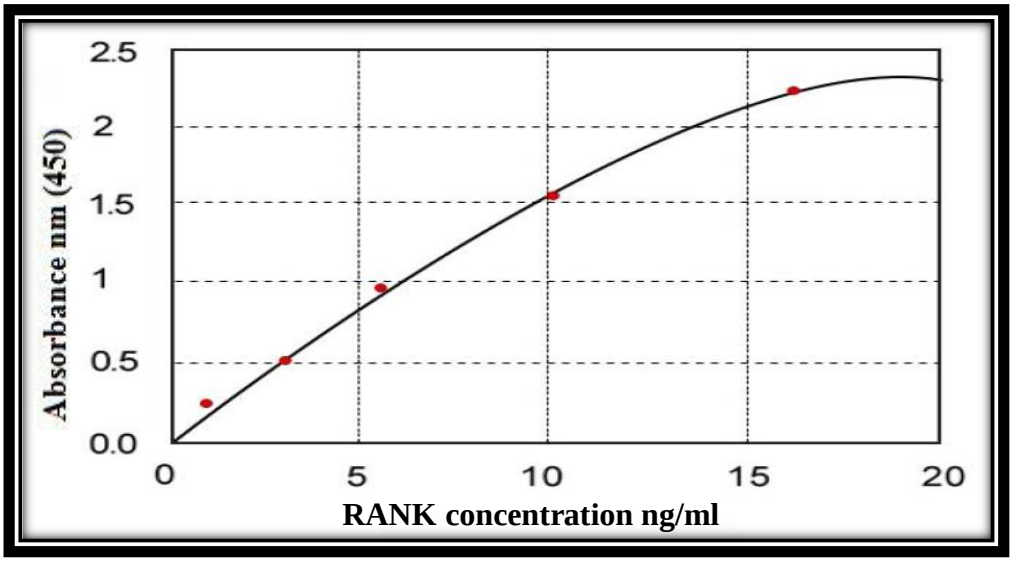
Assay Procedure

1. Prepare all reagents, standard solutions and samples as instructed. Bring all reagents to room temperature before use. The assay is performed at room temperature.

2. Determine the number of strips required for the assay. Insert the strips in the frames for use. The unused strips should be stored at 2-8°C.
3. Add 50µl standard to standard well. **Note**: Don't add antibody to standard well because the standard solution contains biotinylated antibody.
4. Add 40µl sample to sample wells and then add 10µl anti-TNFRSF11A/RANK antibody to sample wells, then add 50µl streptavidin-HRP to sample wells and standard wells (Not blank control well). Mix well. Cover the plate with a sealer. Incubate 60 minutes at 37°C.
5. Remove the sealer and wash the plate 5 times with wash buffer. Soak wells with at least 0.35 ml wash buffer for 30 seconds to 1 minute for each wash. For automated washing, aspirate all wells and wash 5 times with wash buffer, overfilling wells with wash buffer. Blot the plate onto paper towels or other absorbent material.
6. Add 50µl substrate solution A to each well and then add 50µl substrate solution B to each well. Incubate plate covered with a new sealer for 10 minutes at 37°C in the dark.
7. Add 50µl Stop Solution to each well, the blue color will change into yellow immediately.
8. Determine the optical density (OD value) of each well immediately using a microplate reader set to 450 nm within 10 minutes after adding the stop solution.

Stander curve of RANK:

The concentration of serum RANK is obtained from the stander RANK curve.



