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The preventive effect of folic acid on the damage induced by methionine overload on physiology , histology and molecular aspect in male mice

A thesis

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بسم الله الرحمن الرحيم

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Dedication

To my role model, my support and my honorary . to who taught me Letters of the light (Number Nine). Reason of my excellence and success, My Father (God prolong his life)

To the apple of my eye who Her prayers and words were my Companion to success and excel, who stayed up the nights, the Symbol of love and tenderness ... The flower of my life... My mother (God prolong her life).

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Always a source of my pride and encouragement .. always present despite the difficulties .. who was my partner in every step of my research .... my brother (Suhaeb)..

The beautiful angel ... the wonderful smile ... the candle of my father's house .... my sister(Abrar)..

The gentle being ... the symbol of calm ... the warm embrace ... my little brother (Awab)

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Tuqa…

Summary

This study is carried out to investigate the protective role of folic acid on the hepatic and kidney damage induced by methionine overload in male mice .

One hundred fifty (150) mice were divided into five groups (30/group), the first group was intubated with tap water for six weeks and served as control group(G1), mice in the second group were intubated orally with 100 mg/kg B .W of methionine (G2). While animals of the third group were intubated with 0.07 mg/kg B.W of folic acid (G3), mice in the fourth group (G4) were intubated orally with 0.07 mg/kg B.W of folic acid in addition to methionine , animals of the last group (G5) were intubated with 100 mg\Kg B.W. iron .

Fasting blood samples were collected by cardiac punctures technique at six weeks of experimental for measuring :A- biomarker of oxidative stress including serum Glutathione (GSH) and Malondialdehyde (MDA) concentrations. B- serum Alanine Aminotransferase (ALT) , Aspartate Aminotransferase (AST) and Alkaline phosphatase (ALP) activities , albumin and globulin concentrations. C - serum Iron ,Ferritin and transferrin concentration and level of the hormone hepcidin, furthermore kidney and hepatic tissue were assessed for histopathological changes , and a sample of the liver was taken to measure gene expression, the results revealed to a significant ($p<0.01$) increase in MDA concentration in G2 and G5 Compare to control and folic acid treated groups and a significant decrease ($p<0.01$) in G3 and G4 , present a significant decrease ($p<0.01$) in serum GSH in G2 , G5 . A significant ($p<0.01$) increase in serum GSH in G3 and G4 . And a significant decrease ($p<0.01$) in albumin , globulin concentrations in G2 and G5, also showed a significant ($p<0.01$) increase in ALT, AST and ALP activities in G2, G5 and a significant decrease ($p<0.01$) in G3 , appear a significant increase ($P<0.01$) in serum Iron , Ferritin and Transferrin in G2,G5 and appear a significant increase ($P<0.01$) in level hepcidin hormone and bilirubin concentration in (G2 , G5) , our result appear a significant decrease ($p<0.01$) in RBC , Hb and PCV in G2 and G5 Compare to control. In

our study was found the most common gene expression (HAMP) in the group with iron overload G5 rather than other groups . The present study finds decrease of gene expression in the methionine group G2 and folic acid group G3 Compare to the control group . The results of the current study showed intubating mice with methionine and folic acid G4 for six weeks no difference in gene expression compare to the control group, the results shows that there is a damage effect on liver and kidney tissue in groups exposed to methionine showed dilatation in central vein and dilatation in sinusoid aggregations inflammatory cells, degeneration in tunica intima of central vein and hyperplasia Kupffer cells degeneration and hypertrophy in hepatocytes, disappearance of arrangement of the hepatic cords and infiltration of mononuclear inflammatory cells in the portal and pyknotic nucleus in hepatocytes , also showed demonstrated glomerular atrophy, enlargement of the urinary space, distal tubules atrophy, the change in the shape of the cells lining the tubules where their normal shape is cuboid, but in the case of methionine overload the shape became flat, as for the proximal tubules, there is no noticeable change, but note that the color of the cytoplasm has become blue, evidence of damage to the kidney tissue. As it has become basal,Hyaline cast accumulation ,and the presence of congestion in the artery and the collection of mononuclear inflammatory cells and hypertrophy of the tunica intima , sections in the liver of animal treated with (methionine and folic acid) showed infiltration of mononuclear inflammatory cells in per portal area but less than the liver in the case of methionine , decreased hepatocyte , Kupffer cell degeneration. It was observed that the kidney tissues returned to normal and the kidney healing was observed, as mononuclear inflammatory cells invaded and glomerular atrophy was observed, but at a lesser rate than the atrophy case in the methionine group only and the cytoplasm of cells in the proximal tube is pink (acidic) Closer to natural, in the case of 0.07mg/kg B.W. of folic acid showed not accumulation of Hyaline cast Compared with the kidney of animal treated with 100mg/kg B.W methionine .

In conclusion, the results of this study confirm the protective role of folic acid against deleterious effect of liver and kidney caused by methionine overload in male mice.

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List of Abbreviations

Abbreviation	Meaning
AdoMet	S-Adenosylmethionine
ALP	Alkaline Phosphatase
ALT	Serum Alanine transaminase
AST	Serum Aspartate transaminase
BHMT	betaine-homocysteine methyltransferase
CBS	cystathionine β-synthase
CSE	cystathionine γ-lyase
CVD	Cardiovascular diseases
DPX	Dextrin plasticizer xylene
EDTA	Ethylene diamin tetraacetic acid
GSH	glutathione
H&E	Hematoxylin & Eosin
HAMP	Hepcidin antimicrobial peptides
Hb	Hemoglobin concentration
hcy	homocysteine
HHcy	hyperhomocysteinemia
MAT	Methionine Adenosyl Transferase
MAT	methionine adenosyltransferase
MDA	Malondialdehyde
Met	methionine
MS	methionine synthase
MTHFR	Methylene tetra hydrofolate reductase
N-5- MTHF	N-5-Methyltetrahydrofolate
PCV	Packed cell volume
RBC	Red blood cell count
ROS	reactive oxygen spices
SAH	S-Adenosyl homocysteine
SAM	S−Adenosyl Methionine
tHcy	Total homocysteine
THF	tetra hydrofolate
vit	vitamin

Chapter One

Introduction

Introduction

Methionine is defined as a basic sulfur that contains an important amino acid and it is necessary for many vital activities in the body .The common natural sources of this amino acid have traditionally been fish and meat meal, especially for starter chicks and broilers (Hoeler and Hooge , 2003), rice and casein offer potential novel available sources of methionine (Lewis and Baley, 1995).

In addition to the fish meal, the National Research Council recorded 1994 that the crab meal, sunflower seed meal, and corn gluten meal were feed sources with high proportions of methionine (Shoob *et al*., 2001;Salman , 2014).

The daily amount of methionine that the body needs is 13 mg / kg or one gram per day for adult humans and is used in pharmaceutical preparations or food supplements . It is an important amino acid that is included in the poultry diet (Xie *et al*., 2007) , considered a preventive agent for many tissues damage (Rishi and Subramaniam ,2017).

Methionine is used in the medical field to treat medical conditions such as dissolving or preventing the formation of kidney stones, uroliths , urologic syndromes (Fuenfstuck *et al*.,1997) . Also, methionine is used in cases of hepatic lipidosis in treatment or prevention because of its need to mobilize and transfer fat in the body (Van Saun *et al*.,2000).

The active form of methionine is S-adenosyl methionine (SAM) is acts as a major biological donor to methyl and it is important in many cellular processes including the formation of nucleic acids, melatonin, proteins, creatine, phosphatide choline and epinephrine(Stipanuk, 2004).

Scientists have found in a number of researches that methionine supplementation has an effect and benefit in reducing immunological stress (Schroecksnadel *et al* ., 2006).

Although nutritionally necessary, it is more toxic to human, animal and poultry tissues in the event of excessive methionine feeding , in the case of an excess of methionine in food, one of the factors triggering a disorder of homocysteine metabolism and the accumulation of homocysteine in large amounts triggered by a case of hyperhomocysteinemia (HHcy) and other pathological conditions ,the risk factor for digestive system, neurological diseases, strokes, arteriosclerosis, coronary artery inflammation and myocardial infarction involves congestive heart failure and coronary artery disease (Hemanth *etal* .,2017; Barroso *etal*.,2017) as well as Alzheimer's disease (Fernández-Rodríguez *et al*. 2016) and type II diabetes,and causes oxidative stress when accumulating methionine receptors in the case of excess methionine intake and iron regulation in the body later affected (Fukagawa, 2008 ;Al-Hashmy and Khudiar, 2009 ; Micovic *et al*., 2016; Tinelli *et al*., 2019;Al-Okaily *et al* .,2019).

The role of Hyperhomocysteinemia in the induction lipid peroxidation and oxidative damage through way of hydrogen peroxide (H_2O_2) (Delvin *etal* ., 2007) leading to occurrence of different disease condition (Jamison *et al* ., 2007; Tounyz and Schiffrin,2008; Škovierová *etal*.,2016).

iron produces homocysteine from cystathionine, S-adenosylhomocysteine and methionine leading to increases the oxidation of lipoproteins of small density and free oxygen radicals (Micovic *et al*.,2016 : Baggott and Tamura, 2015).

Hepcidin is an important peptide hormone It is excreted from the liver, it is an antimicrobial and plays a major role in iron balance in the body in various conditions, including inflammation, hypoxia, erythropoietic activity, and iron states(Masaratana *et al.,* 2013),the production of Hepcidin decrease in cases of hypoxia and anemia and increases dramatically during inflammation and increased iron intake (Ganz, 2006; Rishi and Subramaniam, 2017).

Hamp1 is the gene responsible for the production of Hepcidin, which regulates and secretes the Hepcidin hormone , when the liver senses high amounts of iron, Hamp gene begins with gene expression and production of hepcidin (Sangkhae and

Nemeth ,2017) , which in turn prevents the absorption of iron from the intestine by inhibiting the iron transporter ferroportin on duodenal enterocytes (Miranda *et al.,* 2019; Yamauchi *et al*., 2019).

Folic acid is a vitamin of the B- complex group and has other synonymous names,which is a water-soluble vitamin (Marry, 2001), folic acid is absorbed in the intestine, is derivatives in food, it is absorbed by specific intestinal enzymes and converted into monoglutamyl folate for absorption (Winkels *et al*., 2007).

In it, folate and vitamin B12 or betaine is required, thus three B-group vitamins are involved in the metabolism of Hcy, and deficiencies in the respective vitamins have been reported to cause hyperhomocysteinemia (Yamamoto *et al* .,2012 ; Belcastro and Striano, 2012; Rajagopal *et al*., 2019).

The protective effects exerted by administration of antioxidant and cofactors (Fernandez-Robredo *et al*., 2005; Al-Okaily *et al* ., 2015; Vijayakumar *et al* .,2017 ; Minich and Brown, 2019), Because of the cell culture and some in vivo studies showing that methionine overload lead to considerable toxic effect mediated by reactive oxygen spices (ROS) formation, Santilli *etal* (2016) suggested that the toxic effect of methionine overload could be treated by natural antioxidant ,like folic acid.

Therefore the experiment was designed to investigate the effect of methionine overload on the liver and kidney, and to study the preventive role of folic acid against adverse effect of overload of methionine by measuring the following parameters :

1-Physiological parameters .

- Measuring the level of the hormone hepcidin.
- Measuring the Serum glutathione GSH concentration.
- Measuring the Serum Malondialdehyde (MDA) concentration .
- Measuring the Serum Globulin and Albumin concentration.
- Measuring the Serum Aspartate transaminase (AST) , Alanine transaminase (ALT) and Alkaline Phosphatase (ALP) activities .

- Measuring the serum Iron ,Ferritin and transferrin.
- measuring serum bilirubin concentration.
- Measuring the level of Red blood cell count , Hemoglobin and Packed cell volume.

2- A histological study to know the changes taking place in the liver and kidney tissue of experimental animals during treatment with overload .

3- Genetic study to determine the changes in the gene expression level of the gene Hamp 1, which is responsible for the production of the hormone hepcidin , through the use of technology RT- PCR

Chapter Two

Literature Review

2 - Literature Review

2.1.Methionine

Synonyms : DL-methionine, D- methionine, L-methionine, Met.

The chemical names for methionine: - 2-amino-4-methyl thiobutyric acid

Or α-amino-γ-methyl mercaptobutyric acid.

ID of the International Union of Pure and Applied Chemistry: 2-amino-4-(methylthio)butanoic acid.

The chemical formula for methionine is ($C_5H_{11}NO_2S$)

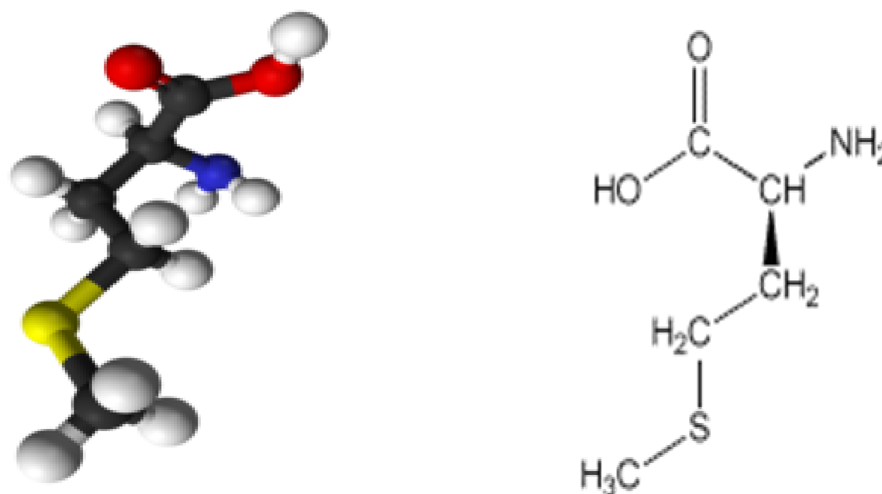


Figure (2-1) chemical structure of methionine (Shoob *et al*., 2001)

Methionine is defined as a basic sulfur that contains an important amino acid and it is necessary for many vital activities necessary in the body, methionine is considered a preventive agent for many liver damage, the daily amount of methionine that the body needs is 13 mg / kg or one gram per day for adult humans (Salman, 2014).

Methionine present in two biologically inactive stereoisomers D-methionine where L-methionine is biologically active for a wide variety of therapeutic purposes, including parenteral nutrition, pharmaceutical adjuvant, electrolyte and pH balance, and other applications (Fufagawa, 2008) .

Studies have also prover that methionine is important for the body but also that high levels of methionine in the food cause damage to the erythrocyte membrane and also trigger delays in tissue growth and injury, steatosis, hepatitis, hypertriglyceridemia and hepatotoxicity (Balkan *et al*.,2004).

It is intracellularly converted to S-adenosyl methionine (SAM), the main biological methyl donor necessary for numerous cellular processes, including nucleic acid formation, melatonin, creatine, epinephrine, phosphatidylcholine and proteins (Al-Hashmy and Khudiar, 2009).

2.1.1. properties of Methionine:

Methionine is a colorless or white crystalline powder soluble in water, mineral acids and alkaline solutions Slightly soluble in alcohol and insoluble in ether, with a slight odor (Hoeler and Hooge, 2003).

2.1.2. Sources of methionine:

The amino acid methionine is naturally present in animal-derived proteins such as beef, fish , poultry, eggs and cheese, it is also present in juices, vegetables and sunflower seeds, crab meals, blood meals, gluten meals and tormented foods, as well as in fruit and casein and rice, which have been established as new sources of methionine or can be developed industrially in pharmaceutical preparation and nutritional supplements (Al-Hashmy and Khudiar ,2009 ; Salman , 2014).

2.1.3. Homocysteine (Hcy)

Homocysteine (Hcy) is a sulfur-containing amino acid formed in the metabolic pathway between methionine and cysteine (Dhonukshe-Rutten *et al*., 2005; Zidan and Elnegris, 2015), and is semi-essential (not forming proteins) (Jordao Júnior *et al*., 2009; Micovic *et al*., 2016), natural homocysteine content ranges from 5-15 μmol / L (Schwab *et al*., 2006) , is metabolized by the two pathways transsulfuration or remethylation , abnormalities of this pathway lead to hyperhomocysteinemia (Brustolin *et al*., 2010).

Elevated levels of Hcy known as hyperhomocysteinemia (HHcy) are associated with various disorder like cardio vascular disease , Alzheimer's disease and bone abnormalities: such as osteopenia and osteoporosis (Dworakowski *et al*., 2006; Ciaccio and Bellia, 2010; Zidan and 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)

Homocysteine is eliminated from the body by converting it into 1-cystothionine through a vit B6 catalyzed reaction and into B9 and B12 catalyzed 2- methionine (Tug *et al*.,2003).

2.1.4. 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 S−Adenosyl Methionine (SAM) which is the most important methyl group donor in biologic methylation and a compound with high-energy that is unusual in that it contains no phosphate (Di Pasquale, 2007; 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 and 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 glutathione (GSH) (Dahlhoff *et al*., 2013; Denise, 2014).

The Remethylation pathway

Is converts Hcy to methionine in this process, Hcy accepts a methyl group from N-5-Methyltetrahydrofolate (N-5- MTHF): is the most biologically active form of the B-vitamin (vit) known as folic acid. It also known generically as 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, firstly tetra hydrofolate (THF) is converted to N-5, 10-methylenetetrahydrofolate by vit B6 dependent serine hydroxyl methyltransferase later, it is reduced to N-5-MTHF by methylene tetra hydrofolate reductase (Bailey and Gregory, 1999; National Center for Biotechnology Information, 2019).

In this reaction Vitamin B12 removes the methyl group from N-5-MTHF and produces THF (Goljan ,2011). 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).

N-5-methyltetrahydrofolate 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 (Castro *et al*., 2006; Škovierová *et al*., 2016).

In fact, around 70% of generated Hcy comes from the Remethylation step depending on the content of Met and choline in the diet (Bailey and Gregory, 1999; Tinelli *et al*., 2019).

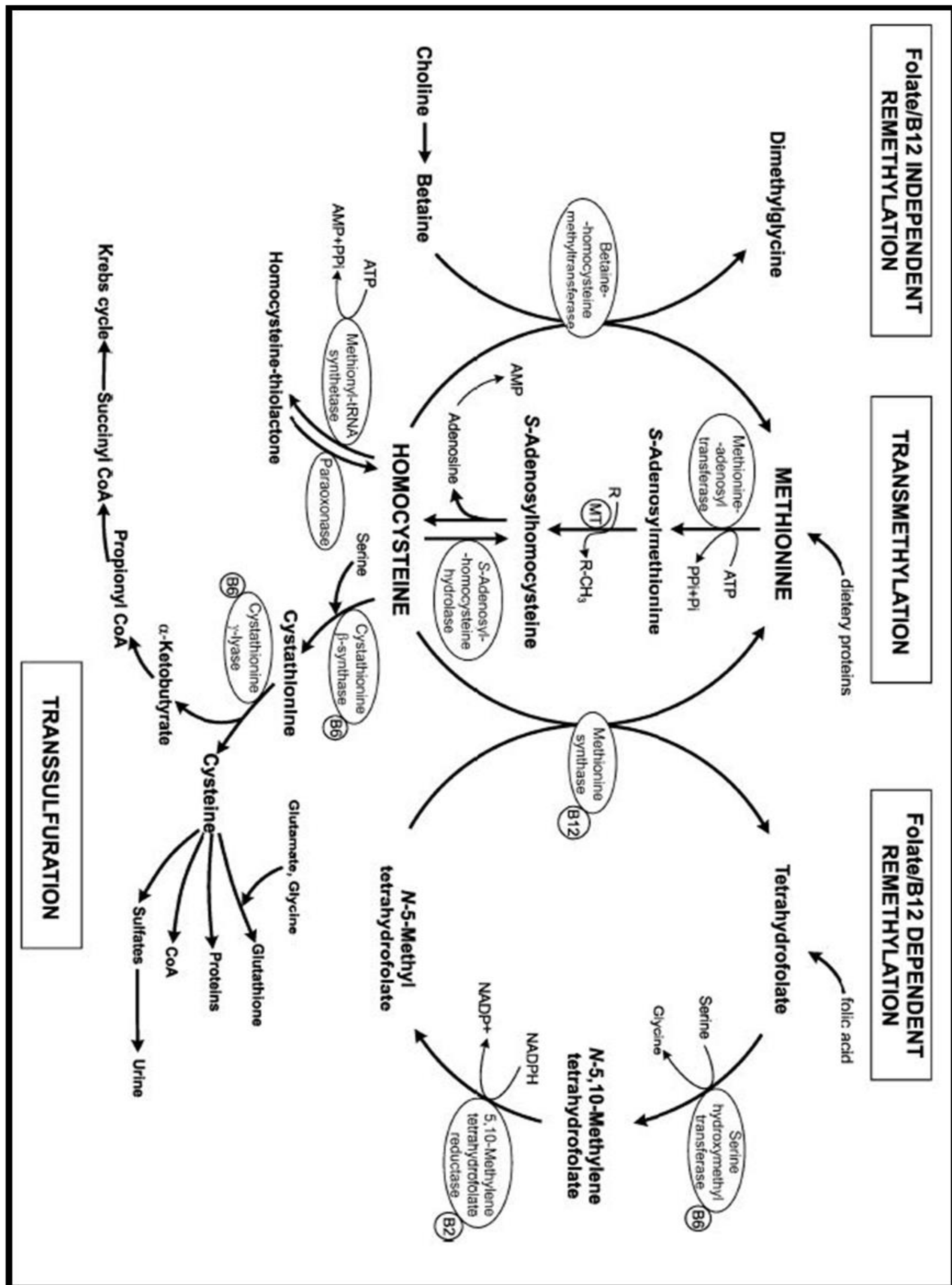


Figure (2-2). The schematic overview of homocysteine metabolism and its relationship with folic acid and vitamins. ATP: adenosine triphosphate; AMP: adenosine monophosphate; PPi: pyrophosphate; Pi: orthophosphate; B2/B6/B12: vitamins B2/B6/B12; CoA: coenzyme A; R: acceptor; R-CH3: methylated product; MT: methyltransferases (Škovierová *et al*., 2016)

Transsulfuration pathway

Transsulfuration of Hcy to cysteine is catalyzed by two vit B6-dependent enzymes, cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE), it has show in Figure (3), CBS catalyzes the condensation of Hcy and serine to form cystathionine, cystathionine is then hydrolyzed by CSE to form cysteine (now containing the sulfur atom from Hcy) . And α-ketobutyrate (from the Hcy carbon chain) plus ammonia (from the amino group of Hcy), 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, oxidative decarboxylation of α-ketobutyrate can be catalyzed by pyruvate and branched-chain keto acid dehydrogenase complexes. 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 and Ueki, 2011; Belalcázar *et al*., 2014; Kumar *et al*., 2017).