Stander curve of Receptor Activator of Nuclear Factor Kappa B (RANK)

Appendix (2)

Measurement of serum Receptor Activator of Nuclear Factor Kappa B Ligand (RANK-L)

Kit contain:-

compound	Quantity
Biotinylated Rabbit RANKL Antibody	1ml x1
Plate Sealer	2 pics
Pre-coated Elisa plate	12*8 well strips x1
Standard Diluent	3ml x1
Standard solution (800ng/L)	0.5ml x1
Stop Solution	6ml x1
Streptavidin-HRP	6ml x1
Substrate Solution A	6ml x1
Substrate Solution B	6ml x1
User Instruction	1
Wash Buffer Concentrate (25x)	20ml x1
Zipper bag	1 pic

Material Required But Not Supplied

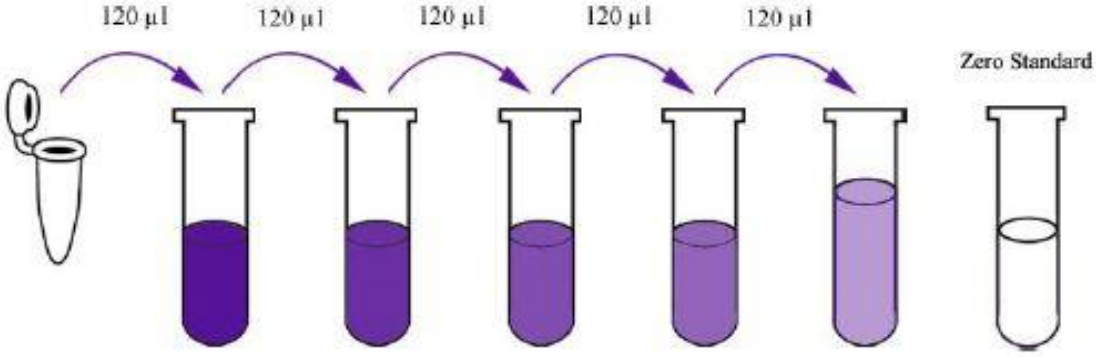
- 37°C±0.5°C incubator
- Absorbent paper
- Precision pipettes and disposable pipette tips
- Clean tubes
- Deionized or distilled water
- Microplate reader with 450 ± 10nm wavelength filter

Reagent Preparation

All reagents should be brought to room temperature before use. Standard Reconstitute the 120µl of the standard (800ng/L) with 120µl of standard diluent to

generate a 400ng/L standard stock solution. Allow the standard to sit for 15 mins with gentle agitation prior to making dilutions. Prepare duplicate standard points by serially diluting the standard stock solution (400ng/L) 1:2 with standard diluent to produce 200ng/L, 100ng/L, 50ng/L and 25ng/L solutions. Standard diluent serves as the zero standard (0. ng/L). Any remaining solution should be frozen at -20°C and used within one month, Dilution of standard solutions suggested are as follows:

400ng/L	Standard No.5	120µl Original Standard + 120µl Standard Diluent
200ng/L	Standard No.4	120µl Standard No.5 + 120µl Standard Diluent
100ng/L	Standard No.3	120µl Standard No.4 + 120µl Standard Diluent
50ng/L	Standard No.2	120µl Standard No.3 + 120µl Standard Diluent
25ng/L	Standard No.1	120µl Standard No.2 + 120µl Standard Diluent



Standard Concentration	Standard No.5	Standard No.4	Standard No.3	Standard No.2	Standard No.1
800ng/L	400ng/L	200ng/L	100ng/L	50ng/L	25ng/L

Wash Buffer

Dilute 20ml of Wash Buffer Concentrate 30x into deionized or distilled water to yield 500 ml of 1x Wash Buffer. If crystals have formed in the concentrate, mix gently until the crystals have completely dissolved

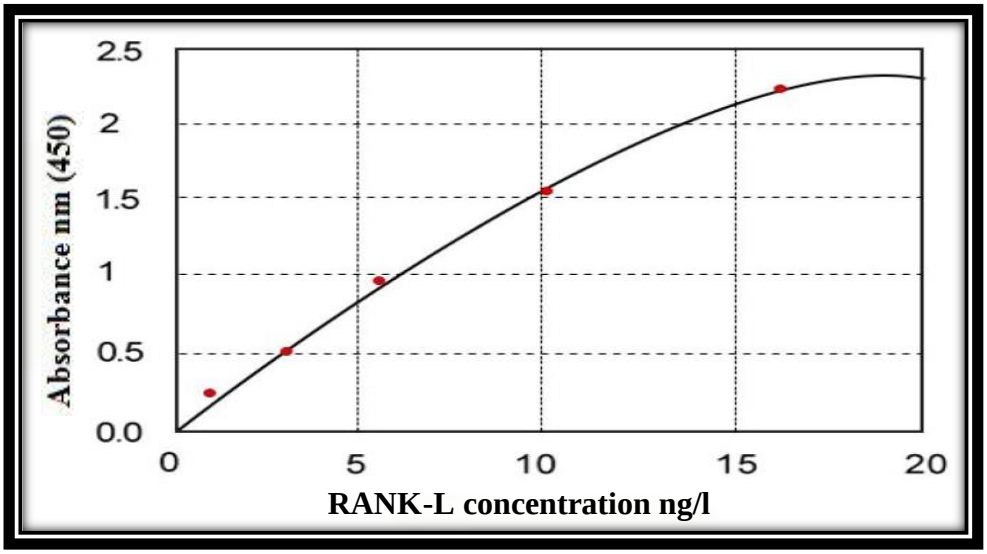
Assay Procedure

1. Prepare all reagents, standard solutions and samples as instructed. Bring all reagents to room temperature before use. The assay is performed at room temperature.

2. Determine the number of strips required for the assay. Insert the strips in the frames for use. The unused strips should be stored at 2-8°C.
3. Add 50µl standard to standard well. **Note**: Don't add antibody to standard well because the standard solution contains Biotinylated antibody.
4. Add 40µl sample to sample wells and then add 10µl anti-RANKL antibody to sample wells, then add 50µl streptavidin-HRP to sample wells and standard wells (Not blank control well). Mix well. Cover the plate with a sealer. Incubate 60 minutes at 37°C.
5. Remove the sealer and wash the plate 5 times with wash buffer. Soak wells with at least 0.35 ml wash buffer for 30 seconds to 1 minute for each wash. For automated washing, aspirate all wells and wash 5 times with wash buffer, overfilling wells with wash buffer. Blot the plate onto paper towels or other absorbent material.
6. Add 50µl substrate solution A to each well and then add 50µl substrate solution B to each well. Incubate plate covered with a new sealer for 10 minutes at 37°C in the dark.
7. Add 50µl Stop Solution to each well, the blue color will change into yellow immediately.
8. Determine the optical density (OD value) of each well immediately using a microplate reader set to 450 nm within 10 minutes after adding the stop solution.

Stander curve of RANK-L:

The concentration of serum RANK-L is obtained from the stander RANK-L curve.



Stander curve of Receptor Activator of Nuclear Factor Kappa B Ligand (RANK-L)

Appendix (3)

Measurement of serum Homocysteine (Hcy)

Elisa Kit contain:-

compound	Quantity
Biotinylated Rabbit Hcy Antibody	1ml x1
Plate Sealer	2 pics
Pre-coated Elisa plate	12*8 well strips x1
Standard Diluent	3ml x1
Standard solution (128ug/ml)	0.5ml x1
Stop Solution	6ml x1
Streptavidin-HRP	6ml x1
Substrate Solution A	6ml x1
Substrate Solution B	6ml x1
User Instruction	1
Wash Buffer Concentrate (25x)	20ml x1
Zipper bag	1 pic