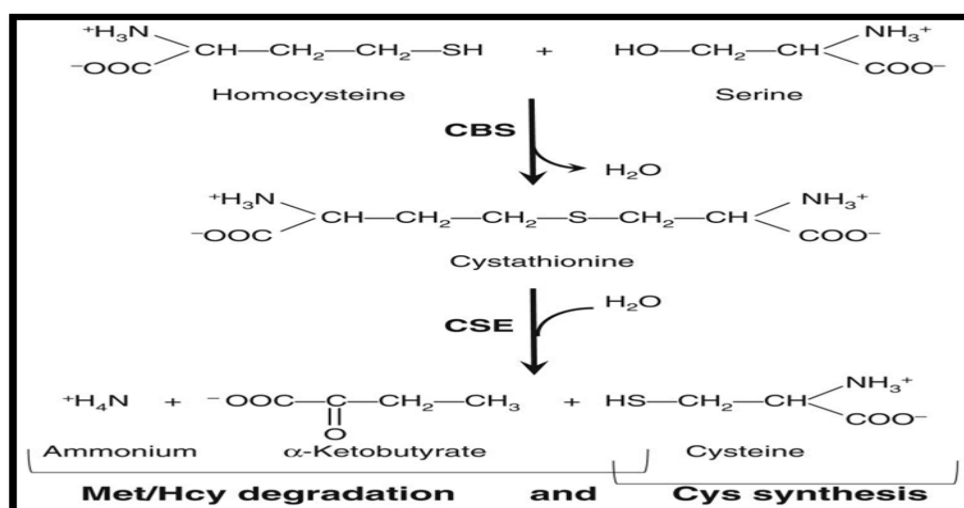


Figure (2-3) Transsulfuration pathway for homocysteine degradation and cysteine synthesis (Stipanuk, and Ueki, 2011)

2.1.5. 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 Hcys metabolism such us Methylene tetra hydrofolate reductase (MTHFR), CBS, Methionine synthase, Methionine synthetase reductase, and Methionine adenosyl transferase (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 recently shown to be an important modulator of Hcys concentration, especially in individuals with TT genotype (Wilson *et al*., 2010).

The MTHFR gene C677T strongly influences the levels of the Hcys 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 trans-sulfuration 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 production, induced by immune system activation, involves a greater demand for antioxidants, such as vitamin 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 Hcys 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, before fortification, folic acid deficit was present in 2.3% of school-age children, 24.5% of adults and 10.8% of the elderly population of the United States, 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 (Selhub *et al*., 1993;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).

2.1.6.Hyperhomocysteinemia as a risk factor

If high Hcy in the blood plasma suggests weakness in cell metabolism and high Hcy is a risk factor for heart disease and atherosclerosis, it is anticipated that changes that occur in Hcy in cases of liver damage, in people heavily addicted to

alcohol, and even in people suffering from alcohol cirrhosis (García-Tevijano *et al* .,2001; Al-Bazii , 2009) .

But in the case of an excess of methionine in food, one of the factors triggering a disorder of homocysteine metabolism and the accumulation of homocysteine in large amounts triggered by a case of hyperhomocysteinemia (HHcy) and other pathological conditions ,the risk factor for digestive system, neurological diseases, strokes, arteriosclerosis, coronary artery inflammation and myocardial infarction involves congestive heart failure and coronary artery disease as well as Alzheimer's disease and type II diabetes and homocysteine (Al-Hashmy and Khudiar, 2009 ; Micovic *et al*., 2016).

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 and 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 W.H.O agreed to consider HHcy a strong contributor for cardiovascular disease (Tinelli *et al*., 2019).

High homocysteine is a contributing factor for brain injuries, strokes, arteriosclerosis, inflammation of the coronary arteries and myocardial infarction (Al-Beer *et al*.,2013; Sirdan *et al* 2014).

It was 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 and Striano, 2012; Ni *et al*., 2014).

It has been found that people with schizophrenia have elevated levels of hcys in the blood, and excessive amino acid in the plasma is considered a risk factor for the central nervous system, and when studying the levels of hcy in people with schizophrenia and mental retardation, it has been found that the levels of hcy in males are higher than in females, and that the level of hcy increases, this causes cardiovascular disease, the number of deaths in patients with schizophrenia is therefore growing due to heart disease and the detrimental impact of hcy on the nervous system, as it acts to promote programmed cell death and neuronal DNA damage (Diez *et al*., 2005; Akanji *et al* .,2007).

High deficiencies in homocysteine and vitamin B9 and B12 aid weaken the central nervous system and also function on depressive disorders (Brustolin *et al*., 2010).

It was found that HHcy induce congenital defects include Down syndrome, congenital heart defect, neural tube defect and nonsyndromic oral clefts (Vollset *et al*., 2000; Perna and Ingrosso, 2016; Škovierová *et al*., 2016).

As homocysteine levels increase, cell damage is caused by high free radicals and oxidative stress and therefore cell apoptosis, because oxidative stress is the main cause of apoptosis (Mangiagalli *et al*., 2004).

Huang *et al* found that high homocysteine increases the production of H_2O_2 and homocysteine is toxic and affects endothelial cells due to high levels of H_2O_2, as it acts negatively on antioxidant defense systems and is also associated with an increase in malondialdehyde levels and the loss of vitamin B from the liver increases oxidative stress (Huang *et al*., 2001).

Total homocysteine (tHcy) levels in people with all forms of diabetes and those with nephropathy have elevated tHcy levels relative to diabetics without nephropathy (Wijekoon *et al*., 2005).

Reviewing the studies revealed that the increased evidence that oxidant stress caused by Hcy can induce insulin resistance, in addition, in vitro studies have recently found that oxidant stress by interrupting insulin signals reduces insulin responsiveness, in addition, another in vivo study highlighted the fact that the rise in Hcy levels may not be a general complication of the syndrome of insulin resistance, as healthy volunteers with altered insulin-mediated glucose disposal have no increased Hcy levels (Abbasi *et al*.,1999).

Folate deficiency and HHcy effects on oocyte quality and maturation, implantation, placentation, fetal growth, and organ development, correlating with sub fertility and negatively correlated with embryo quality (Ebisch *et al*., 2006; Boxmeer *et al*., 2009).

The HHcy is a newly recognized as risk factor for osteoporosis (Behera *et al*., 2017).

The elevated level of homocysteine in pregnant women results in a reduction in the total surface of the blood stream, resulting in endometriosis disorder (Tug *et al*.,2003), homocysteine homeostasis was found to be partly regulated by dietary (vitamin B12, vitamin B6 and folate) and hormonal and genetic factors through a variety of experiments (El-Saleh *et al*.,2004) .

Several studies have shown that the level of hcy increases in postmenopausal women and polycystic ovarian syndrome, suggesting that hcy is correlated with the condition of estrogen (De Leo *et al*., 2000) .

The HHcy plays 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/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) , researchers find that infant mortality rises during the first weeks of pregnancy due to congenital malformations and coronary artery disease, and found that high hyperhomocysteinemia is a risk factor for pregnant women and raises the chance of having children with heart disease and heart defects (Brustolin *et al*., 2010).

One research dealt with vegetarians who consume a plant protein in which homocysteine is converted via the retransformation process, who require methyltetrahydrofolate as a substratum, and who use B12 as an supplement when a vitamin B12 deficiency is either absent or inadequate, as a vegetarian susceptible to hyperhomocysteinemia as a result of consuming large amounts of vegetables and fruits is rich in vit B9, however, it is low in vit B12, which is only available in meat in its most essential form, so the percentage of hyperhomocysteinemia increases so that the risk of developing these people increases with Vascular and cardiac diseases and endothelial injury (Ambroszkiewicz *et al*., 2006).

High homocysteine enhances the development of collagen in smooth muscle cells and DNA synthesis (Tsai *et al*.,1994; Majors *et al*.,1997).

Taking methionine over long periods has a detrimental effect on iron regulation and its metabolism, and the related proteins contribute to a decrease in blood serum levels of transferrin and iron (Mori and Hirayama, 2000).

Iron produces homocysteine from cystathionine, S-adenosylhomocysteine and methionine and increases the oxidation of lipoproteins of small density and free oxygen radicals (Micovic *et al*.,2016).

The researchers found that the association between homocysteine and iron was positive, researchers found that when a group of healthy people got iron researchers had a high level of homocysteine, there is proof that complete homocysteine is indirectly linked in plasma and iron stores, evidence and data also suggest that

methionine is rendered homocysteine, which is based on iron (Baggott and Tamura , 2015).

Numerous studies have shown that people who smoke have higher homocysteine relative to non-smokers because smoking increases oxidative stress and the degradation of antioxidants, and thus high hyperhomocysteinemia (Brustolin *et al*., 2010) , and that excessive alcohol intake results in metabolic disorder and also leads to high hcy (Huliberg *et al*., 2005), and that consuming coffee in large amounts on the same day often raises the amount of hcy in the blood plasma. (Verhoef *et al* ., 2007) , eating such pharmaceutical items on an ongoing basis results in the absorption of folate and a metabolic condition such as metformin, sulfasalazine, methotrexate and oral contraceptive, which also results in a high percentage of hcy (Norri *et al*. , 2001; Desouza *et al* .,2002; Westphal *et al* .,2003).

2.1.7.The effect of methionine overload administration on the liver.

Methionine is a protective factor and lipotropic agent against various kinds of hepatic damage and improves bile flow (Sahi *et al*., 2006).

Methionine is beneficial for the liver, but for dietary excess methionine, liver damage due to methionine overload leads to increased levels of aspartate amino transferase (AST) , alanine aminotransferase (ALT) and alkaline phosphatase (ALP) in blood circulation due to cell death and injury, studies have shown that overdose of methionine results in multifocal granulomatous lesion characterized by mononuclear aggregations in the blood vessel and parenchyma in the liver and hepatocytic necrosis (Al-Bazii , 2009 ;Al-Shammry and Al-Okaily, 2009).

Patients with liver disease also with high blood homocysteine may work to inhibit cell proliferation and produce hypomethylation, leading to DNA destabilization in these patients and increased lipid peroxide production, collagen synthesis and decreased glutathione in the cells (Ferré *et al*., 2002).

The researchers found that HHcy causes several liver disorders including hepatic compromise, cirrhosis of the liver, fat accumulation, and necrosis of the liver,signs of necrosis include high levels of AST and ALT, high levels of

inflammatory cells and collagen, where they accumulate in the tissues, and high levels of lipid peroxidation (Matte *et al*., 2009) .

2.2. folic acid

Folate is defined as a water-soluble vitamin of the complex group B and has other equivalent names such as (vitamin B9, pteroylglutamic acid , Folic acid) .

Basically, Vitamin B9 is made up of the base pteridine attached to one molecule each of P-aminobenzoic acid and glutamic acid. In plants, folic acid exists as a polyglutamate conjugate consisting of γ-linked polypeptide chain of 7glutamate residues (Al- Bazii,2009).

In 1941 they discovered a compound which was known to be useful in the treatment of anemia and extracted from spinach leaves called folic acid (De -lea *et al* ., 2002) .

Folate derivatives are cleaved to monoglutamyl folate for absorption by different intestinal enzymes in the diet, most of this is reduced by the enzyme folate reductase to tetrahydrofolate (H4folate) in the intestinal cells, which uses NADPH as a donor to minimize equivalents. Polyglutamate tetrahydrofolate are potentially the actual coenzymes in the tissue (Winkels *et al*., 2007).

Vitamin B9 is absorbed by the small intestine and then passes through proteins into the bloodstream, the body uses what it needs and what is added is excreted through the urine, and the body stores a large amount of folate in the liver and other tissues around 7 mg where the body can use this stock for a few months in case of folic acid deficiency (Al- Bazii,2009).

Folic acid is essential to sustain all cells as critical co-enzymes of normal DNA and RNA synthase for natural growth and participates in many important metabolic processes in the body and is essential for nucleic acids as it protects the protection of cell division and maintains genetic codes (Stopper *et al* ., 2008) , red blood cells and white blood cells must also be developed and matured, as well as protein metabolism and energy production (Ambrosczhiemicz *et al* ., 2006) .

It maintains the roles of the digestive system and the nervous system and creates neurotransmitters such as serotonin, which regulates mood, appetite and sleep by the presence of folic acid, it is also used by the body to break down and synthesize amino acids Precursor to DNA remythelation and can have an effect on mood by lowering homocysteine levels in the blood (Al- Bazii,2009).

Folic acid alone or folic acid combined with other B-vitamins have all been shown to reduce tHcy concentration in patients on chronic treatment with antiepileptic drugs(Belcastro and Striano, 2012).

Folate and vitamin B12 or betaine is required, thus three B-group vitamins are involved in the metabolism of Hcy, and deficiencies in the respective vitamins have been reported to cause hyperhomocysteinemia, in addition, dietary methionine content is reported to be another parameter in hyperhomocysteinemia , Yamamoto *et al* have reported that vitamin B6 deficiency in a 70% casein diet did not always cause severe hyperhomocysteinemia in rats, thus the way methionine levels affect Hcy levels, under B6-deficient conditions is an issue of concern, the involvement of the three B-group vitamins in Hcy metabolism led us to speculate that additional dietary folate should contribute toward improving hyperhomocysteinemia under B6-deficient dietary conditions (Yamamoto *et al* .,2012) .

2.2.1. Effect of folic acid on body system

When menopause in women is marked by a physiological rise in Hcy levels and the weakening of various metabolic processes, and by taking folate supplementation it enhances the hepatic metabolism processes Perhaps by introducing general improvements in the processes of catabolic and anabolic states Vitamin B9 improves insulin sensitivity and lipid metabolism in women after menopause (Villa *et al* .,2005).

Studies have shown that vitamins and folic acid play a role in mitigating damage to poultry from heat stress and environmental stress (Gursu *et al* .,2004).

Folic acid is an important vitamin cofactor needed to re-methylate Hcys to methionine in the metabolism of Hcys ,research has been carried out on women with

diabetes after menopause and found that after giving them folic acid, there was a decrease in Hcy levels and an improvement in the health of the blood vessels and the heart, as well as an improvement in lipid levels where lipid parameters were lowered while using folic acid (Vijayakumar *et al* .,2017).

Using vitamin B has lowered growth hormone levels in obese children with elevated growth hormone levels (Peña *et al* .,2007).

It was found that consuming vitamin B12 with B6 and B9 decreases the effect of hyperhomocysteinemics due to methionine deficiency and that using such vitamins together is better than using folic acid alone, and that vitamin B 9 is better than B6 or B12 when used separately to decrease Hhcy (Al-Beer *et al*.,2013).

Folic acid decreases blood homocysteine levels and reduces the risk of heart disease and is also essential for cell proliferation in the fetus and works to reduce homocysteine levels through vitamin B12 work in combination with vitamin B9 to turn homocysteine into methionine (kerkeni *et al* ., 2006).

Clinical studies indicate that vitamin B9 has an impact on cancer and its effect depends on the timing and dosage of the drug and that it has been found to protect and strengthen against rectum and colon cancer, folic acid is common in patients with chronic liver disease as vitamin B is used as a compulsory addition to food in Canada and the United States, neural tube deficiencies recorded in newborns decreased, using folic acid with CCl_4 affects liver function and related diseases (Marsillach *et al* .,2008).

Paracetamol is a widely used analgesic antipyretic medication, but it is generally considered a safe medication in large single-dose ingestion as an intentional overdose that causes severe hepatic necrosis and prolonged fasting that lead to hepatotoxicity and cute liver failure, the researchers found that the use of folic acid in cases of paracetamol-related hepatotoxicity using vitamin B9 to play a preventive role is of great benefit in reducing and treating the harm done by acute paracetamol overdose (Al-Sowyan,2009).

2.3. iron

Iron is an essential element for microbes, plants and higher animals, as it plays an essential role in many pathways of integrative metabolism, as it works on blood formation, energy metabolism, DNA synthesis, or oxygen transport , mitochondrial biogenesis , oxidative phosphorylation, and other enzymatic functions , when an overabundance of iron in the body causes a disease called hemochromatosis, where iron atoms work to obtain Fenton interactions and caused the production of toxic reactive oxygen species, and when a shortage in the amount needed by the body occurs anemia ,when iron is deposited in the liver in abnormal amounts, cases of cirrhosis and chronic hepatitis occur (Anderson and Shah, 2013;Yamauchi *et al*.,2019), and iron is the most abundant metal in the human body and it is an essential element for all life forms, iron (Fe) has important functions in the body as a component of hemoglobin and numerous other iron containing proteins such as myoglobin and cytochrome-C, iron is stored mainly in the liver within the iron storage proteins, ferritin and hemoglobin (Umran *et al*., 2013).

The human body has an average of 2–4 g of iron, of which 80% is bound in hemoglobin, there is tight control of systemic iron levels by means of iron absorption, storage, and recycling, iron deficiency causes a decrease in hemoglobin production and can consequently lead to anemia, while iron overload burdens the body and results in excess tissue iron, which can cause cell damage(Muñoz *et al*. ,2009; Wang and Pantopoulos, 2011).

The daily losses of iron, 1–2 mg in adults, represent <0.1% of the 3–4 g of total iron in the human body, and must be replaced from dietary sources to maintain iron balance, iron losses occur predominantly through desquamation of epithelial cells in the intestine and the skin, and through minor bleeding, importantly, the iron loss cannot substantially increase through physiologic mechanisms, even when iron intake and stores are abundant ,most of the iron in the body is in hemoglobin of red cells, which contain about 1 mg of iron per milliliter of cells, or about 2– 2.5 g of iron total, in contrast, blood plasma contains only 2–3 mg of iron, bound to

transferrin, the plasma iron carrier that is the exclusive source of iron for erythropoiesis, the life span of human erythrocytes is about 120 days, so every day the oldest1/120 of erythrocytes are degraded by macrophages and their iron content is returned to plasma transferrin, the recycling of erythrocytes generates a stream of 20–25 mg of iron a day, causing plasma iron to turn over every two hours or so, most of the iron-transferrin is destined for erythrocyte production in the bone marrow; other cells contain and require much less iron, with some able to utilize non-transferrin-bound iron as well (Ganz and Nemeth, 2011).

Iron is an essential element and its deficiency is a nutritional problem in both developed and developing countries, consequently, many types of dietary iron supplementation are used, some researchers have suggested that the general population is consuming excess iron due to these supplements, they found that several types of diseases are provoked by short- or long-term exposure to iron in quantities above the capacity of the organism to protect itself against iron’s reactivity and that iron’s role in pathological processes is related to its ability to catalyze reactions that lead to the formation of oxygen free radicals(Weinberg , 1990 ; Crawford , 1995).

The majority of functional iron within the body is present in haem proteins, such as hemoglobin ,myoglobin and cytochromes, although iron is an essential nutrition element for all lifes forms, iron overload may lead to various diseases caused by the accumulation of iron in the body (Patterson and Marschall , 2006) , iron as we know stored mainly in the liver within the iron storage proteins ,ferritin and hemosiderin ,many of key biological functions of iron in living system rely on the high redox potential, in contrast ,other researchers have reported that parenteral iron overload in rats increased the liver iron content mainly by deposition of iron in mitochondria that affected on oxygen transport or mitochondria electron as potentially harmful in terms capacity of oxidative damage to cellular component such as fatty acid ,proteins and nucleic acid (Umran *et al.* ,2013).