Material Required But Not Supplied

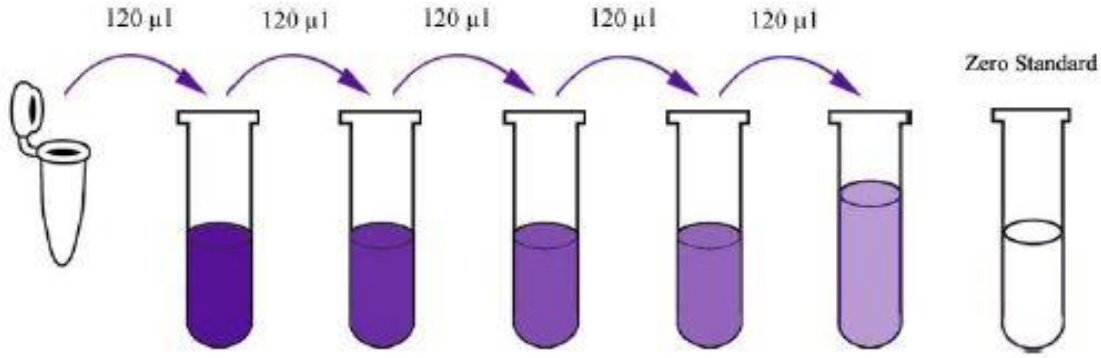
- 37°C±0.5°C incubator
- Absorbent paper
- Precision pipettes and disposable pipette tips
- Clean tubes
- Deionized or distilled water
- Microplate reader with 450 ± 10nm wavelength filter

Reagent Preparation

All reagents should be brought to room temperature before use. Standard Reconstitute the 120µl of the standard (128µg/ml) with 120µl of standard diluent to generate a 64µg/ml standard stock solution. Allow the standard to sit for 15 mins

with gentle Agitation prior to making dilutions. Prepare duplicate standard points by serially diluting the standard stock solution (64µg/ml) 1:2 with standard diluent to produce 32µg/ml, 16µg/ml, 8µg/ml and 4µg/ml solutions. Standard diluent serves as the zero standards (0µg/ml). Any remaining solution should be frozen at -20°C and used within one month. Dilutions of standard solutions suggested are as follows:

64µg/ml	Standard No.5	120µl Original Standard + 120µl Standard Diluent
32µg/ml	Standard No.4	120µl Standard No.5 + 120µl Standard Diluent
16µg/ml	Standard No.3	120µl Standard No.4 + 120µl Standard Diluent
8µg/ml	Standard No.2	120µl Standard No.3 + 120µl Standard Diluent
4µg/ml	Standard No.1	120µl Standard No.2 + 120µl Standard Diluent



Standard Concentration	Standard No.5	Standard No.4	Standard No.3	Standard No.2	Standard No.1
128µg/ml	64µg/ml	32µg/ml	16µg/ml	8µg/ml	4µg/ml

Wash Buffer

Dilute 20ml of Wash Buffer Concentrate 25x into deionized or distilled water to yield 500 ml of 1x Wash Buffer. If crystals have formed in the concentrate, mix gently until the crystals have completely dissolved.

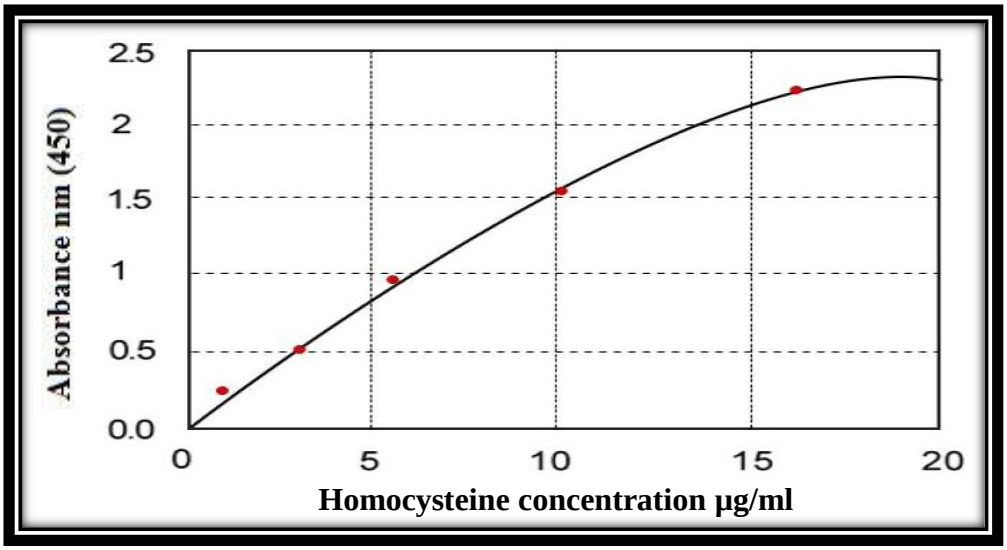
Assay Procedure

1. Allow serum to clot for 10-20 minutes at room temperature. Centrifuge at 2000-3000 RPM for 20 minutes.

2. Prepare all reagents, standard solutions and samples as instructed. Bring all reagents to room temperature before use. The assay is performed at room temperature.
3. Determine the number of strips required for the assay. Insert the strips in the frames for use. The unused strips should be stored at 2 8°C.
4. Add 50µl standard to standard well. **Note**: Don't add antibody to standard well because the standard solution contains biotinylated antibody.
5. Add 40µl sample to sample wells and then add 10µl anti-Hcy antibody to sample wells, then add 50µl streptavidin-HRP to sample wells and standard wells (Not blank control well). Mix well. Cover the plate with a sealer. Incubate 60 minutes at 37°C.
6. Remove the sealer and wash the plate 5 times with wash buffer. Soak wells with at least 0.35 ml wash buffer for 30 seconds to 1 minute for each wash. For automated washing, aspirate all wells and wash 5 times with wash buffer, overfilling wells with wash buffer. Blot the plate onto paper towels or other absorbent material.
7. Add 50µl substrate solution A to each well and then add 50µl substrate solution B to each well. Incubate plate covered with a new sealer for 10 minutes at 37°C in the dark.
8. Add 50µl Stop Solution to each well, the blue color will change into yellow immediately
9. Determine the optical density (OD value) of each well immediately using a microplate reader set to 450 nm within 10 minutes after adding the stop solution.

Stander curve of Hcy:

The concentration of serum Hcy is obtained from the stander Hcy curve



Stander curve of Homocysteine (Hcy)

Appendix (4)

Measurement of Malondialdehyde (MDA):

Serum MDA concentration was measured by the thiobarbiutric acid (TBA) assay. One ml serum was added to 3ml of 1% phosphoric acid, 1 ml of 0.6% TBA and 0.15 ml of 0.2% hydroxyl butylated toluene (HBT) in 95% methanol. The samples was heated in boiling water for 45 minutes, MAD reacted with thiobarbituric acid under high temperature (90-100 °C) and acidic condition. The reaction yield MDA-TBA adducts (pink color).

Thiobarbituric acid + Malondialdehyde $\longrightarrow$ MDA-TBA adduct + H_2O

The solution was left to cool and 4ml of butanol was added the butanol phase was separated by centrifuge at 3000 rpm for 10 minutes and absorbance was measured at 532 nm with an extinction coefficient. Absorbance $_{532}$=1.53x10^5 M $^{-1}$ cm $^{-1}$ **(Jetawattana, 2005).**

Appendix (5)

Determination of serum reduced glutathione concentration (GSH)

Reduce glutathione determined using the disulfide chromogenic compound 5,5 -dithiobis-(2-nitrobenzoic acid) (DTNB) which is readily reduced by sulfhydryl group of GSH to an intensely yellow compound as shown. The absorbance of the reduce chromogen is measured at 412 nm directly proportional to the concentration of GSH **(Burtis & Ashwood, 1999)**.