For most living organisms, iron is essential but potentially toxic, making the maintenance of systemic iron homeostasis critical, this homeostasis is orchestrated by the hormone hepcidin ,which regulates the amount of the cell membrane iron exporter ferroportin , hepcidin binds to ferroportin ,inducing its degradation and leading to decreased iron availability and hypoferremia, hepcidin regulation is complex and depends on various signals, among the most important is body iron level (both circulating and intracellular iron) that upregulates hepcidin through the bone morphogenic protein6 (Willemetz *et al*.,2014).

2.3.1. iron overload

Iron overload is a serious chronic condition that develops when your body absorbs too much iron over many years ,when the body stores excess iron, this is referred to as iron overload, iron overload can have many causes, the most common being genetic, other causes include too much iron in the diet (particularly from supplementation, chronic transfusion therapy, iron injections, chronic hepatitis, and other disorders , and Organs commonly affected by iron overload disorders are liver, heart, endocrine glands ,pancreas ,bones and joints (Umran et al., 2013).

Term iron overload represent a condition result from increase total iron store in the body which cause impaired in organs function , iron is take up from food in duodenum and superior part of jejunum, utmost food have two essential types of iron: ferrous iron protoporphyrin from the red meat and ferric iron from grain and vegetable, living beings have developed mechanism to keep iron homeostasis, comprising the planned direction of iron ingestion, reusing iron and utilization of iron stored, in any case, in spite of these mechanisms, creatures have a restricted capacity to discharge abundance iron, likely because of absence of developmental powers for this ability , the iron is lost from body through uncontrolled losses of the blood normally gastrointestinal, additionally huge iron lost via menstrual bleeding in ladies of kid bearing age, kidney epithelial cells, skin and by excretions for instance sweat, tears and gastrointestinal discharges (Takea and Mhsen, 2019).

Iron overload indicates accumulation of iron in the body due to any cause hemochromatosis, hemochromatosis is mostly defined as iron overload with a hereditary/primary close (Pietrangelo and Antonello , 2010) or originating from metabolic disorders, in general ,the term hemochromatosis is used to indicate the pathological effect of iron accumulation in any given organ, organs commonly affected by hemochromatosis are the liver ,heart and endocrine glands and on longer term lead to various diseases (Umran *et al*., 2013 ; Miranda *et al*., 2019).

The metabolism of iron is therefore tightly regulated to prevent tissue damage, however, iron overload can occur in subjects with genetic disorders such as hereditary hemochromatosis and beta thalassemia, or secondary to iron overload during blood transfusion and hemolysis (Fleming and Ponka , 2012).

Excessive concentration of iron in cells induces oxidative stress with per oxidative decomposition of polyunsaturated fatty acids in membrane phospholipids, thereby altering vital organelle integrity and cell function (Philippe *et al*.,2007).

Iron excess has been linked to risks of development of certain chronic disorders such as diabetes, glucose intolerance, and cardiovascular diseases , high intracellular iron concentrations are associated with an increase in free radicals that can cause oxidative damage and trigger pathologic processes (Silva *et al*.,2008).

When using ferrous sulfate, it increases the level of iron, and therefore works to decrease the level of body weight, the level of estrogen, the weight of the uterus, the weight of the ovaries, the number of mature ovarian follicles, and it increases the level of blood hemoglobin and the level of malondialdehyde (Takea and Mhsen, 2019).

2.3.2. Hepcidin

Hepcidin is a peptide hormone (a 25 - amino acid disulfide rich peptide synthesized in the liver which is primarily expressed in the liver and to a smaller extent in other tissues) and a primary mammalian iron metabolism regulator (a master systemic iron homeostasis regulator) that accelerates body iron excretion , serum hepcidin elevation is thought to be a compensatory response to iron overload,

hepcidin is protected from hepatic iron accumulation. (Yamauchi *et al*.,2019; Miranda *et al*. ,2019).

Hepcidin, a liver-secreted antimicrobial peptide, has been shown to play a central role in controlling systemic iron homeostasis in response to hypoxia, iron status, erythropoietic activity and inflammation (Masaratana *et al*.,2013; Hentze *et al*.,2010).

The peptide hormone hepcidin controls body iron homeostasis by evaluating the level of dietary iron release from enterocytes and stored iron release from reticuloendothelial macrophages, and functions as a systemic iron- regulating hormone by regulating iron transfer from iron - exporting tissues to plasma (Ganz , 2006; Ganz , 2011).

Hepcidin is encoded as an 84-aa prepropeptide, containing an N-terminal 24-aa endoplasmic reticulum-targeting signal sequence, the 60-aa prohormone contains a consensus furin cleavage motif, and only the mature peptide, but not the prohepcidin, was shown to be secreted from cells , the mature hormone circulates in plasma and its binding to α 2- macroglobulin has been reported, while this interaction was shown to promote hepcidin activity in vitro, the effect on hepcidin clearance is still unknown, a major route of hepcidin clearance is renal excretion, when kidney function is normal, However, based on the comparison between serum and urinary concentrations, it appears that only 5% of hepcidin from plasma filtered in the kidneys ends up intact in the urine , suggesting that hepcidin may not be freely filtered in the glomerulus and/or that filtered hepcidin is reabsorbed and degraded in proximal tubules similarly to other small peptide hormones, hepcidin may also be cleared by receptor-mediated endocytosis in tissues expressing its receptor ferroportin, as indicated by the accumulation of radiolabeled hepcidin in ferroportin-rich tissues and the degradation of the endocytosed ferroportin-hepcidin complex in cultured cells, how much hepcidin catabolism occurs by renal clearance or by degradation in target tissues remains to be determined (Nemeth and Ganz , 2009).

Hepcidin decreases iron exports of ferroportin on the surface of duodenal enterocytes and macrophages, thus preventing iron release from these cells (Corradini *et al*.,2011).

More hepcidin is released when iron is plentiful, which suppresses more iron absorption and release and creates less to no hepcidin in iron deficiency, allowing more iron to enter the plasma (McLachlan *et al*.,2017).

Hepcidin synthesis is raised by loading iron, and reduced by anemia and hypoxia, alternatively, hepcidin synthesis is substantially increased during inflammation, the results of hepcidin overload in mice showed that hepcidin-1 was overexpressed under the influence of a particular hepatic promoter ,carrying iron through the basolateral membrane of duodenal enterocytes by ferroportin determines whether the iron is delivered to the plasma transferrin or removed from the body when the enterocytes shed into the intestinal lumen, when iron stocks are adequate or high, the liver releases hepcidin which circulates to the small intestine, there hepcidin helps ferroportin to become internalized, blocking the only way to move iron from enterocytes to plasma, when iron levels are small, hepcidin production is suppressed, ferroportin molecules are displayed on the enterocyte basolateral membranes and iron is transferred to the plasma transfer from the enterocyte cytoplasm (Ganz, 2006).

The axis of hepcidin ferroportin is the principal health and disease regulator for extracellular iron homeostasis (Ganz and Nemeth, 2011).

2.3.3. Hepcidin antimicrobial peptides (HAMPs)

Humans express one hepcidin gene, HAMP but mice express two hepcidin genes, Hamp1 and Hamp2, human hepcidin gene, HAMP is located on the long arm of chromosome 19 at position 13.1. Unlike humans and rats, mice have two hepcidin genes, Hamp1 and Hamp2, which are both located on mouse chromosome 7. Similar to HAMP, iron induces the expression of both Hamp1 and Hamp2, Based on predicted structure analysis, and studies using Hamp1 knockout or Hamp2 overexpressing mice, Hamp1 has been suggested to be the equivalent of HAMP

regarding the regulation of iron metabolism , the role of Hamp2 is however unknown (Lu *et al*.,2015; McLachlan *et al*.,2017).

Hepcidin was first isolated from human urine through cation exchange chromatography, and has since been found in a number of other mammals and in fish. Mammals have only a single copy of the hepcidin gene (with the exception of the mouse) (Hilton and Lambert, 2008).

genes have also been identified in vertebrates including mice, rats, pigs and several species of fish (Ganz, 2006).

Both mice and pigs have a second hepcidin gene that encodes a peptide less similar to human hepcidin, overexpression of hepcidin-2 in mice, however, had no effect on iron metabolism, and the function of the second hepcidin gene is still unclear (Ganz, 2005).

Two studies examined the role of strain and sex in the expression of hepcidin genes and showed that female mice have higher hepcidin levels than male mice of the same strain and that the differences between the strains are mainly due to the differences in Hamp2 expression, they also showed that females had higher levels of liver, spleen, and serum iron, but no differences in transferrin saturation, than males of the same strain, and there was a significant difference between the strains in liver and spleen iron levels in males (McLachlan *et al*.,2017).

When the liver senses high levels of circulating iron, the HAMP gene (Hepcidin) is upregulated and secreted into the blood stream, which prevents absorption of dietary iron by inhibiting the iron transporter ferroportin on duodenal enterocytes (Miranda *et al*., 2019).

Iron excess is regulated through a pathway involving the cell surface receptor hemojuvelin that stimulates expression of the hepcidin encoding gene (HAMP) (Truksa *et al*.,2009).

Found that serum hepcidin -1 levels correlated well with liver Hamp1 expression (McLachlan *et al*., 2017).

HAMP expression increases in response to inflammation and iron overload and decreases under conditions of anemia or hypoxia, during infection and inflammation HAMP transcription is induced by interleukin-6 (IL-6) ,and the presence of hepcidin in a variety of tissues suggests that it plays an important and wide-ranging role in innate immune defenses ,however, hepcidin's primary role appears to be hormonal, serving as a negative regulator of iron homeostasis. In mammals, the liver is the primary site of HAMP expression (Hilton and Lambert, 2008).

The regulation of the hepcidin (HAMP) gene by iron status involves the participation of the extracellular signaling molecule bone morphogenetic protein 6, Previous studies identify two distinct physiological pathways through which iron status regulates hepcidin: one is relatively acute (and associated with changes in circulating iron concentrations), and the other is chronic (and associated with changes in liver iron stores) (Feng *et al*.,2012).

Iron overload prompted a down regulation of ferroportin, associated with an up regulation of hamp1, whereas an opposite response was observed during anemia, with no changes in hamp2 in either situation. During infection, ferroportin expression decreased, indicating iron withholding to avoid microbial proliferation. In vivo administration of Hamp1 but not Hamp2 synthetic peptides caused significant reduction in ferroportin expression, ferroportin activity is mediated through the iron-regulator Hamp1, and not through the dedicated antimicrobial Hamp2 (Neves *et al*.,2017).

Ferroportin protein response to the diet in Hamp-/- mice was tissue specific with increased expression in the duodenum and spleen, but decreased expression in the liver,iron-deficient diet increased the expression of iron transport machinery in Hamp-/- duodenum through hepcidin-independent mechanisms (Masaratana *et al*.,2013).

Ferroportin is the only known iron exporter in the cells, playing a critical role in maintaining iron homeostasis, its regulation is tightly connected with the antimicrobial peptide and key regulator of iron metabolism hepcidin, which causes

its internalization and degradation, effectively blocking iron export from the cells. However, whereas in mammals a single hepcidin gene exists (with the mouse being the sole known exception), many teleost fish have a large number of hepcidin genes that can be divided in two types, with markedly different functions (Neves *et al.*,2017).

Chapter Three

Materials and Method

3.Materials and Methods

3.1. Chemicals

Table (3.1) Used chemicals according to the company and origin

No.	Materials	Company	Origin
1	AccuZol™ (Trizol 100ml)	Bioneer	Korea
2	AccuZol™ Total RNA Extraction Kit	Bioneer	Korea
3	Acidic alcoholic eosin	Riedel-dehaenag	Germany
4	Actin, R Actin, F	Integrated DNA Technologies	Belgium
5	Agarose	Promega	USA
6	Aluminum and potassium sulphate	BDH	England
7	Chloroform	BDH	England
8	D.P.X	Thomas Baker	India
9	DEPC water	ABM	Canada
10	Eosin Stain	Himedia Lab Put. Ltd	India
11	Ethanol	Labort	India
12	Ethidium Bromide	Promega	USA
13	ferrous sulfate	HIMEDIA	India
14	Folic acid	HIMEDIA	India
15	Formalin	BDH	England
16	Glacial Acetic Acid	BDH	England
17	Hematoxylin Stain	Himedia Lab Put. Ltd	India

18	Hepcidin Kit	Biocellular Company	China
19	Hepcidin R Hepcidin F	Integrated DNA Technologies	Belgium
20	Isopropanol	Labort	India
21	L-Methionine	HIMEDIA	India
22	Normal saline	Labort	India
23	Nuclease free water	Bioneer	Korea
24	Onestep Evagreenstar™ qPCR PreMix	ABM	Canada
25	Paraffin Wax	Histo- Line Lab,OWax	Italy
26	Red Mercuric oxide	BDH	England
27	RNase free water	ABM	Canada
28	Tris-Borat-EDTA (TBE)	BIO BASIC INC	USA
29	Xylene	Scharlau	Spain

3 . 2. Devices and instruments

Table (3.2) the instruments and devices used in present study with manufacture company and Origin

No.	Devices	Company	Origin
1	Anticoagulant tube(EDTA (tube	(AFMA-Dispo)	Japan
2	Autoclave	TOMY® Vertical Autoclave	Germany
3	Balance	Sartorius	Germany
4	Beaker	HAILAO	Italy

5	Centrifuge	Heraeus Christ	Germany
6	Cold Eppendorf Centrifuge	Hermle	Germany
7	Compound Light microscope with camera	MEIJI	Japan
8	Cylinder	Boeco	Italy
9	digital camera	Mettler	Germany
10	Disposable Syringes	Medical ject	S.A.R.
11	Dissecting tools	S.I.E.	Pakistan
12	ELISA - Reader and washer	BioTek	USA
13	Eppendorf centrifuge	Fisons	England
14	Eppendorf tube	----------	---------
15	Filter Paper	Turck 0.33 Zelpa	Belgium
16	Staining Gar	Harshman	Germany
17	Hematocrit centrifuge	Heraeus Christ	Germany
18	Hemocytometer	Superoior	Germany
19	Hot magnetic stirrer	HYSC	Korea
20	Hot Plate	Lassco	India
21	Incubator	Memmert	Germany
22	Insulin Syringe	Medical ject	S.A.R.
23	Jell test tube	Gold Star	Jordan
24	Latex gloves	Great glove	Malaysia
25	Liquid nitrogen	Rockefeller	USA
26	MAGLUMI Ferritin 800	Snibe diagnostic	Aruba
27	Microcentrifuge tubes 1.5ml	Eppendorf	Germany
28	Micro pestles	Genaid	USA
29	Micropipette 1 - 50 µL	CYAN	Belgium
30	Mindray BS-240	Mindray Medical	China

		International Limited	
36	Mixer	Exispin	Korea
37	Morter and pestle	Fisher	USA
39	Nano drop	Optizen	Korea
40	Oven	Daihan- Lab. Tech	Korea
41	Pipette tips and Pipette filter tips 10 , 200, 1000 ul	Axy Gen	USA
42	Printer	Epson	Japan
43	Pyrex	Volac	England
44	Real time PCR	Realplex 4	USA
45	Refrigerator	concord	lebanon
46	Rotary Microtome	Histo-Line Lab. Mod. MRS 3500	Italy
47	Sahli apparatus	CrisTA Hawksley	Japan
48	Sensitive Balance	Sartorius	Germany
49	Slides and cover slip	China MHECO	China
50	Spectrophotometer	Apple 203	Japan
51	Spin down mixer centrifuge	Bioneer	Korea
52	Vortex	CYAN	Belgium
53	Water Bath	Memmert	Germany

3.3. Physical examination methods

3.3.1. Experimental protocol

One hundred fifty (150) mice weight of mice (20-40g) were used in the current study were taken from the Cancer Research Center in Baghdad, Iraq. model selected for present study is Albeno pulp-c and their ages between(12-16) weeks and the animals were placed in the animal house of the College of Veterinary Medicine / University of Kerbala in special plastic cages and

provided the animals with the appropriate conditions In terms of temperature around (25 ±5 C°) and ventilation and The light system was 14/10 hrs light/dark cycle with a relative humidity of 50±5%. They were kept for 2 weeks for adaptation with standard experimental condition.

3.3.1. 1.Experimental Design

One hundred fifty (150) adult mice were randomly divided into 5 groups (thirty each group) and treated as follows for six weeks.

1.Group I :- Animals in this group were intubated orally tap water , serving as control.

2.Group II :- mice of this group were intubated orally 100mg/kg B.W of L-methionine (Seshadri & Robinson, 2000)

3.Group III:- Animals in this group were intubated orally 0.07 mg/kg BW of folic acid (woo,2007)

4.Group IV:- Animals in this group were intubated 100 mg/kg BW of methionine plus 0.07mg/kg B.W of folic acid. (Woo,2007)

5.Group V:- Animals in this group were administered orally 100 mg/kg B.W of ferrous sulfate (hameed , 2019).

3.3.1. 2. Measure the level of iron in the diet

The percentage of iron in the bush was measured using the spectrophotometer in the laboratories of the College of Pharmacy / University of Kerbala. Three samples were sent from the diet, which was given as a daily feeding to the mice, and we found the percentage of iron in the diet (5.56 μg/kg).

3.3.1.3. Body weight

The body weight of all male mice and of all groups was recorded before the start of the experiment in order to determine the correct dose to be given to each animal and was measured using the electric scale.

3.3.1.4. Collect of the blood samples

Blood samples were drawn after starving the animals throughout the night after Six weeks of the experiment, the animals anesthetized by chloroform inhalation in order to control and calm the animal before the blood draw . where 3 ml of blood was drawn from the heart by means of a heart puncture directly by the way the animal lay lying on its back, and sterile medical syringes of 3 ml were used, then the blood was placed In special tubes containing anticoagulant Ethylene diamin tetraacetic acid (EDTA) and in gel tubes not containing an anticoagulant, the serum was separated by a centrifuge at a speed of 3000 r / min for 15 minutes, and the sera were kept in a freeze at -20 ° C until the completion of the measurements.

3.3.1.5. Collection of tissue samples for RNA extraction

As previously mentioned above , the study involved taking 150 male mice taken from the Cancer Research Center in Baghdad, Iraq. model selected for present study is Albino pulp-c . And their ages ranged between (12-16) weeks

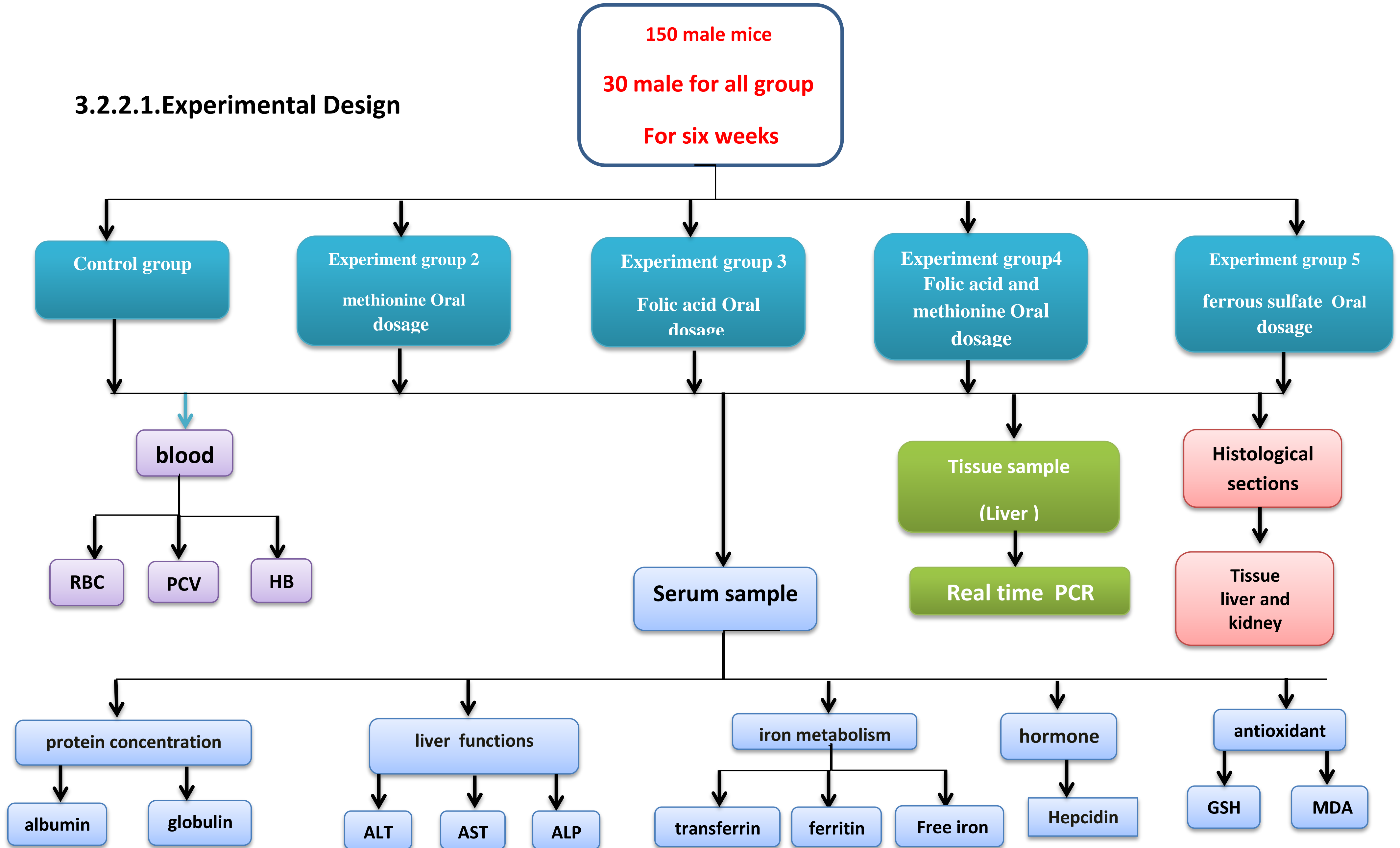
Were taken(10) mice From each group in a random manner In order to collect samples from them for genetic analysis . After completing the collection of blood samples from the heart, a portion of the liver tissue was taken it weighed around (100 mg), put in Eppendorf tube contains 1 ml of Trizol® reagent. and kept in the freezer at a temperature of -20 to prepare for the RNA extraction process .