Preparation of reagents:-

1. **Precipitating solution 50% Tricholroacetic acid (TCA).**
   50mg of TCA are dissolved in a final volume of 100ml of distal water (DW).
2. **Ethylenediamine tetra acetic acid – disodum (EDTANa2).**
   148.9 gm of EDTA – Na are dissolved in a final volume of 1 liter of DW.
3. **Tris- EDTA Na buffer (0.4) PH 8.9.**
   48.458 gm of Tris are dissolved in 800 ml of DW. 100ml of 4 M EDTA solution are added and bring to a final volume of 1 litter with DW. The PH was adjusted to 8.9 by the addition of 1ml of HCL.
4. **5,5 Dithiobis (2-nitrobenzoic acid) (DTNB) reagent.**
   0.099 gm of DTNB was dissolved in absolute methanol and brings to a final volume of 25ml (this reagent is stable for at least 13 weeks at 4c.
5. **GSH standards.**
   Stock standard solution of GSH (0.001M) is prepared by dissolving 0.0307 gm of GSH standard in a final volume of 100ml of 0.4M Tris-EDTA-Na buffer PH 8.9. From stock solution 2,5,10,20,40,50 and 60 Um of standard GSH were prepared by formula N1.V1=N2.V2 Normality of stock solution must be converted to micro mol by (10^6).

Procedure:-

Serum GSH concentration was determined by using a modified procedure utilizing Ellman's reagent (DTNB), which is summarized as follows standard and sample were prepared into test tube as follows:

Reagent	Sample ul	Reagent blank ul	Standard ul
Serum	100		
Standard			100
DW	800	900	800
TCA	100	100	100

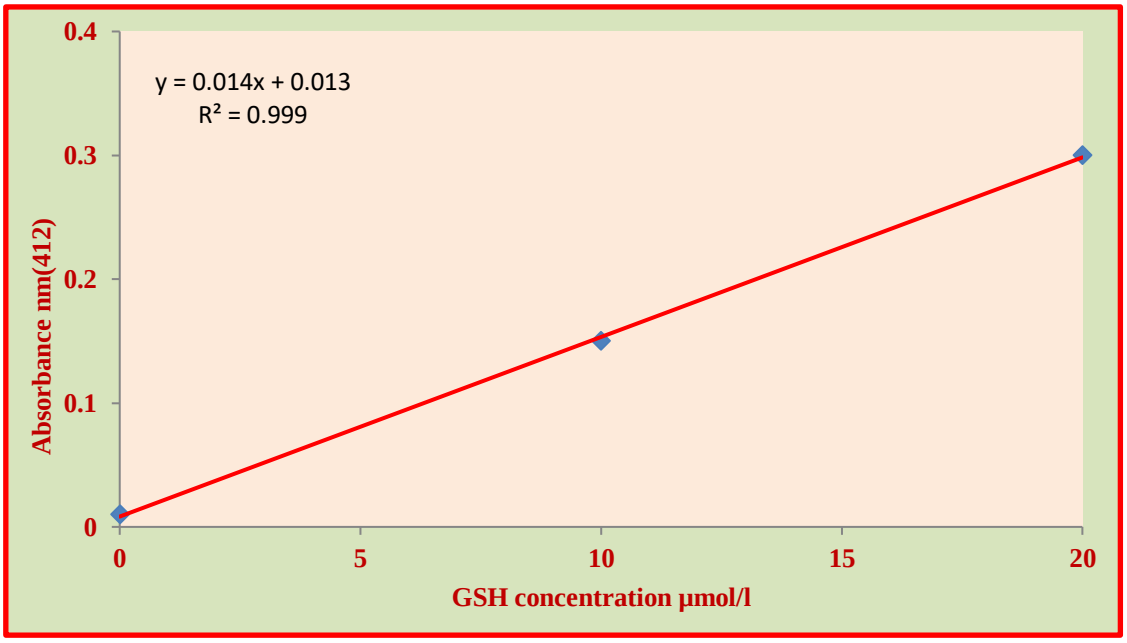
Distal water: Tubes were mixed in vortex intermittently for 10-15 minutes, and centrifuged for 15 minutes at 3000x g**,** then pipette into test tube as follows

Reagent	Sample ul	Reagent blank ul	Standard ul
Supernatant	400	400	400
Tris- EDTA buffer	800	800	800
DTNB reagent	20	20	20

Tubes were mixed in vortex. The spectrophotometer was adjusted with reagent blank to read zero absorbance (A) at 412 nm, and the absorbance of standers and samples were read within 5 minutes of addition of DTNB.