3.3.1.6. Organs collection for Histological section

After the end of the experiment, animals (male mice) were sacrificed by anesthesia using chloroform, and the animals were dissected to remove samples (liver and kidney), and each of them was isolated separately, and the parts connected to the excised organ were removed, and the organs were

preserved in formalin at a concentration of 10% in clean plastic containers after numbering them for until Perform the Histological section.
The two stain , Eosin and Hematoxylin , were prepared by following the steps recommended by the researcher (Suvarna *et al*., 2013) and the steps used for staining according to the method (Suvarna *et al*., 2013).

3.2.2.1.Experimental Design



3.3.2 . Measurement of biochemical parameters

3.3.2.1. Determination of serum concentration of malondaidehyde (MDA)

Malondialdehyhe was estimated by Thiobarbituric acid (TBA) assay method of (Buege and Aust, 1978) on spectrophotometer, As Show appendix I .

3.3.2.2. Determination of serum Reduced Glutathione concentration (GSH)

Reduced glutathione was measured following the method of (Sedlak and Lindsay ,1968) , As Show appendix I I .

3.3.2.3 . serum protein concentration

3.3.2.3.1. Serum albumin concentration

According to mindray company used the Bromcresol green (BCG) method, as shown appendix III.

3.3.2.3.2. Serum globulin concentration

The total globulin concentration was estimated indirectly by measuring of albumin in serum and then subtract it form the result of total protein , as shown appendix IV.

3.3.2.4. Serum determination of Alanine Aminotransferase (ALT) , Alkaline phosphatase (ALP) and Aspartate Aminotransferase (AST)

The mindray apparatus was used to examine the enzymes, and the method for examining the enzymes was carried out according to the instructions of the manufacturer producing the device , as shown appendix V .

3.3.2.5. Determine the level of iron in the serum

Also, the mindray device was used to determine the percentage of iron, and the instructions of the producing company were followed in the method of examination.

3.3.2.6. Measuring the level of ferritin in the serum.

The examination was carried out using a device MAGLUMI Ferritin 800 and the method of examination was carried out according to the instructions of the producing company.

3.3.2.7. Calculate the percentage of saturation of the iron carrier Transferrin.

The percentage of transferrin was calculate based on a mathematical equation was estimated according to (Young, 2000), as shown appendix VI.

3.3.2.8. Measurement of the level of bilirubin in the serum

The serum bilirubin level was measured depending on Dual wave length total Bilirubin meter Using a device Spectrophotometer depending on the recommendations and instructions of the producing company.

3.3.2.9. Measuring the level of the hormone Hepcidine in the serum

In the serum, the level of the hormone hepcidine was measured using a Kit Specific to measure the hormone level in mice depending on the method of using Kit According to the instructions of the producing company , as shown appendix VII.

3.3.3. Blood test

3.3.3.1. Hemoglobin measurement (Hb):

Hemoglobin was measured using the Sahli method (Jain, 1986) , as shown appendix VIII .

3.3.3.2. Pack cell volum (PCV):

used the Microhaematocrit method According to (Coles, 1986; Jain, 1986) , as shown appendix IX .

3.3.3.3. Red blood cells count :

The Haemocytometer method was used according to (Gregg, 2000), as the number of red blood cells per 1 cubic millimeter of blood was calculated .

3.4. Method of examining gene expression

3.4.1. Primers

Two set primers were used in this study, one used for B-actin gene as Housekeeping gene and one used for hepcidin gene as target genes. These primers were Designed using a source (Huang H *et al.* , 2009) . Primers used in quantification of gene expression using q RT-PCR techniques based on SYBER Green DNA binding dye, was provided by Bioneer, Korea.

Table (3.3): The Primers, sequences, gene bank accession number, and references.

Primer		Sequence	Reference
hepcidin	F	5′-AGAGCTGCAGCCTTTGCAC-3′	Huang H *etal* , 2009
	R	5-′GAAGATGCAGATGGGGAAGT-3′	
β-actin	F	5′-AGTGTGACGTTGACATCCGTA- 3′	Huang H *etal* , 2009
	R	5′-GCCAGAGCAGTAATCTCCTTCT-3′	

3.4.2. Quantitative Reverse Transcriptase Real-Time PCR Kits

Table (3.4): All kits which used in quantification of gene expression levels by qRT PCR with their companies and countries of origin:

No.	Kit	Company	Country
1.	AccuZol™ Total RNA Extraction Kit	Bioneer	Korea
	Trizol 100ml		
2.	Onestep Evagreenstar™ qPCR PreMi	ABM	Canada
	-PreMix		
	-RocketScript Reverse Transcriptase (200u)		
	- SYBER Green fluorescence		
	- Exicycler™ 20 µL reaction		
	- 8Well strips × 12 each		
	- DEPC – D.W. 1.8 ml × 4 tubes-		

3.4.3. Molecular analysis

3.4.3.1 Quantitative Reverse Transcriptase Real-Time PCR

Q RT-PCR technique was used for quantification of *Hepcidin* gene expression levels relative to Housekeeping gene *B-actin* gene expression levels in rat liver cell treated with Methionine and folic acid and iron .

3.4.3.2. Total RNA extraction

Total RNA were extracted from rat tissues (liver) by using (TRIzol® reagent kit) and done according to company instructions, as shown appendix X .

3.4.3.3. DNase inactivation (DNase I) Treatment

The extracted total RNA were treated with DNase I enzyme to remove the trace amounts of genomic DNA from the eluted total RNA by using samples (DNase I enzyme) and done according to method described by promega company, USA instructions , as show appendix XI .

3.4.3.4. Assessing RNA yield and quality

There are three quality controls were performed on isolated RNA. First one is to determine the quantity of RNA (ng/µL) that has been isolated by used Nanodrop UV/VIS spectrophotometer (OPTIZEN POP. MECASYS Korea), the second is the purity of RNA by reading the absorbance in spectrophotometer at 260 nm and 280 nm in same Nanodrop machine, and the third is the integrity of the RNA by prepared gel electrophoresis, as show Appendix XII.

3.4.4. Real Time-quantitative Polymerase Chain Reaction:

3.4.4.1. Reverse transcriptase (cDNA synthesis) using PCR technique:

Synthesis and amplification of cDNA were performed with the master amplification reaction (ABM, Canada), as show appendix XIII .

3.4.4. 2. Data analysis of q RT-PCR

The data results of q RT-PCR for target and housekeeping gene were analyzed by the relative quantification gene expression levels (fold change) Livak method that described by (Livak and Schmittgen, 2001) , as show appendix XIV .

3.5. Histological section method

3.5.1. preparation Histological section

The method described in (Luna, 1968) was used to prepare parts of tissue. Waxy molds were used for the dehydration, clearing and molding.

3.5.2. Histological Sectioning

Samples preserved in woven tissue parts were cut by rotary microtome and loaded onto glass slides and put on a hot plate at a temperature of 40-37 ° C until dry and the textile slides were then stained according to the method mentioned in (Luna, 1968).

3.5.3. Microscopy

The textile parts were filmed using a Digital Camera Eyepiece (DCE-pw1) high-resolution digital camera linked to a computer.

3.6. Statistical analysis

Statistical analysis of data for five experiments of present study was performed on the basis of one way analysis of variance (ANOVA) using significant level of ($P<0.01$) and ($P<0.05$).

Differences were determined using least significant differences (LSD) (Steel and Torries, 1980).

Chapter Four

Result

4.Results :-

4.1. physiological and biochemical parameters

4.1.1. Serum Glutathione (GSH) and Malondialdehyde (MDA) concentration.

Table (4-1) illustrated the serum GSH concentration in the control and four treated groups along the experimental period , the table showed a significantly ($P<0.01$) increase after six weeks in G3 and G4 as compared to control group ,the result also showed that oral intubation of (100mg/kg B.W) from methionine and iron overload G2 and G5 in mice caused significant ($p<0.01$) decrease in serum GSH concentration as compared to control group .

The result also showed serum MDA concentration in male mice after oral intubation of (100mg/kg B.W/daily) for 6 week and also denoted the effect of oral intubation of folic acid and iron on serum MDA concentration.

The serum MDA concentration was significantly($p<0.01$) increased after intubation of methionine (G2) and in G5 as compared to control, and significant ($p<0.01$) decrease in G3 as compared to control group .

Table (4-1) Effect of daily oral intubation of methionine and folic acid and iron for six weeks on Glutathione (GSH) concentration (µmol /l) and on Malondialdehyde (MDA) concentration (µmol/d l) in male mice .

Groups \ parameters	GSH µmol/L	MDA µmol /dl
G1 control group	5.01 ± 0.64 B	5.86 ± 0.75 B
G2 Methionine group	2.93 ± 0.12 C	9.78 ± 0.08 A
G3 Folic acid group	8.18 ± 0.24 A	3.39 ± 0.24 C
G4 Folic acid and methionine group	8.83 ± 0.47 A	4.89 ± 0.31 BC
G5 Iron group	2.52 ± 0.15 C	10.79 ± 0.75 A

expressed as mean ± SE. n=30/ group .G1= considered as control group. G2 = mice intubated (100mg/kg B.W/day) of methionine for six weeks . G3= mice intubated Folic acid (0.07mg/kg B.W) for six weeks .G4 = mice intubated Folic acid (0.07mg/kg B.W) and methionine (100mg/kg B.W/day) for six weeks . G5 = mice intubated ferrous sulfate for six weeks.
Means with the same latter are not significantly different (p<0.01) vs.

4.1.2. Serum Globulin and Albumin concentration

The effect of oral intubation of methionine ,folic acid and iron loading mice on serum Globulin and Albumin concentration were demonstrated in table(4-2), a significant (p<0.01) decrease in serum albumin concentration were detected after six weeks of experiment in methionine treated groups (G2) and iron treated group(G5) comparing to G1 , the result also showed a significant increase (P<0.01) in Albumin concentration in folic acid treated group G3 in comparison with G1 ,was appear in table (4-2) non-significant (p<0.01) effect in G4 in comparing to G1.

The result also showed a significant (p<0.01) decrease in serum Globulin concentration in methionine treated groups (G2) and iron treated group(G5) comparing to G1 Statistical differences were absent between G4 and G1 groups during experimental period in serum Globulin .

Table (4-2) Effect of daily oral intubation of methionine and folic acid and iron for six weeks on albumin (g/dl) and Globulin concentration (g/l) in male mice.

parameters / Groups	Globulin (g/dl)	ALB (g/l)
G1 control group	3.15 ± 0.26 B	3.16 ± 0.40 B
G2 Methionine group	2.15 ± 0.26 D	2.00 ± 0.12 C
G3 Folic acid group	3.84 ± 0.51 A	4.30 ± 0.21 A
G4 Folic acid and methionine group	3.29 ± 0.13 B	3.05 ± 0.08 B
G5 Iron group	2.89 ± 0.12 C	2.15 ± 0.03 C

expressed as mean ± SE. n=30/ group .
Means with the same latter are not significantly different (p<0.01) vs.

4.1.3. Aspartate transaminase (AST) Alanine transaminase (ALT) and Alkaline Phosphatase ALP activity .

Table (4-3) demonstrated serum AST activity in all treated and control groups. AST activity showed highest significant (p<0.01) elevation after six weeks of methionine over load G2 and iron overload G5comparing to G1 . Meanwhile , significant (p<0.01) reduction in serum AST activity were observed during 42 days after intubation of folic acid and folic acid - methionine treated mice comparing to G1 .

The result also showed presence significant increase (p<0.01) in ALT activity were appeared in mice (100mg/kg B.W) of methionine (G2) daily and iron (G5) overload for 42days with comparing to control group , also significant decrease (p<0.01) in serum ALT activity were observed after intubation of folic acid (G3)for six weeks comparing to G1, and non-significant difference in activity ALT in G4 compared to G1.

While there were significant ($p<0.01$) increment in ALP was shown at the end of experiment in methionine treated group G2 and in G5comparing to G1. While combined intubation of folic acid(G3) treated mice caused significant ($p<0.01$) decrement in ALP activity comparing to G1, and non-significant ($p>0.01$) differences in serum ALP activity between G4 and G1

Table (4-3) Effect of daily oral intubation of methionine and folic acid and iron for six weeks on Aspartate transaminase (AST) concentration (IU/L)and on Alanine transaminase (ALT) activity (IU/L) and Alkaline Phosphatase(ALP) activity (IU/L) in male mice.

parameters / Groups	AST (IU/L)	ALT (IU/L)	ALP (IU/L)
G1 control group	307.00 ± 3.07 C	71.01 ± 2.66 B	120.00 ± 1.15 C
G2 Methionine group	360.00 ± 4.07 B	80.25 ± 1.92 A	184.50 ± 2.43 B
G3 Folic acid group	214.30 ± 5.56 E	55.20 ± 1.22 C	110.00 ± 0.96 D
G4 Folic acid and methionine group	236.00 ± 2.32 D	70.01 ± 1.35 B	123.00 ± 1.53 C
G5 Iron group	437.94 ± 3.77 A	82.80 ± 1.34 A	262.00 ± 3.09 A

expressed as mean ± SE. n=30/ group.
Means with the same latter are not significantly different

4.1.4. Serum Iron and Ferritin concentration and Transferrin and hepcidin Hormone level :-

Table (4-4) illustrated the mean value of serum iron level in male mice after oral intubation of methionine , folic acid and iron overload for 6 weeks

The serum iron level was significantly($p<0.01$) increased after intubation of G2, G4 and G5 as compared to G1, at the same time combined intubation of G3 to mice for six weeks showed non-significant in serum iron level comparing to G1.

The result also showed that intubation of methionine and iron overload significantly($P<0.01$) increased in Ferritin concentration in G2 and G5 comparing to G1 ,and showed non-significant differences in Ferritin in G3 and G4 comparing to G1.

At the end of experiment , transferrin level significantly($P<0.01$) increased after combined intubation of iron overload in G5 comparing with G1,on the other hand this parameter was significantly($P<0.01$) decreased in G2, G3, and G4 groups comparing to G1.

Table (4-4) illustrated that there is a significant ($P<0.01$) increase in the level of hepcidin Hormone concentration in intubation of methionine overload (G2) and intubation of iron (G5) for six weeks compared with G1 and there was showed non-significant ($p>0.01$) differences in hepcidin in G3 and G4 comparing to G1.

Table(4-4) Effect of daily oral intubation of methionine and folic acid and iron for six weeks on Iron concentration (µg/dl) and on Ferritin concentration(ng\ml) and on transferrin (mg\dl) and on hepcidin Hormone in male mice.

Groups \ parameters	Iron (µg/dl)	Ferritin (ng\ml)	Transferrin (mg\dl)	hepcidin Hormone (pg\ml)
G1 control group	175.20 ± 4.49 C	0.62 ± 0.01 C	698.50 ± 0.86 B	48.37 ± 0.62 CD
G2 Methionine group	212.07 ± 1.58 B	1.20 ± 0.2 B	462.50 ± 4.33 C	56.35 ± 0.91 B
G3 Folic acid group	162.4 ± 3.97 C	0,81 ± 0.06 C	287.50 ± 1.44 E	46.52 ± 0.55 D
G4 Folic acid and methionine group	214.4 ± 3.75 B	0.79 ± 0.02 C	363.50 ± 2.02 D	51.01 ± 0.79 C
G5 Iron group	495.60 ± 10.79 A	5.82 ± 0.14 A	724.84 ± 2.47 A	217.02 ± 3.39 A

expressed as mean ± SE. n=30/ group.
Means with the same latter are not significantly different.

4.1.5.Levels of Red blood cell count, Hemoglobin and Packed cell volume.

Table (4-5) illustrated the mean value of Red blood cell count in the G1 and G4 along the experimental period. The table showed a general trend of Red blood cell count a significant decrease in G2and G5 comparing to control group, While there were non-significant (p>0.01) differences in Red blood cell count in G3 and G4 comparing to control group.

The results also showed that oral intubation of methionine ,folic acid and iron caused significant(p<0.01) decrease in HB concentration in methionine treated mice (G2),methionine-folic acid (G4)and iron treated mice(G5) comparing to G1,and there was non-significant difference between G3 and G1.

While there were a significant(p<0.01) decrease in PCV were detected after six weeks of experiment in G2 and G5 comparing to G1 , and non-significant (p >0.01) differences in PCV between G3, G4 and G1.

Table (4-5) Effect of daily oral intubation of methionine and folic acid and iron for six weeks on Red blood cell count , Hemoglobin and Packed cell volume in male mice.

parameters / groups	RBC 10^{12}\ L	Hb g\dl	PCV %
G1 control group	8.13 ± 0.20 A	13.50 ± 0.17 A	44.01 ± 0.99 A
G2 Methionine group	6.37 ± 0.15 B	10.16 ± 0.13 C	32.60 ± 1.12 B
G3 Folic acid group	8.32 ± 0.11 A	12.80 ± 0.23 A	42.22 ± 0.43 A
G4 Folic acid and methionine group	7.98 ± 0.20 A	11.51 ± 0.30 B	42.65 ± 1.82 A
G5 Iron group	5.42 ± 0.61 B	9.06 ± 0.20 D	28.14 ± 0.63 C

expressed as mean ± SE. n=30/ group.
Means with the same latter are not significantly different

4.1.6.Serum bilirubin concentration

Table (4-6) illustrated the mean value bilirubin there were a significant increase (P< 0.01) in the bilirubin level in G2 treatment with excessive administration of methionine and in G5 treatment with excessive administration of iron compared to G1(table 4-6) .

The result also showed non-significant (p>0.01) differences in the mean value of serum bilirubin concentration between G3 and G4 compared to G1 during experimental period.

Table (4-6) Effect of daily oral intubation of methionine and folic acid and iron for six weeks on bilirubin concentration (mg\dL) in male mice.

Groups / Parameters	G1	G2	G3	G4	G5
Bilirubin mg\dL	0.30 ± 0.01 C	1.20 ± 0.14 B	0.33 ± 0.02 C	0.34 ± 0.10 C	2.61 ± 0.33 A

expressed as mean ± SE. n=10/ group . G1= considered as control group. G2 = mice intubated (100mg/kg B.W/day) of methionine for six weeks . G3= mice intubated Folic acid (0.07mg/kg B.W) for six weeks . G4 = mice intubated Folic acid (0.07mg/kg B.W) and methionine) (100mg/kg B.W/day) for six weeks . G5 = mice intubated ferrous sulfate for six weeks. Capital letter denote difference between groups (p<0.01) vs.
Means with the same latter are not significantly different

4.2 results of Real- Time PCR

After collect samples from liver mice for genetic analysis and Put in Eppendorf tube contains 1 ml of Trizol® reagent and when completed the RNA extraction process in our study was found the most common gene expression (HAMP) in the group with iron overload (T C) have (4.658) rather than other groups , on the other hand , the present study was fond decrease of gene expression in the TA and TB as (0.356) and (0.361) Compared to the control group, and The results of the current study showed that when intubating mice with methionine and folic acid for six weeks resulted in no difference in gene expression compared to the control group ,as shown in the following figure (4-1) (4-2) and table (4-7).

Table (4-7)Data analysis results of relative gene expression of HAMP gene in exposure to methionine and Folic acid and iron in male mice

Group	CT (HAMP1)	CT (HKG)	ΔCT	ΔΔCT	2-ΔΔCT
control	15.82	14.89	0.93	-0.238	1.074
Methionine	17.34	14.25	2.45	1.495	0.356
Folic acid	19.93	17.15	2.78	1.828	0.361
Methionine and folic acid	15.64	15.79	-0.15	-1.108	2.156
Iron	13.86	15.1	-1.23	-2.191	4.658

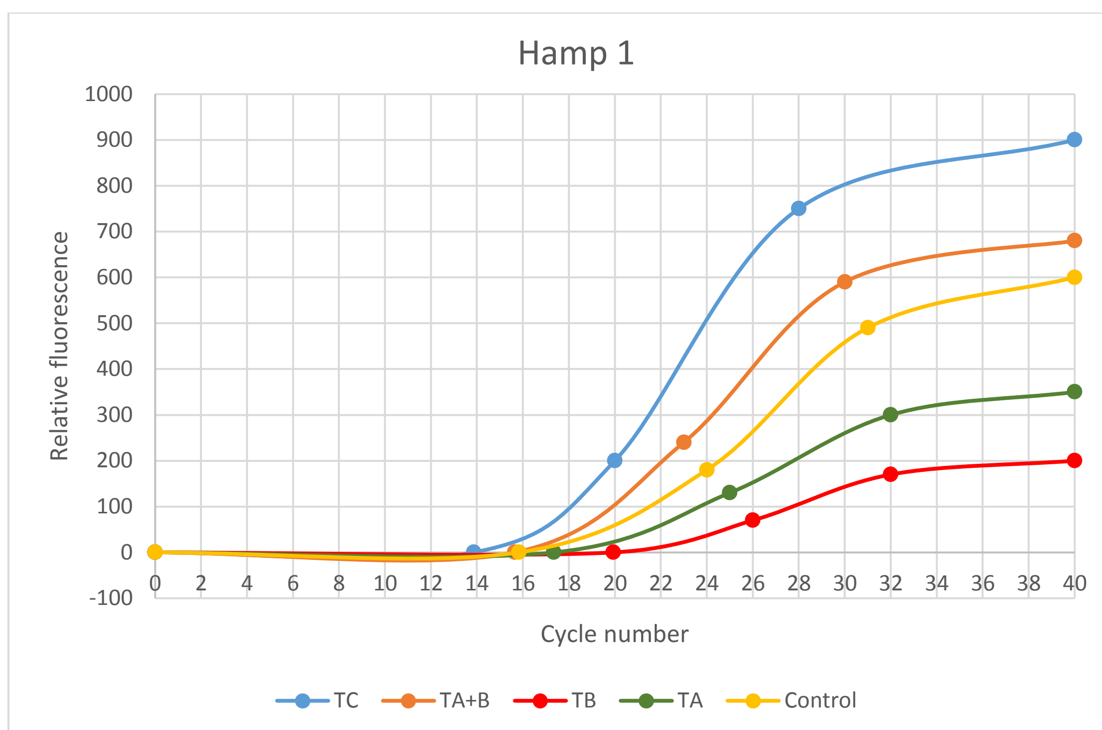


Figure (4-1) Peak analysis of duplex gen(HAMP1) showing translocation of SYBER Green overtime

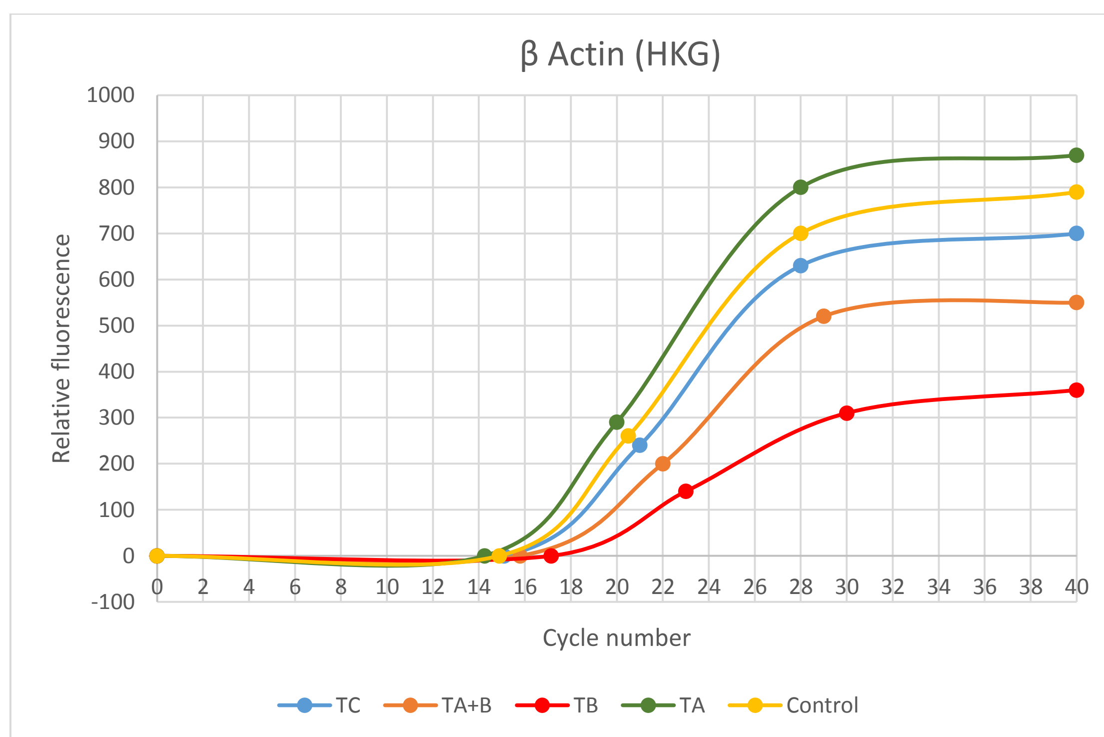


Figure (4-2) Peak analysis of duplex gen (housekeeping) showing translocation of SYBER Green overtime

4.3 results of Histological study :

Histological sections of liver and Kidney of methionine overload mice

Histological section in the liver of animal in Control group show the radial hepatic cord in liver that arranged around vein (4-3) and show Control group of liver show portal area located between hepatic lobes which consist of bile duct and hepatic artery and portal vein (4-4)

Histological section in the liver of animal treated with (100mg /kg B.W of methionine) showed dilatation in Central vein and dilatation in Sinusoid aggregations inflammatory cells, degeneration in tunica intima of Central vein and Endothelial cell death aggregations and hyperplasia Kupffer cells degeneration and hypertrophy in hepatocytes, other histological sections showed Disappearance of arrangement of the hepatic cords and edema in central vein and in Sinusoid and infiltration of mononuclear inflammatory cells in the portal and Pyknotic nucleus in hepatocytes(figure(4-5)(4-6)(4-7)) .

While other sections in the liver of animal treated with (methionine and folic acid) showed infiltration of mononuclear inflammatory cells in per portal area but less than the liver in the case of methionine and decreased hepatocyte and Kupffer cell degeneration figure (4-8).

Histological section in the liver of animal treated with 100 mg/kg B.W of ferrous sulfate In this section show the hemosiderin deposited in liver parenchyma around the portal vein and showed liver cells suffer from cloudy swelling and showed apoptotic cells , and showed sever deposited of hemosiderin around the hepatic artery in the same time and sever deposited of hemosiderin around the bile duct and moderate deposited of hemosiderin around portal vein, and showed liver parenchymal cells with sever deposition of hemosiderin in the outer edge or border of liver, compared to the center of the liver as in the figure (4-9) (4-10) (4-11).

During our 42-day experiment in which male mice were intubated with an overload of methionine demonstrated glomerular atrophy, enlargement of the urinary space, distal tubules atrophy, and the change in the shape of the cells lining the tube where their normal shape is Cuboid, but in the case of methionine overload the shape became flat, as for the proximal tubules, there is no noticeable change, but we note that the color of the cytoplasm has become blue, evidence of damage to the kidney tissue, as it has become basal, and we notice Hyaline cast accumulation ,and the presence of congestion in the artery and the collection of mononuclear inflammatory cells and hypertrophy of the tunica intima , as in the figure(4-12) (4-13) (4-14)(4-15) (4-16).

In the case of 0.07mg/kg B.W of folic acid showed note not accumulation of Hyaline cast Compared with the kidney of animal treated with 100mg/kg B.W methionine as in the figure (4-17).

But in the case of methionine and folic acid dosing together, it was observed that the kidney tissues returned to normal and the kidney healing

was observed, as mononuclear inflammatory cells invaded and glomerular atrophy was observed, but at a lesser rate than the atrophy case in the methionine group only and the cytoplasm of cells in the proximal tubules is pink (acidic) Closer to natural, as in the figure (4-18).

Histological section in the Kidney of animal treated with 100 mg/kg B.W of ferrous sulfate in the section showed renal tubules (proximal and distal tubules) filled with cast due toxic materials (nephritis) with lymphocyte infiltration around the tubules and in kidney tissue , and showed the shrinking glomeruli with deposition of hemosiderin in the glomeruli and renal tubules that lead to renal nephritis with lymphocyte infiltration around the glomeruli, and showed proximal and distal renal tubules filled cast of toxic materials that lead to renal nephritis as in the figure (4-19) (4-20) (4-21).

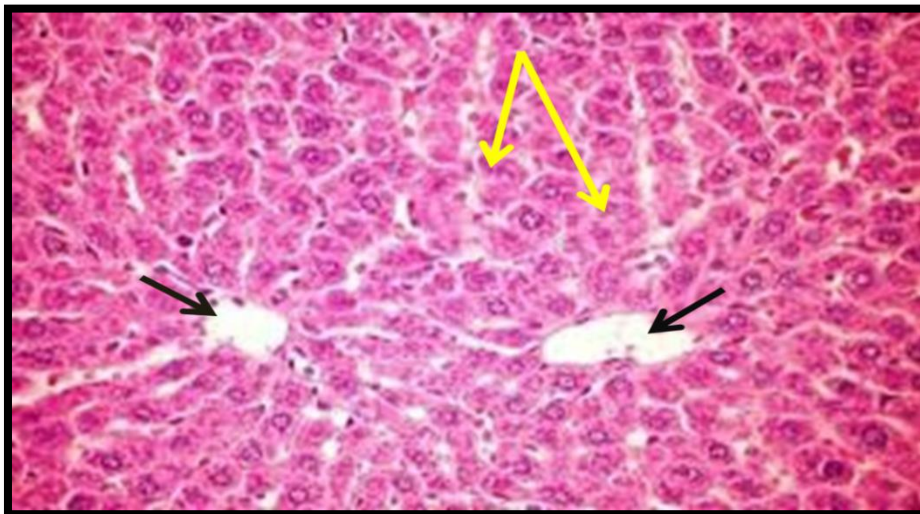


Figure (4-3) Histological section in the liver of animal in Control group show the radial hepatic cord in liver that arranged(yellow arrow) around vein (black arrow) (H&E X 40)

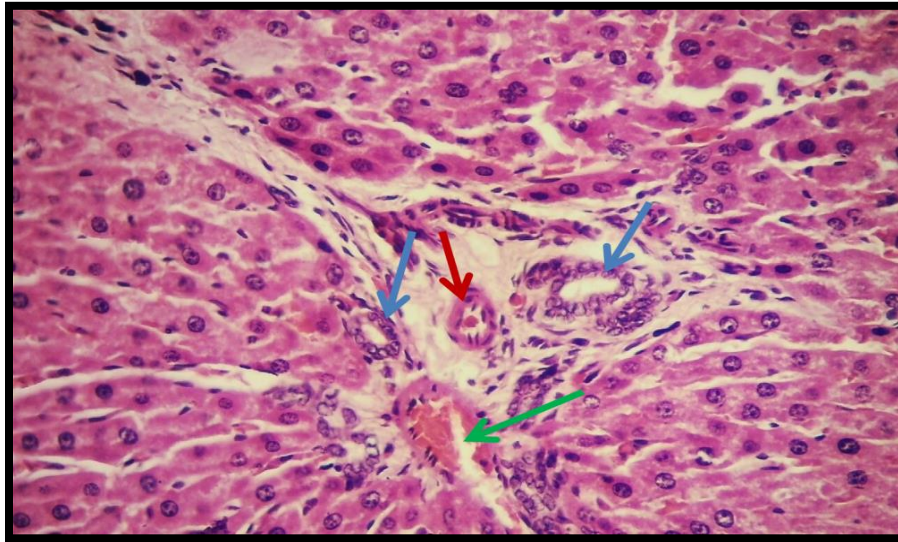


Figure (4-4) Control group of liver show portal area located between hepatic lobes which consist of bile duct (blue arrow), hepatic artery (red arrow) and portal vein (green arrow) (H&E X 40).

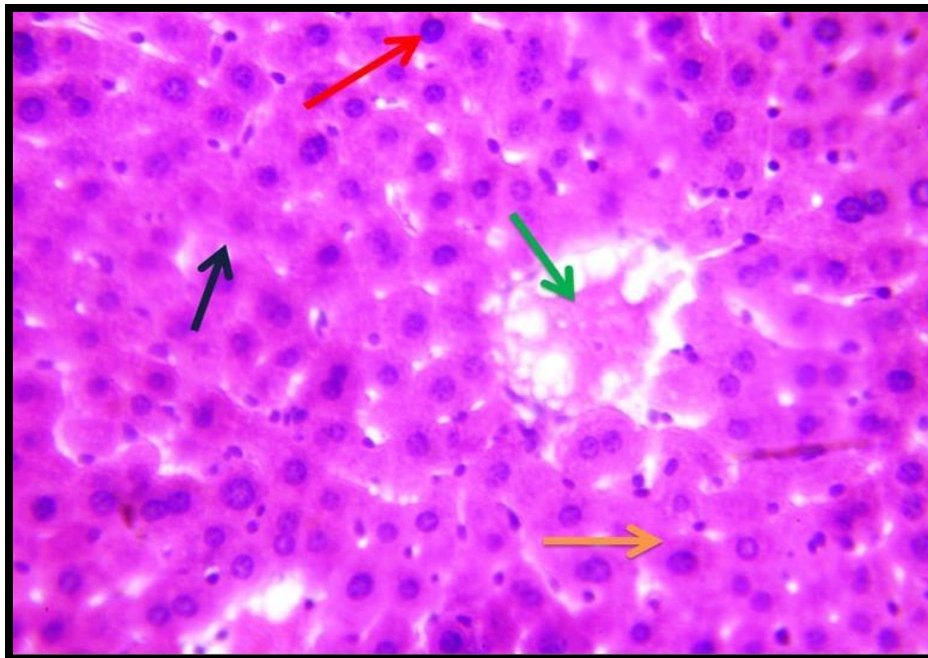


Figure (4-5) Histological section in the liver of animal treated with 100mg/kg B.W methionine note hepatocytes spread randomly, edema in central vein and in Sinusoid (green arrow), hypertrophy in hepatocytes (orang arrow), Pyknotic nucleus (red arrow) and Hepatocyte degeneration (black arrow) (H&E X 40).

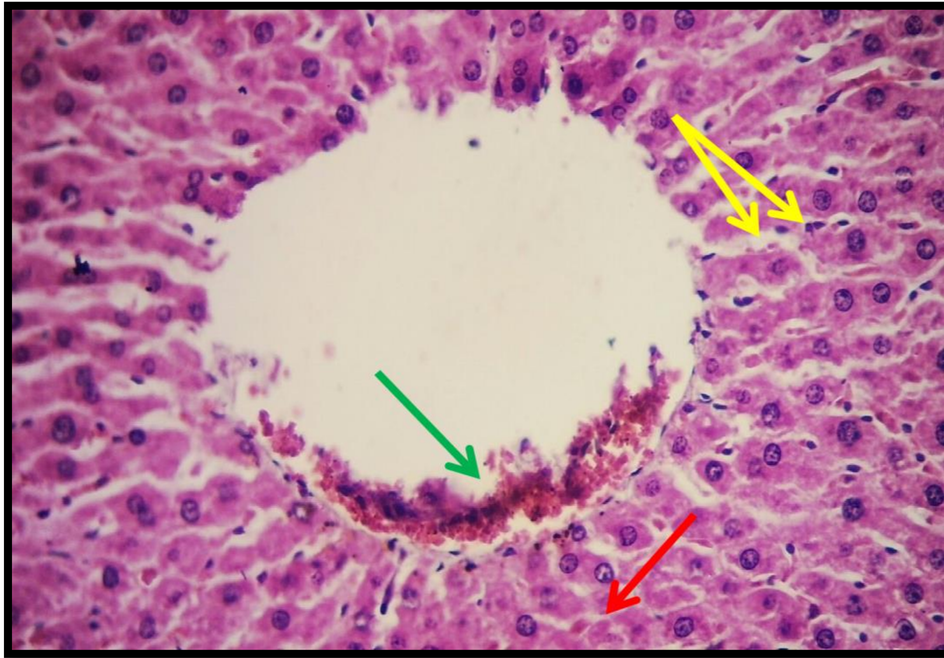


Figure (4-6) Histological section in the liver of animal treated with 100mg/kg B.W methionine note the dilation in central vein (green arrow), aggregations inflammatory cells, degeneration in tunica intema, aggregations and hyperplasia Kupffer cells (yellow arrow)and hypertrophy in hepatocytes (red arrow) (H&E X 40).

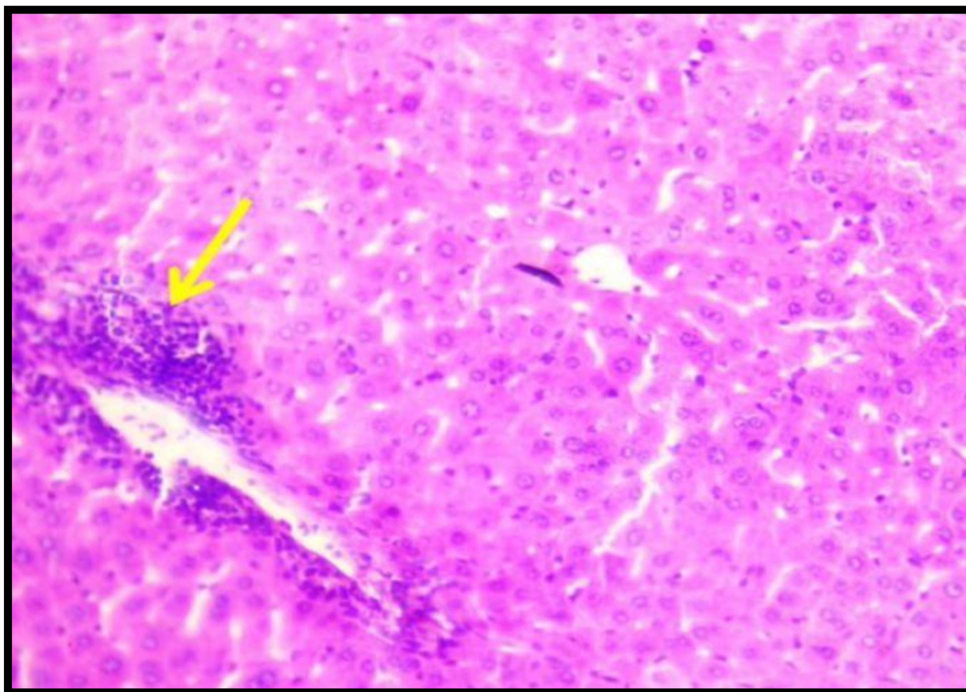


Figure (4-7) Histological section in the liver of animal treated with 100mg/kg B.W methionine note the infiltration of mononuclear inflammatory cells in portal area (yellow arrow) (H&E. X20)

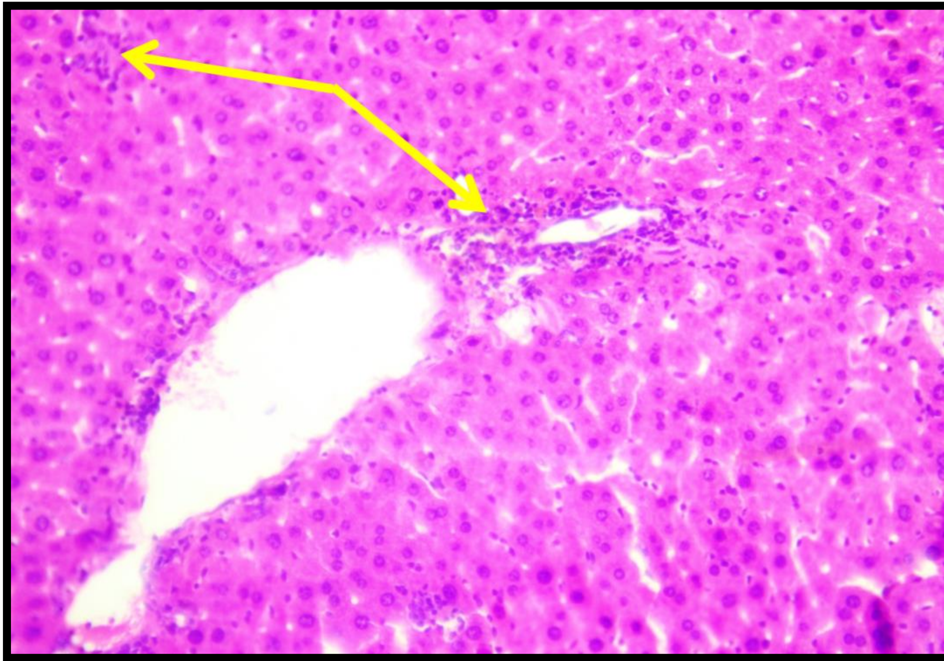


Figure (4-8) Histological section in the liver of animal treated with 100mg/kg B.W methionine and 0.07mg/kg B.W of folic acid note infiltration of mononuclear inflammatory cells in portal area (yellow arrow) less than methionine group also, showing decreased of hepatocytes degeneration and Kupffer cells (H&E X 20).