Stander curve of GSH

The concentration of serum GSH micromole/liter was obtained from the stander glutathione curve



Calibration curve of Glutathione (GSH) determination

Appendix (6)

Determination of serum Iron concentration

The concentration of serum Iron is obtained by using Furuno CA-180 fully automated chemistry analyzer that can measure different types of minerals, protein and enzymes for different types of samples serum, plasma and urine.

Each parameter has special reagent kit that is ready to use and load to the highly stable refrigerated reagent tray (removable carousel) of machine while tubes of samples load to the multifunction sample tray. The Furuno CA-180 depends on barcode system to identify sample tubes and reagent plastic containers to reduce system error.

The iron reagent kit composition: (Follow instruction manual according to the manufacturer)

Reagent R1: F245: 12 x 16 ml (liquid) blue cap and F400: 8 x 40 ml (liquid) blue cap. Composition: acetate buffer 500 mM pH 4.50, thiourea ≥ 50 mM, guanidine hydrochloride ≥ 100 mM and surfactant.

Reagent R2A: F245: 2 x 24 ml (liquid) red cap and F400: 2 x 40 ml (liquid) red cap. Composition: ferrozine 6 mM.

Reagent R2B: F245: 2 vials powder for 24 ml and F400: 2 vials powder for 40 ml. Composition: sodium ascorbate ≥ 50 mM.

Standard: iron (III) solution 200 µg/dl - 5 ml

Reagent preparation:

Reagent R1: ready to use.

Reagent R2: add all the content of reagent R2B to reagent R2A and let to stay 20 minutes, mixing occasionally by inversion. Do not shake. Stable 90 days at 2-8°C.

Sample size that require of test is 250 µl of serum or plasma and the absorbance step at wavelength: 578 nm. The time spent for completing test is about 10 minutes.

Appendix (7)

Determination of serum Parathyroid hormone (PTH) concentration

The levels of PTH in serum of experimental subjects were determents by Fluorescence Enzyme Immunoassay technique through using (TOSOH-AIA-360 fully Automated Immunoassay Analyzer).

The AIA-360 close system device accompanied by Tosoh's AIA Reagent test cup, each test cup completely self contain single test, no preparation is required and dry reagent require no refrigeration. Relative to the measurement of PTH we used PTH test cup with assay range from (1.0 to 2000 pg/mL).

The Assay operations begin with the loading of specimens and reagent cups into the carousel by loading test tubes first (or designated sample cups) into the sample holder and reagent cups (numbers correspond to analytes) into the reagent cup holder. The AIA-360 has sample capacity 25 tube. The minimum sample value for tube is 500 µl and 100 µl for sample cup. The AIA 360 use barcode system to identify subject sample and test cub to reduce system error.

The total assay time for Intact PTH on the AIA-360 is ~20 minutes which involve breaking of the aluminum top seal of test cup, addition of serum sample to the test cup, incubation of immune reaction for about 10 minutes, wash step and free separation, addition of substrate and fluorescence kinetics detection, after about 20 minutes the result appear on device screen and can be record on roll paper.

الخلاصة:

أجريت هذه الدراسة لمعرفة تأثير فرط الهوموسيستين في الدم في نشاط ناقضات العظم والمستحدث بفرط المثيونين في ذكور الأرانب .

تم أستخدام (20) من ذكور الأرانب البالغه والتي قسمت عشوائيا الى مجموعتان (عشر حيوانات/مجموعة) جرعت المجموعة الأولى الماء العادي فمويا, مجموعة السيطرة , اما أرانب المجموعة الثانية فقد جرعت (100ملغم/كغم و.ج) من المثيونين يوميا ولمدة 12 أسبوعا.