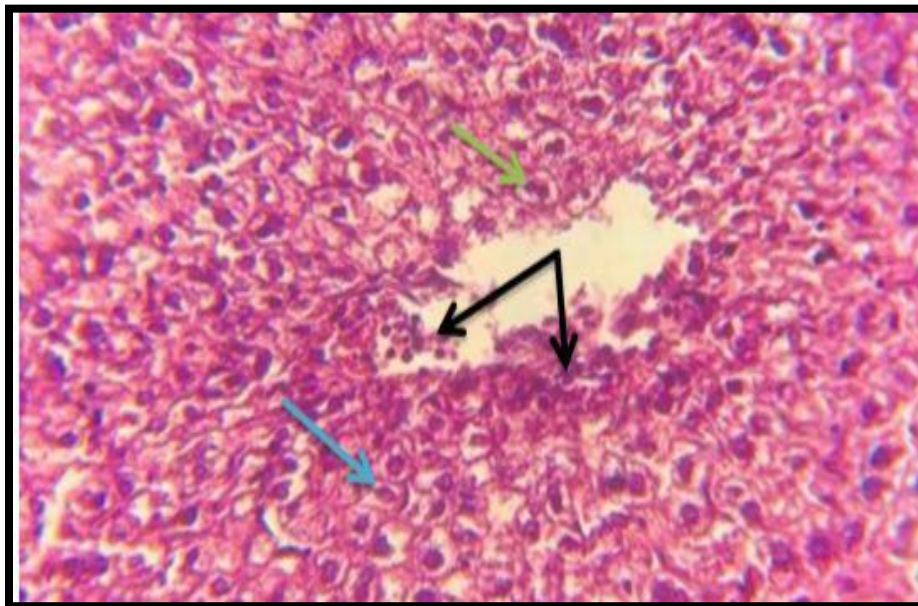


Figure (4-9) Histological section in the liver of animal treated with 100 mg/kg B.W of ferrous sulfate in this section show branch of portal vein and the black arrow show the hemosiderin deposited in liver parenchyma around the portal vein and the blue arrow showed liver cells suffer from cloudy swelling and the green arrow showed apoptotic cells. (H&E X 40)

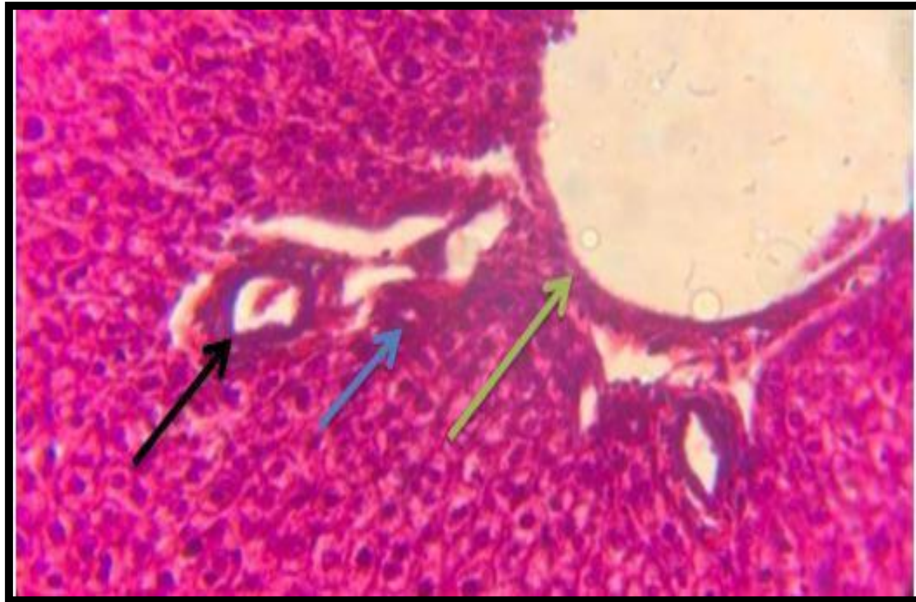


Figure (4-10) Histological section in the liver of animal treated with 100 mg/kg B.W of ferrous sulfate In this section show branch of portal vein and the black arrow show sever deposited of hemosiderin around the hepatic artery in the same time the blue arrow show sever deposited of hemosiderin around the bile duct and the green arrow shows moderate deposited of hemosiderin around portal vein (H&E X 40)

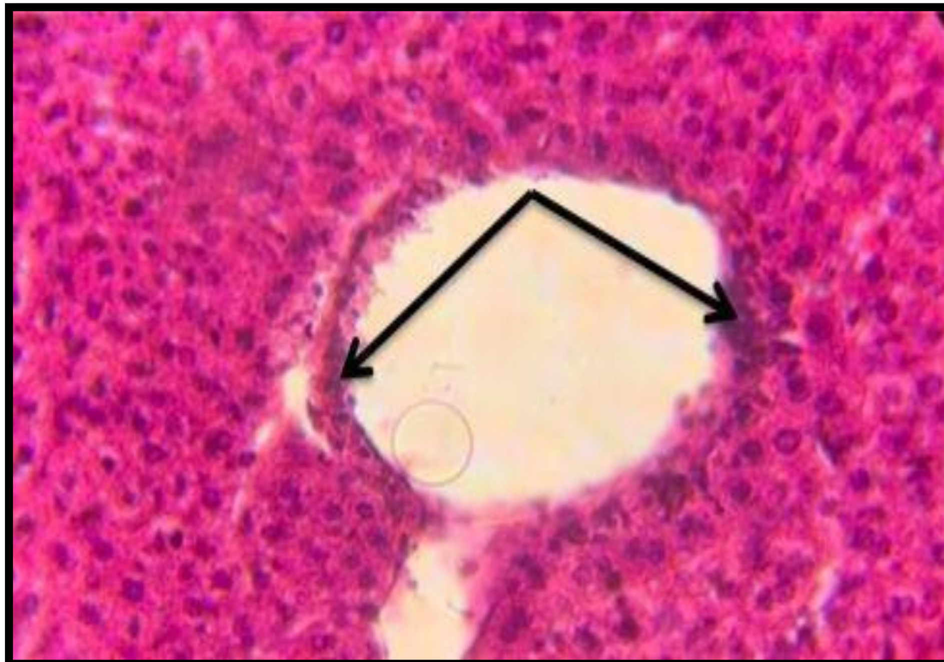


Figure (4-11) Histological section in the liver of animal treated with 100 mg/kg B.W of ferrous sulfate In this section show liver parenchymal cells with hemosiderin deposition and the black arrow shows hemosiderin deposition around the portal vein. (H&E X 40)

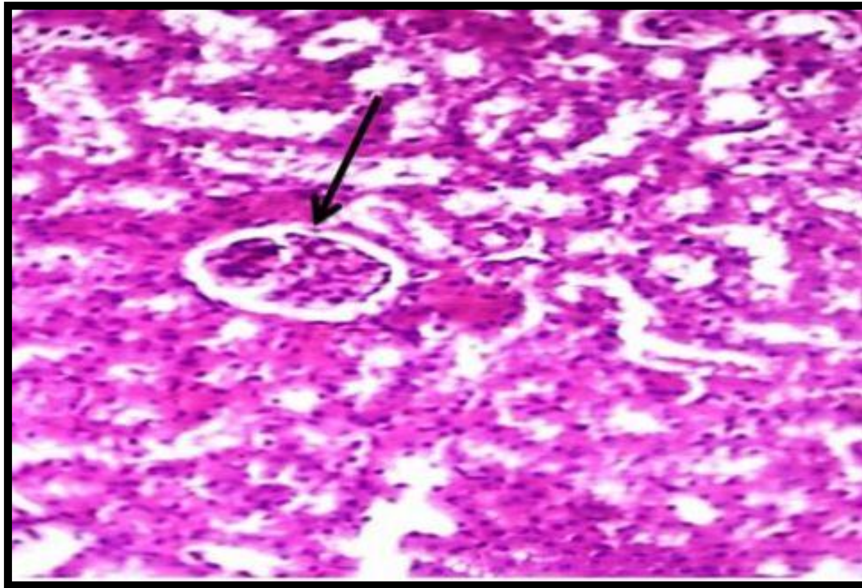


Figure (4-12) Histological section in the kidney of animal in Control group show normal size of renal corpuscle with small corpuscle space (black arrow) (H&E. X 20).

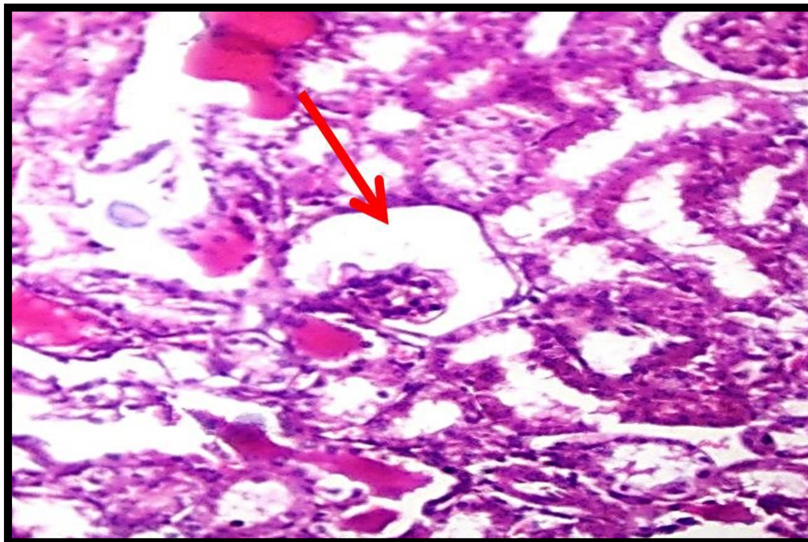


Figure (4-13) Histological section in the kidney of animal treated with 100mg/kg B.W methionine note Glomerular atrophy and enlargement in urinary space (red arrow) (H&E X 20).

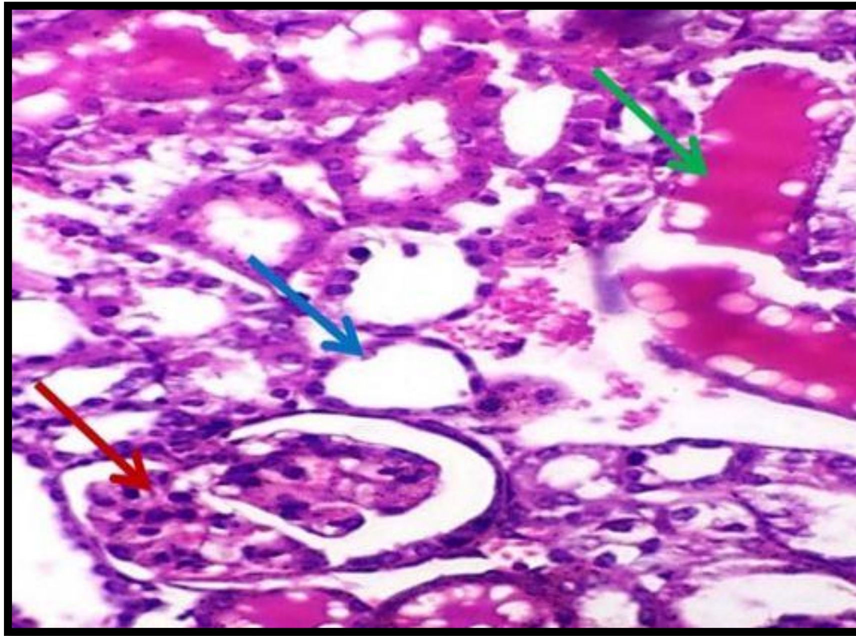


Figure (4-14) Histological section in the kidney of animal treated with 100mg/kg B.W methionine note atrophy in distal tube (blue arrow), accumulation of Hyaline cast (green arrow) enlargement in urinary space (red arrow) (H&E X 40)

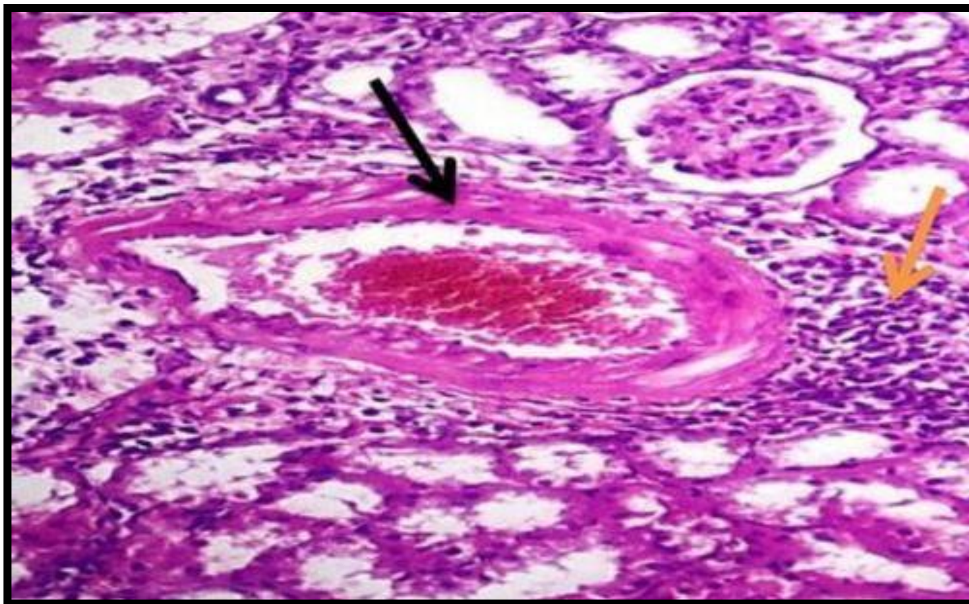


Figure (4-15) Histological section in the kidney of animal treated with 100mg/kg B.W methionine note infiltration of mononuclear inflammatory cells (orang arrow) and congestion artery with enlargement in tunica intima (black arrow) (H&E X 40).

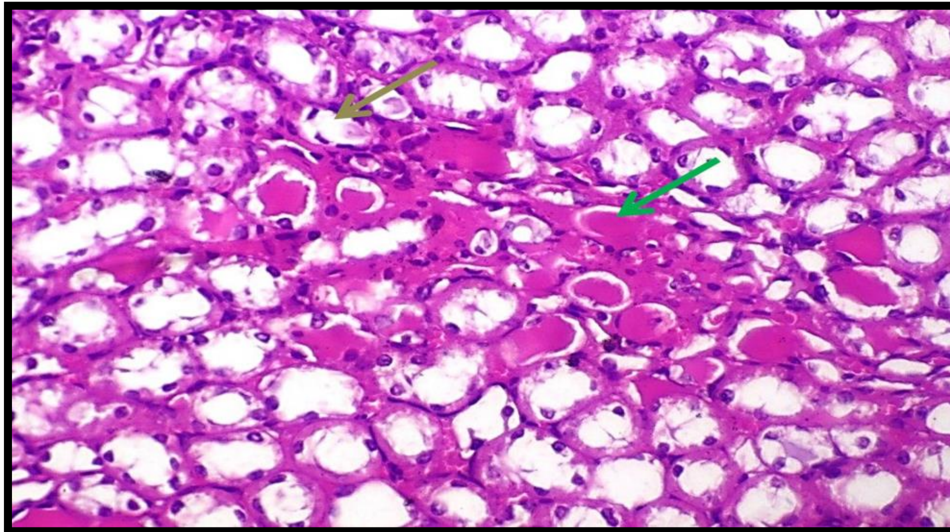


Figure (4-16) Histological section in the kidney of animal treated with 100mg/kg B.W methionine note accumulation of Hyaline cast (green arrow) and atrophy of the thin part of Helne loop (grey arrow) (H&E X 40)

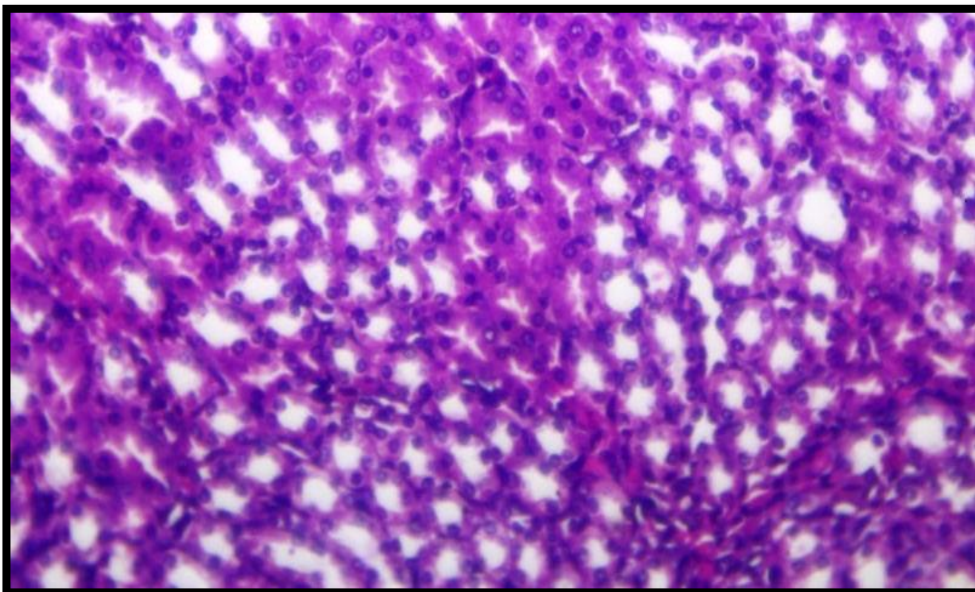


Figure (4-17) Histological section in the kidney (medulla)of animal treated with 0.07mg/kg B.W of folic acid note not accumulation of Hyaline cast Compared with the kidney of animal treated with 100mg/kg B.W methionine (H&E X 20).

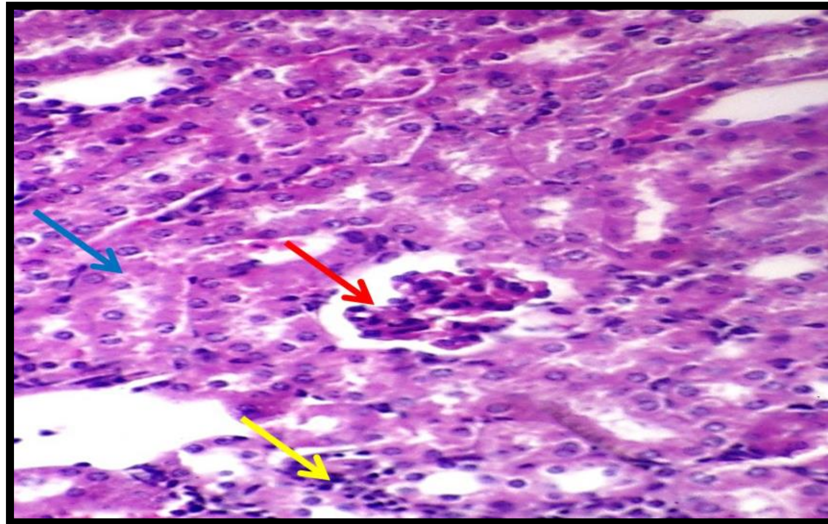


Figure (4-18) Histological section in the kidney of animal treated with 100mg/kg B.W methionine and 0.07mg/kg B.W of folic acid note Glomerular atrophy but less than that of the methionine group (red arrow), infiltration of inflammatory cells (yellow arrow), the cytoplasm of the proximal tubules return to pink (acidic) in color indicating a state of healing (blue arrow) (H&E X40).

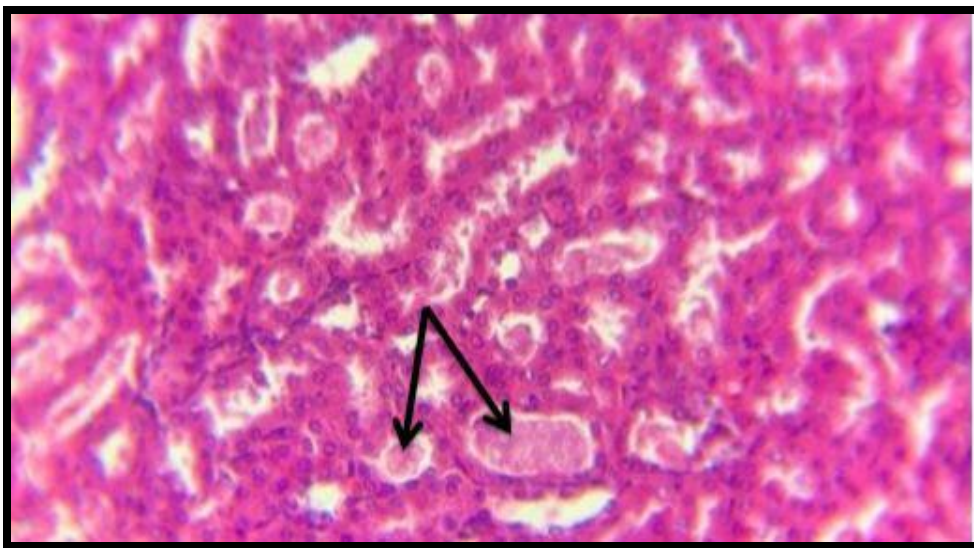


Figure (4-19) Histological section in the Kidney (medulla) of animal treated with 100 mg/kg B.W of ferrous sulfate in this section the black arrow shows renal tubules (proximal and distal tubules) filled with cast due toxic materials (nephritis) with lymphocyte infiltration around the tubules and in kidney tissue (H&E X 40)

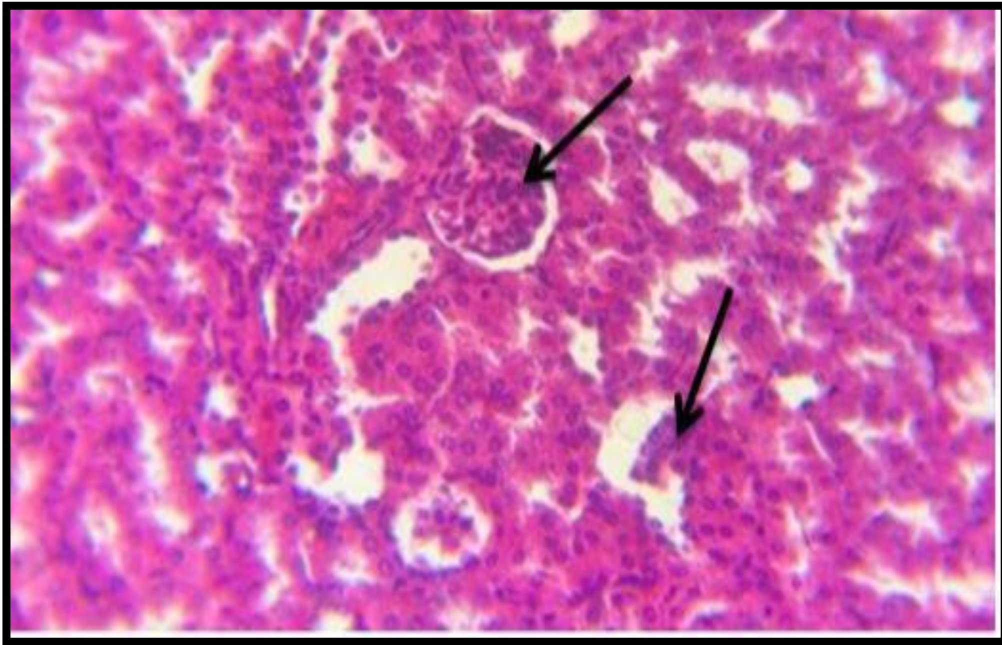


Figure (4-20) Histological section in the Kidney of animal treated with 100 mg/kg B.W of ferrous sulfate in this section the black arrow shows the shrinking glomeruli with deposition of hemosiderin in the glomeruli and renal tubules that lead to renal nephritis with lymphocyte infiltration around the glomeruli and around the renal tubules. (H&E X 40)

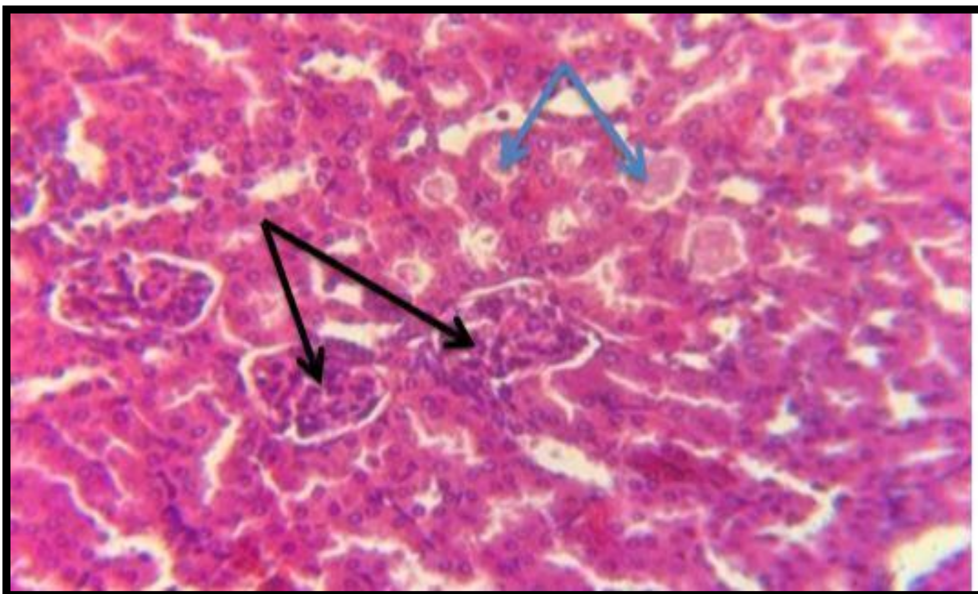


Figure (4-21) Histological section in the Kidney (medulla) of animal treated with 100 mg/kg B.W of ferrous sulfate in this section the black arrow shows the glomeruli with deposition of hemosiderin in the glomeruli and the blue arrow shows proximal and distal renal tubules filled cast of toxic materials that lead to renal nephritis with lymphocyte infiltration around the glomeruli and around the renal tubules (H&E X 40).

Chapter Five

Discussion

5.1. Effect of methionine and iron overload on Physiological parameters

5.1.1. Effect of methionine and iron overload on the antioxidants status.

Studying the effect of methionine and folic acid and iron overload on the antioxidant status of the male mice in the present study ,showed a significant decrease in serum GSH concentration in methionine treated group and in iron group such changes may be attributed to HHcy induced after methionine overload. ,the result of the present study was correlated with(Ventura *etal*.,2000; Huang *etal* .,2001; Hayden and Tyage ,2004; Jordao Júnior *etal*.,2009; Albazi ,2009; Waly *etal* .,2011; Hsu *etal*.,2015; Çelik *etal*.,2017;Cacciapuoti,2019)

Many authors recognized that methionine overload caused HHcy through disturbing remethylation pathway, preventing normal conversion of SMTH to methionine and subsequent stress of transulfration pathway ,such HHcy will lead to formation of homocysteine S-S mixed disulfide conjugates which inhibit the superoxide radical scavenging activity of metallothionine (Barbato *etal*., 2007).

The suppression effect of methionine overload on the antioxidant status of the rats in the present study may be attributed to hyperhomocysteinemia (HHCY) induced after methionine overload led to a decrease in the level of GSH (Al-Hashmy and Khudiar, 2009; Kim *etal* .,2015).

Accordingly, it can hypothesized that a depression in scavenging activity of metalothionine after methionine overload may lead to superoxide production and decrease in the antioxidant production including GSH in this study.

Moreover, such predicted HHcy after methionine overload may decrease the ability of the cells to detoxify H_2O_2 and other lipid peroxide (Petrak *etal.*, 2007; Tounyz and Schifrin, 2008; Sanchez-Roman *etal* .,2012 ; Liu *etal* .,2013) and it might indirectly produce oxidative reduction in the activity of intracellular antioxidant enzyme.

Low serum levels of GSH and total antioxidant capacity are considered to be indicators of oxidative stress, a metabolic condition that involves high level of HCY, under oxidative stress, the cellular antioxidants capacity is not counterbalancing the oxidative damage induced by various insults including, free radicals and environmental toxins (Waly *etal* .,2011).

Concerning TBA reactions (the biomarker of oxidative stress), serum MDA level was significantly increased as results of methionine overload and iron group, although MDA as a maker of oxidative damage has been studied extensively , generally, very variable and conflicting results have also been reported in various tissues and plasma of animals model and human exposed to methionine overload (Tug *etal*.,2006; Karamouzis *etal*.,2008 ; Liu etal.,2013; Feng *etal*.,2018).

The subsequent increase in serum MDA concentration in the current study may be attributed to high sensitivity of mice to free radicals production by Hyperhomocysteinemia. an elevation in generation of lipid peroxidation (MDA level) was postulated to cause a gradual cell injury by free radicals liberating lipoxygenase enzymes which oxidized unsaturated membrane fatty acids and subsequent production of MDA ,overwhelming endogenous scavenging system including GSH resulting in oxidative stress (Weiss *etal*.,2001; Handy and Zhang, 2006; Stark, 2005; Albazi ,2009; Kim *etal* .,2015).

MDA is an end product of lipid peroxidation that belongs to the compounds reacting with thiobarbituric acid. MDA diffuse to distant cellular structures where it can cause further cellular damage, including DNA damage, MDA level was used as a marker of lipid peroxidation (Hosseini *et al*., 2011; Al-Beer,2013)

The increase of MDA level may be due to an increase in the production of free radicals more than ability of the scavenging system to remove them increased serum MDA and decrease GSH levels ,this findings is in agreement with many laboratory studies which indicated alteration in the antioxidants status of different tissue as a results of an increase in lipid perooxidation of these tissue

after induction oxidative stress (Khudiar , 2000 ; Jordao Júnior *etal*.,2009; Albazi,2009;AL-Zubaidi , 2007; Hsu *etal*.,2015 ; Al-Okaily *etal*.,2015).

Intubation of folic acid to methionine treated mice for six weeks showed a significant increase in serum GSH and decrease in serum MDA concentration.

The antioxidant effect of folic acid is documented, such effect may be due to reduction of blood homocysteine levels and then depression of lipid peroxidation and free radicals production by folic acid(Pastore *etal*.,2006;Panigua *etal* .,2007;Abazi,2009). One other possible pathway is that folic acid may effectively inhibit NADPH oxidase mediated superoxide production leading to reduction of lipid peroxidation and subsequent decrease in MDA level (Yong and Zou , 2003).

There is a strong correlation between increase GSH levels and decrease in lipid peroxidation (MDA production) after folic acid intervention , these biomarkers of oxidative stress are interrelated , and they assure that folic acid induced suppression of oxidative stress of affected mice .

Moreover , GSH regarded as a major cellular antioxidant so it is elevation after folic acid intubation augmented cellular protection against free radicals damage (Kitamoto *etal*., 2004;Villa *etal*., 2004;Sharma *etal*.,2006) , induced by methionine overload.

Homocysteine increases oxidative stress as reflected by oxidative (malondialdehyde and carbonyl levels) and antioxidative parameters (plasma sulphydril, reduced glutathione, superoxide dismutase and catalase levels) (Çelik *etal* .,2017).

The current study indicated that intubation of iron to adult male mice for six weeks caused a significant increase in MDA concentrations and a significant decrease in GSH concentrations the result of the present study was Agreement with (Takea and Mhsen, 2019).

While some sources indicated the role of iron overload in the release of free radicals, which leads to inhibition of glutathione released from the liver, and this substance works to maintain the level of Hemoglobin in the red blood cell and

also works to inhibit the enzyme glutathione peroxidase, which is important in the removal of peroxide of hydrogen H_2O_2, so the inhibition of this the enzyme works to reduce the lifespan of the haze, and this was agreed with (Al-Ghreaty , 2011).

Iron and hydrogen peroxide are capable of oxidizing a wide range of substrates and causing biological damage. The reaction, referred to as the Fenton reaction, is complex and capable of generating both hydroxyl radicals and higher oxidation states of the iron. The mechanism and how it is affected by different chelators (Winterbourn, 1995).

The Haber-Weiss reaction is a specific example of the Fenton reaction. This term refers to the reaction between hydrogen peroxide and ferrous salts to produce a reactive species capable of oxidising a wide variety of organic substrates, if Fe^{2+} can be recycled from Fe^{3+} ,the iron can act catalytically (Winterbourn, 1995).

5.1.2. Effect of methionine and iron overload on serum Albumin and Globulin

The current study indicated that intubation of methionine and iron to adult male mice for six weeks caused a significant decrease in serum albumin and globulin concentrations The result of the present study was Agreement with (Al-Bazii, 2009; Liu *etal* .,2013; Micovic *etal* .,2016).

Methionine overload and subsequent Hyperhomocysteinemia markedly suppressed voluntary food intake , and micronutrient deficiency that lead to growth retardation (Jolin *etal* ., 2005; AL-qanbar *etal* .,2020) .

Protein molecules are essential target for free radicals attack, both intracellularly and extracellularly (Faraci and Lentz,2004; Au-Yeung *etal*.,2004) where under conditions of severe oxidative stress, free radicals generation at inappropriate sites could lead to protein modification. Proteins are also modified indirectly with reactive carbonyl compounds formed by autoxidation of carbohydrate and lipid, with eventual formation of advanced glycation and lipid

peroxidation (Inagi and Miyata, 1999; Mangiagalli *etal*., 2004; Sans *etal*.,2006). The consequence of such damage may impair enzymatic activity, modified membrane and cellular function.

Sulfhydral group of serum proteins, including serum albumin, have been suggested to be a sacrificial antioxidant in plasma and extravascular space, thus free radicals produced after methionine overload may mediated oxidation and poor degradation of albumin leading to its depletion (Halliwell, 2001; Al-kenany, 2010).

Apart from activating the unfolded protein response, Hcy may be potentially cleave critical protein disulfide bound resulting in the alteration of structure and /or function of proteins (Majors *etal*.,2002). This might also lead to protein misfolding / unfolding with suppression of serum protein concentration.

Oral intubation of 0.07mg /kg BW/day of folic acid to for six weeks to mice which that to methionine over load caused a significant elevation in serum albumin and globulin The result of the present study was Agreement with (Al-Bazii, 2009; Al-kenany, 2010) .

folic acid treatment significantly increased plasma total protein and globulin ,and then plays a role of protection of toxicity and treatment with folic acid resulted in an increase in protein concentrations accompanied by changes in the protein synthesis rate (Komatsu and Tsukamoto,1998; El- Demerdash *etal* .,2006).

Folic acid is necessary to reduce the level of total protein, bilirubin and globulin and to reduce AST, ALT and ALP levels . and used folic acid reduction of inflammatory cell number and less degenerative changes (Al-Sowyan,2009).

Hypoalbuminemia has been reported in hyperhomocystenemic patients, which serve to reduce the overall antioxidant protection against disease development (Herlin *etal*., 2001; Rondal, 2002; Santilli *etal* ., 2016) including albumin, therefore the hypohomocysteinemic effect of folic acid, may participate in elevation of serum albumin level (Stadman *etal*.,1998).

Besides , folic acid supplementation may suppress the formation of protein content of carbonyl (Solini *etal*., 2006) leading to reduction in protein glycation and peroxidation end product , alleviate the damage in cellular function leading to maintenances of protein content of liver cells and extracellular fluid (Mimic-Oka *etal* .,2001; Al-Okaily *et al* ., 2015).

5.1.3. Effect of methionine and iron overload on ALT, AST and ALP activities.

Transaminases (ALT and AST) are two closely related enzymes of clinical significance, particularly in the assessment of liver function (Tiikkinen *et al*., 2004). In this study a significant elevation in both transaminase enzymes in animals treated with methionine for six weeks were observed , indicating occurrence of liver disorder (Ferre *et al* . , 2002 ; Ebbesen and Ingersity , 2005; Albazi ,2009;Kim *etal*.,2015; Micovic *etal* .,2016).

Methionine overload may caused oxidative stress in the most of the body cells causing release of free radicals in tissue cells leading to damaging the cell membrane and central portal liver cells with subsequent release of AST and ALT enzymes (Garcia-Terijano *etal* .,2001;Sahi *et al* ., 2006).

Homocysteine metabolism and its synthesis occur in the liver as the liver plays a significant and essential role in Hcy metabolism, the liver is important in metabolism as it produces an important enzyme in Met and Hcy metabolism , and any defect that occurs in the liver tissue or enzymes that are responsible for the metabolism of Met or Hcy, as well as any genetic defect that occurs for the genes that are responsible for certain enzymes that enter the metabolic process (Significant decrease in expression in major genes) that contributes to disruption of blood Hcy levels (García-Tevijano *et al*., 2001).

The liver produce a large amount of ALT and AST which are secreted to the circulation with injury or death , where leakage enzymes escape from the cystol causing elevation in the serum level of these enzymes , in addition , release of

liver enzyme from cytosol can occur secondary to cellular necrosis with membrane damage (Al-Bazii, 2009) . This fact is documented in this study by the oxidative stress induced by methionine overload leading to cell damage and increase in these enzymes(Wolff , 2006; Kim *etal*.,2015)

Methionine overload in this study may lead to abnormal methionine metabolism and decrease in vitamin B6 this leading to elevation in ALT and AST concentration in the serum (Scott *et al*., 2005). Besides HHcy caused by folic acid deficiency may lead to increase in ALT and AST concentration (Suzuki *et al*., 1999).

On the other hand, Serum alanine aminotransferase and aspartate aminotransferase are widely used as markers for acute and chronic hepatocellular damage due to various causes (Dufour *et al*., 2000).

The correlation between hepatic tissue damage and elevation of liver enzymes activities is well documented (Sidho *et al*., 2004; Sukayra , 2005; Abrahaim *et al* ., 2006 ; Al-Hazza , 2008; Jordao Júnior *etal*.,2009).

The present study revealed that administration of folic acid effectively improved liver function by return to normal levels AST and ALT enzymes activities. The observed restorations in the activity of previous enzymes after folic acid intubation are in agreement with previous studies (Ferit-Cursu *et al*., 2004; Al-Bazii, 2009; Santilli *etal* ., 2016)

It has been recently found that hepatic NADPH oxidase was activated during HHcy leading to increased superoxide anion production and peroxynitrite formation in the liver resulting in hepatocellular damage (Adinolfi *et al* ., 2005) , accordingly folic acid supplementation may be effectively inhibit NADPH oxidase –mediated liver dysfunction , with subsequent restoring aminotranaminase activities (Ungvari *et al*.,2003).

Similarly Ekaidem and his colleaques (2007) reported that serum ALT and AST activities were return to normal activity by folic acid therapy. It has been found that folic acid supplementation quickly and efficiently normalized hepatic

function and reversed the sustained elevation in aminotransferase activities in patients with rheumatoid arthritis (Niesen *et al* .,2004; Keech *etal.* ,2005) , and liver cirrhosis (Ferre *etal* ., 2002).

The hypohomocysteinemia effect of folic acid and the resultant improvement of functional activity of damaged hepatocyte may be responsible for the depressor effect of folic acid on ALT and AST activities(Kim *etal* .,2004).

The current study indicated that intubation of iron to adult male mice for six weeks caused a significant increase in AST and ALT level. These results are in agreement with (Stal *etal*.,1996;Olynyk *etal* .,1995; Mohammad and Al-Doski , 2012) .

5.1.4.Effect of methionine and iron overload on iron parameters (serum iron ,transferrin saturation and Ferritin)

Oral intubation of 100mg /kg B.W/day of methionine to adult male mice for six weeks led to a significant increase in serum iron level with a slight increase in ferritin and a decrease in blood serum levels of transferrin , these results are in agreement with (Mori and Hirayama, 2000; Micovic *etal* .,2016).

The results showed that intubation of iron to adult male mice for six weeks led to a significant increase in the average serum iron levels, transferrin saturation and Ferritin in the experimental animals, these results are in agreement with (Argyropoulou *et al* , 2000; Turbino-Ribrro *et al*., 2003; Mahachoklertwattana *et al* , 2003; Jasim ,2008; ; Al-Ghreaty , 2011; Al-salame , 2011;Mohammad and Al-Doski ,2012; Takea and Mhsen, 2019).

Iron (Fe) catalyses the formation of Hcy from methionine, S-adenosylhomocysteine and cystathionine, thus increasing the circulating tHcy levels. Furthermore, free Fe catalyses the production of free oxygen radicals and the oxidation of small density lipoproteins, which is a known risk factor for vascular damage, while the tendency for increased iron levels was observed in methionine group, where the homocysteine levels were proportionally higher,

which is a very important fact. Accordingly, (Lee *et al*.,2015) noted that the increased values of iron in blood were related to mitochondrial dysfunction, which might result in increased ROS production; therefore, in the conditions of iron overload, N-acetylcysteine could be used as an antioxidant with the capacity to reduce (ROS). this knowledge of the significance of Fe as a diagnostic and prognostic parameter, especially in conjunction with other biochemical parameters, is thus of great importance given that hyperhomocysteinemia is increasingly described in the literature as an independent risk factor for cardiovascular diseases (Baggott and Tamura, 2015; Lee *etal*.,2015; Micovic *etal* .,2016) .