تم جمع عينات الدم بعد 12 أسبوع من التجربة , وتم سحب الدم بطريقة الوخز القلبي لقياس المعاييرالتالية: أ- قياس تركيز الهوموسيستين (Hcy) في مصل الدم. ب- قياس المؤشرات البايلوجية لنشاط ناقضات العظم المتضمن: قياس تركيز الرانك (RANK) و قياس تركيز الرانكل (RANK-L) في مصل الدم. ج- قياس مستوى الاملاح والمعادن المتضمن : قياس تركيز الكالسيوم (Ca^{+2}) و قياس تركيز الصوديوم (Na^{+}) وقياس تركيز البوتاسيوم (K^{+}) وقياس تركيز الحديد (Fe^{+2}) في مصل الدم. د- قياس تركيز هرمون جار الدرقية (PTH) في مصل الدم. ذ- قياس انزيم الفوسفاتيز القلوية (ALP) في مصل دم. هـ- دلالات الاجهاد التأكسدي والمتضمنة قياس تركيز الكلوتاثيون المختزل (GSH) و قياس تركيز مالونديالديهايد (MDA) في مصل الدم. بالاضافة لدراسة التغيرات المرضية النسيجية لعظم الفخذ.

أظهرت نتائج هذه التجربة ان التجريع الفموي للمثيونين (100ملغم/كغم و.ج) يوميا لذكور الأرانب ولمدة 12 أسبوع قد تسبب بحدوث أرتفاع معنوي ($p\leq0.001$) في تركيز Hcy و تركيز RANK و تركيز RANK-L و تركيز PTH و تركيز ALP و تركيز MDA و تركيز Fe^{+2} و انخفاض معنويا ($p\leq0.001$) في تراكيز GSH و Ca^{+2} و Na^{+} بالاضافة الى انخفاض معنوي ($p\leq0.05$) في تركيز K^{+} بالمقارنة مع مجموعة السيطرة. فضلا عن ذلك اظهر الفحص النسيجي الى ان التجريع الفموي للمثيونين اظهر زيادة في اعداد ونشاط خلايا ناقضات العظم مصحوبة بتأكل وتنخر في التركيب الاسفنجي لعظم الفخذ.

اما بالنسبة للعلاقات فقد تم ربط Hcy مع مجموعة من المؤشرات الحيوية واظهرت النتائج علاقة معنوية ($p\leq0.01$) موجبة بين Hcy مع كل من (ALP , Fe^{+2} , RANK-L) , الا ان Hcy أظهر علاقة سلبية معنوية ($p\leq0.01$) مع (Ca^{+2} و GSH). كما أظهر RANK-L علاقة موجبة معنوية ($p\leq0.01$) مع RANK. وكذلك أظهر PTH علاقة موجبة معنوية ($p\leq0.01$) مع RANK-L , الا انه اظهر علاقة سلبية معنوية ($p\leq0.01$) مع Ca^{+2}.

بأختصار , يستنتج من الدراسة الحالية أن فرط الهوموسيستين في الدم المستحدث بفرط المثيونين قد تسبب في زيادة مستويات RANK و RANK-L , الامر الذي ترتب عليه زيادة في أعداد ونشاط خلايا ناقضات العظم مانتج عنه تنخر وتأكل في التركيبة الأسفنجية لعظم الفخذ , يتبين من ذلك ان ارتفاع الهوموسيستين في الدم يعد عامل خطورة لحدوث هشاشة العظم.

جمهورية العراق

وزارة التعليم العالي والبحث العلمي

جامعة كربلاء/كلية الطب البيطري

فرع الفسلجة والكيمياء الحياتية والأدوية

تأثير فرط الهوموسستين في الدم على بعض الدلائل الحيوية و نشاط ناقضات العظم في ذكور الأرانب المستحدث بالمثيونين

رسالة مقدمة الى

مجلس كلية الطب البيطري- جامعة كربلاء وهي جزء من متطلبات نيل درجة الماجستير في علوم الطب البيطري / الفسلجة

من قبل

محمد ماجد حميد القنبر

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