Taking methionine over load periods has a detrimental effect on iron regulation and its metabolism, and the related proteins contribute to a decrease in blood serum levels of transferrin (Mori and Hirayama, 2000; Micovic *et al*.,2016).

Iron produces homocysteine from cystathionine, S-adenosylhomocysteine and methionine and increases the oxidation of lipoproteins of small density and free oxygen radicals(Micovic *etal* .,2016).

The increase in free iron may be due to the high oxidative stress in animals in which the iron liberates free radicals, which leads to a depletion of the antioxidant reserve (Jawad, 2005) as iron accumulates as a result of continuous injection and as a result of the absence of a major physiological pathway to be excreted from the body, which leads to This element is collected in all parts of the body, especially in the liver, heart and spleen (Al- salame , 2011). While the increase in the transferrin is due to the increase in the levels of iron in the plasma stimulates the liver to transferrin in order to get rid of iron.

Iron is an important and essential element in all living organisms and has a major role in a variety of complementary metabolic pathways. It also enters in hematopoiesis, energy metabolism, DNA synthesis, oxygen transportation, and mitochondrial biogenesis. In the case of taking extra iron, it causes disease.

hemochromatosis and one of the causes of chronic liver and cirrhosis cases is iron deposition in the liver Because it is considered an exacerbating factor(Yamauchi *et al*., 2019).

Excessive iron can be toxic because free ferrous ion reacts with peroxides to produce free radicals, which was highly reactive and can damaged DNA, protein, lipids and other cellular components (Celik *et al* ., 2009).

5.1.5.Effect of methionine and iron overload on hepcidin level.

The results showed that intubation of iron and methionine to adult male mice for six weeks led to a significant increase in the average serum hepcidin level in the experimental animals, these results are in agreement with (Nemeth and Ganz , 2009; D'angelo, 2013; Anderson and Shah ,2013) .

Hepatocytes have a crucial role in the mobilization of iron to satisfy metabolic requirements , The liver produces the majority of proteins involved in iron metabolism, including hepcidin and transferrin. The main characteristic of transferrin is its ability to reversibly bind iron, which allows it to be a cellular iron donor or iron acceptor (Muñoz *et al*. ,2009; D'angelo, 2013).

Researchers found that When feeding mice with a diet containing a low percentage of iron, hepcidin hormone activity decreased (Truksa *etal* .,2009; Masaratana *etal* .,2013) . Hepcidin is also homeostatically regulated by iron loading. Dietary iron or transfusions increase hepcidin synthesis, however, the molecular mechanisms of this regulation are still unclear (Ganz , 20015) .

hepcidin synthesis is increased by iron loading and decreased by anemia and hypoxia. Additionally, hepcidin synthesis is greatly increased during inflammation the transport of iron by ferroportin across the basolateral membrane of duodenal enterocytes determines whether the iron is delivered to plasma transferrin or removed from the body when the enterocytes shed into the intestinal lumen. When iron stores are adequate or high, the liver produces hepcidin, which circulates to the small intestine. There hepcidin causes ferroportin to be internalized, blocking the sole pathway for the transfer of iron

from enterocytes to plasma. When iron stores are low, hepcidin production is suppressed, ferroportin molecules are displayed on basolateral membranes of enterocytes, and there they transport iron from the enterocyte cytoplasm to plasma transferrin (Ganz, 20016).

More hepcidin is released when iron is plentiful, which suppresses more iron absorption and release and creates less to no hepcidin in iron deficiency, allowing more iron to enter the plasma (McLachlan *et al*.,2017)

hepcidin is homeostatically regulated by iron and erythropoietic activity. Iron excess stimulates hepcidin production, and increased concentrations of the hormone in turn block dietary iron absorption thus preventing further iron loading Conversely, hepcidin is suppressed in iron deficiency, allowing increased absorption of dietary iron and replenishment of iron stores. Increased erythropoietic activity also suppresses hepcidin production. Apart from enhancing iron absorption, this enables the rapid release of stored iron from macrophages and hepatocytes and augments the supply of iron for erythropoiesis. The molecular mechanisms underlying hepcidin regulation by iron and erythropoiesis are areas of intense investigation but are still incompletely understood (Nemeth and Ganz , 2009) .

Hepcidin performs its different functions via a single biochemical mechanism: hepcidin-ferroportin interaction , Intestinal epithelial cells and reticuloendothelial macrophages use the same transporter, ferroportin, to transport iron in the plasma. Moreover, macrophages and enterocytes exhibit strong upregulated ferroportin expression in the erythropoietic response in an iron-restricted state (D'angelo, 2013).

The results of the current study showed that when intubating folic acid to mice for six weeks, there was no significant difference in the level of hepcidin compared to the control group, due to its antioxidant effect and due to the absence of an increase in the level of iron in the serum.

5.1.6.Effect of methionine and iron overload on red blood cell counts, pack cell volume and Hemoglobin concentration:

Important associations were found between serum homocysteine and markers of iron deficiency. Monitoring homocysteine levels might be essential to understand the development of different clinical conditions including anemia ,They found the hemoglobin level decreased when anemia and high homocysteine occurred(Liu *et al* .,2013;Sirdah *et al* .,2014).

The current study indicated that intubation of iron and methionine to adult male mice for six weeks caused a significant decrease in the number of red blood cells (RBC) compared to the control group. As for the Red blood cell count , Hemoglobin and on Packed cell volume , their values were also observed to decrease, The result of the present study was Agreement with (Dabbagh *et al*., 1997; Afroditi, 2006; Al-Ghreaty , 2011; Al- salame , 2011; Mohammad and Al-Doski , 2012; Abu-Taweel *et al*., 2013 ;Takea and Mhsen, 2019).

It seems that iron stores in the blood cells (Hemoglobin) have been saturated since the beginning of the intubated with iron overload and that repeated injections lead to adverse damage to the red blood cells as a result of the release of free radicals that affect the membranes of the red blood cells, which leads to their breakdown and decomposition in the bloodstream, thus reducing The rates of blood cell preparation, the volume of the stack and the percentage of Hemoglobin, as there is a direct relationship between hemoglobin and the volume of compacted blood cells, and this confirms what (Cornejo *etal*.,2005; Takea and Mhsen, 2019), indicated that there is a direct relationship between the rate of hemoglobin levels and the volume of compacted blood cells and that each confirms the other in their levels in Blood, and this decrease may be due to transferrin saturation, as there is an increase in iron (III), which is liberated in the serum as a result of continuous intubated of iron and participates in uncontrolled reactions (irregular) as the ferric ion does not have the ability to

transport oxygen, so there will be a decrease in oxygen levels. The red blood cells that circulate in the bloodstream, which in turn lead to a reduction in survival rate, and this is consistent with what(Kaneko *etal*.,1997; Al-Ghreaty ,2011) mentioned. a deficiency in the red blood cell preparation causes blood loss and thus dilution, thus reducing the PCV values.

On the other hand, Demir and Öner (1995) note that the decrease in Red blood cell count , Hemoglobin and Packed cell volume level occurs as a result of the erythrocyte wall being broken through the effect of heavy elements on the lipids and proteins that make up the wall, as well as the effect on the permeability of the membrane.

While some sources indicated the role of iron overload in the release of free radicals, which leads to inhibition of glutathione released from the liver, and this substance works to maintain the level of Hemoglobin in the red blood cell and also works to inhibit the enzyme Glutathione peroxidase, which is important in the removal of peroxide of hydrogen H_2O_2, so the inhibition of this the enzyme works to reduce the lifespan of the haze, and this was agreed with (Al-Ghreaty , 2011)

Exposure to Iron overload can cause toxic effect on many tissues, however the first to be influenced is blood, as reactive oxygen species combined with RBCs membrane then transferred to the liver. RBCs are the most common pointers of oxidative stress because of their cell membranes sensitivity and the sensitivity of its enzymes to free radicals (Takea and Mhsen, 2019).

The current study indicated that intubation of folic acid and methionine-folic acid to adult male mice for six weeks caused a non-significant in the number of RBC compared to the control group , Folic acid is many important for red blood cells and white blood cells must also be developed and matured, as well as protein metabolism and energy production (Ambrosczhiemicz *et al* ., 2006) , Folic acid alone or folic acid combined with other B-vitamins have all been

shown to reduce hHcy concentration in patients on chronic treatment with antiepileptic drugs(Al-Ghreaty , 2011;Belcastro and Striano, 2012) .

5.1.7. Serum bilirubin concentration

Studying the effect of methionine and folic acid and iron overload on the bilirubin concentration of the male mice in the present study ,showed a significant increases in Serum bilirubin concentration in methionine treated group and iron group the results was agreement with(Abu-Taweel *et al*., 2013 ;Takea and Mhsen, 2019) , when mice treated with methionine overload due to an increase in the processes that produce oxidants

As homocysteine levels increase, cell damage is caused by high free radicals and oxidative stress and therefore cell apoptosis, because oxidative stress is the main cause of apoptosis (Mangiagalli *et al*., 2004).

Intubation of folic acid to methionine treated and in group Intubation of folic acid only to mice for six weeks showed non-significant Serum bilirubin concentration due to the effect of folic acid as an antioxidant and It does not cause degeneration , Folic acid is essential to sustain all cells as critical co-enzymes of normal DNA and RNA synthase for natural growth and participates in many important metabolic processes in the body and is essential for nucleic acids as it protects the protection of cell division and maintains genetic codes (Stopper *et al* ., 2008) , red blood cells and white blood cells must also be developed and matured, as well as protein metabolism and energy production (Ambrosczhiemicz *et al* ., 2006) .

level of serum bilirubin was increased carefully in mice administrated iron for six weeks were observed and this refers to an increases in oxidative stress which may be due to a decrease in antioxidant defenses or due to an increase in the processes that produce oxidants , The result of the present study was Agreement with (Abu-Taweel *etal*.,2013; Umran *etal*., 2013; Younis, 2017;Takea and Mhsen, 2019).

5.2. Effect of methionine and iron overload on gene expression

Through our experience, it was observed that when a high dose of iron was given, it led to an increase in the gene expression of the Hamp 1 gene, and this result was in agreement with (Ilyin *et al* .,2003; Willemetz *et al*.,2014; Lu *etal*.,2015; McLachlan *et al*.,2017).

HAMP1 is the gene responsible for the production of Hepcidin, which regulates and secretes the Hepcidin hormone , When the liver senses high amounts of iron, Hamp gene begins with gene expression and production of hepcidin, which in turn prevents the absorption of iron from the intestine by inhibiting the iron transporter ferroportin on duodenal enterocytes (Miranda *et al*., 2019).

Iron excess is regulated through a pathway involving the cell surface receptor hemojuvelin that stimulates expression of the hepcidin encoding gene (HAMP) (Truksa *et al*.,2009).

iron overload prompted a down regulation of ferroportin, associated with an up regulation of hamp1, whereas an opposite response was observed during anemia (Neves *etal* .,2017)

When feeding mice with a diet containing a low percentage of iron, hepcidin hormone activity decreased and hepcidin gene expression was found (Masaratana *etal* .,2013) .

The effects of hepcidin excess were shown in mice that overexpressed hepcidin-1 under the control of a liver-specific promoter Excessive hepcidin also blocked intestinal iron uptake (Ganz, 2006).

Found that serum hepcidin -1 levels correlated well with liver Hamp1 expression (McLachlan *et al*., 2017).

When the liver senses high levels of circulating iron, the HAMP gene (Hepcidin) is up regulated and secreted into the blood stream, which prevents absorption of dietary iron by inhibiting the iron transporter ferroportin on duodenal enterocytes (Miranda et al., 2019).

the presence of hepcidin in a variety of tissues suggests that it plays an important and wide-ranging role in innate immune defenses ,however, hepcidin's primary role appears to be hormonal, serving as a negative regulator of iron homeostasis. In mammals, the liver is the primary site of HAMP expression (Hilton and Lambert, 2008).

HAMP expression increases in response to inflammation and iron overload and decreases under conditions of anemia or hypoxia(Hilton and Lambert, 2008).

Taking methionine over long periods has a detrimental effect on iron regulation and its metabolism, and the related proteins contribute to a decrease in blood serum levels of transferrin and iron (Mori and Hirayama, 2000).

5.3. Effect of methionine and iron overload on histological section

The present study pointed that intubation of male mice with methionine (100mg/kg B.W/daily)for six weeks , while histological sections of liver showed multifocal granulomatous lesion indicating occurrence of oxidative stress by methionine overload and corresponding Hyperhomocysteinemia .

Concerning liver damage observed after methionine overload in this study , excess methionine intake induce tissue damage including liver enlargement , fatty liver and erythrocyte membrane damage. Hepatic dysfunction is a common clinical complication in HHcy The result of the present study was Agreement with (Al-Bazii, 2009; Al-Kinany, 2010) , although its pathogenesis remains largely unknown , HHcy induce the generation of hydroxyl radical (OH-) in the liver which may be responsible for the induction of oxidative stress (Shunhe and Nobuhiro , 2006) and consequently hepatic damage ,furthermore , the mechanism of methionine overload induced hepatic damage may be through distributing iron intake leading to iron accumulation in the liver (Mori and Hirayama , 2000) .Subsequently , the excess iron might accelerate the production of reactive oxygen species , which may contribute to the observed liver damage (Kovaceic and Richardson, 2006).

Moreover, the ability of Hcy to promote cholesterol biosynthesis in hepatocytes would provide the plausible mechanisms of HHcy associated liver pathology and suggested that HHcy induced oxidation in the liver cells , leading to fatty liver , apoptosis and possibly inflammation(Mario *etal* .,2006).

also folic acid has protective effects against liver damage through restoration of glutathione level and enhancing the activities of antioxidant enzymes (Ambrosczkiemicz *etal*., 2006) as it was documented by this study.

Taking methionine in large quantities leads to a large increase in the proportion of blood urea nitrogen, and the high rate leads to kidney disorders , particularly glomerular function disturbance, and the increase in hyperhomocystinemia leads to a decrease in the glomerular filtration rate, kidney damage and increased concentration of creatine and urea in the blood and increased production and release of glomerulus reactive oxygen species, resulting in damage and weakness of the urinary system (Al-Hashmy and Khudiar, 2009).

Methionine overload works on degeneration of the epithelial cells lining the renal tubes and severe vacuolar degeneration of the renal tubular epithelial lining cells and atrophy of glomerular tufts and infiltration of mononuclear cells between renal tubules. Antioxidant has been found to act to reduce oxidative stress and scavenge free radicals and reduce fat peroxide from excessive methionine processing, and to reduce methionine damage to the urinary system (Salman , 2014).

Excessive homocysteine in the blood will be followed by people with kidney failure which results in high levels of antioxidants and lipid peroxidation, this condition can be corrected if folic acid is given as a treatment (Jordao Júnior *et al* .,2009), Folic acid is an important vitamin cofactor needed to re-methylate Hcys to methionine in the metabolism of Hcys (Vijayakumar *et al* .,2017).

The results of the current study showed that iron deposition was hemosiderin in hepatocytes with inflammatory cells in the tissues in animals intubation of ferrous sulfate to adult male mice for six weeks, these results are in agreement

with (Olynyk, *et al*., 1995; Khan *et al*., 1999; Özgüner and Sayın, 2002; Al-Salame, 2011; Al-Ghreaty,2011) .

Iron excess induces cellular injury and functional abnormalities in hepatocytes by the process of lipid peroxidation . Because the liver has a central role in the maintenance of lipid homeostasis, excess iron may alter the concentration of serum lipids (Turbino- Ribeiro *et al*.,2003; Silva *et al*.,2008).

The effects of excess iron in the tissues and its accumulation may be due to an increase in the production of hemosiderin and the two fractions present in large amounts in the visceral tissues due to the continuous injection that allows it to accumulate in the liver , spleen and the rest of the tissues (Al-Salame, 2011).

The excess iron also has toxic effects as it promotes the production of ROS which can cause a series of chemical reactions in many biological molecules such as lipids, proteins , and DNA, as well as contribute to oxidation of membrane fats and association with unsaturated fats. Fatty acids(Lieu *et al*., 2001) and such reactions can result in tissue or organ damage that is obvious in iron-bearing disease (Wong and Richardson, 2003; Al-Ghreaty,2011) .

Excessively accumulated iron is phagocytosed by liver Kupffer cells and macrophages . the macrophage accumulation expanded from the portal triad into peripheral regions such that the macrophage distribution paralleled that of iron deposition. This suggests that the macrophage accumulation was a compensatory response to the iron deposition, and that the macrophages were there to phagocytose excess iron. On the other hand, macrophage accumulation can lead to chronic inflammation and organ damage. The greatly enhanced macrophage accumulation may thus reflect the development of chronic inflammation. , real-time PCR analysis revealed increased expression of genes related to inflammation, oxidative stress and fibrosis (Yamauchi *et al*.,2019).

Abnormal liver function is a well recognize complication of clinical states of iron overload such as many hematological disease , results revealed increasing in liver and kidney weight of almost all animals administrated in iron dextran either

treated or un treated with pomegranate extracts caused by accumulation of iron in liver which finally lead to fibrosis and improvement in kidney structure (Ahmed and Ali, 2012) , the iron accumulation damages the lysosomal membrane, releasing acid hydrolases into the cytoplasm and thus initiating cell damage (Umran *etal*.,2013).

The increase in ALT and AST may be due to damage to the tissues and organs of the body and exudation of enzymes from the cells, given that the liver and spleen are among the organs targeted for iron carrying due to iron deposition in hepatocyte cells and macrophage and thus leads to necrosis and fibrosis in the tissues or as a result of the breakdown of red blood cells in the bloodstream Due to the oxidation potential of iron, it is in agreement with the results of research (Ku *etal*., 1983; Bonkovsky, 1996) who observed that blood transfusions in thalassemia patients lead to iron storage in kupffer and phagocytic cells and thus damage the cells.

Chapter six

Conclusion and recommendation

Conclusions

From the results and discussion obtained from this study , it could be can concluded that :

A-Oral intubation of 100mg/kg B.W. of methionine for six weeks caused a case of oxidative stress and significant elevation in the serum MDA, ALT , AST ,ALP activities and significant increase in Iron level ,Ferritin , hepcidin Hormone and bilirubin concentration ,significant depression in serum GSH ,albumin and globulin concentrations, Transferrin ,RBC, Hemoglobin and PCV, and Kidney and hepatic damage was documented histologically , through formation of marked Kidney and hepatic lesions.

B-Oral intubation of Iron overload for six weeks caused a case of oxidative stress and significant elevation in the serum MDA, ALT , AST and ALP activities , a significant elevation in Iron level ,Ferritin , Transferrin, hepcidin Hormone and bilirubin concentration, significant depression in serum GSH, albumin , globulin concentrations ,RBC, Hemoglobin and PCV and Kidney and hepatic damage was documented histologically , through formation of marked Kidney and hepatic lesions .

C- give effective dose of folic acid with methionine for six weeks to mice caused regression of Kidney and hepatic damage (functionally and structurally) .

D- in our study was found the most common gene expression(HAMP) in the group with iron overload rather than other groups , and in the present study was fond decrease of gene expression in methionine only and folic acid only group and The results of the current study showed that when intubating mice with methionine and folic acid for six weeks resulted in no difference in gene expression .

Recommendation

To assure the harmful effect of methionine overload on different organs , current further studies are required :-

1. Electron microscopic study of different organs subjected to methionine and iron overload .
2. Effect of methionine overload on nervous system .
3. Effect of methionine and iron overload on the reproductive system and hormones .
4. Conducting a study to know the damage of iron on the immune system by measuring the immunoglobulin and interleukins.
5. Conducting a study to find out the role of other trace elements such as (chromium 6, mercury) as substances that cause oxidative stress in animal models.
6. Conducting a histopathological study of the heart and blood vessels of iron loads in animal models.
7. Use of multivitamin preparation consist of vitamin B6, vitamin B12, and folic acid for the prevention of hyperhomocysteinemia in animal models.

References

References

Abbasi, F., Facchini, F., Humphreys, M. H., & Reaven, G. M. (1999). Plasma homocysteine concentrations in healthy volunteers are not related to differences in insulin-mediated glucose disposal. Atherosclerosis, 146(1), 175-178.

Abrahaim, M. (2006). Elevation of liver function tests in patients of rheumatoid arthritis. MSC. Thesis, College of education, University of Baghdad. (Arabic)

Abu-Taweel, G; Ajarem, J.S and Ahmad, M. (2013). Protective effect of curcumin on anxiety, learning behavior, neuromuscular activities, brain neurotransmitters and oxidative stress enzymes in cadmium intoxicated mice. J. Behav. Brain Sci., 3:74–84.

Adinolfi, L.E.;INgrosso, D.;Cesaro, C. and Cimmino, A. (2005). Hyperhomocysteinemia and the MTHFR C6771 polymorphism promote steatosis and fibrosis in chronic hepatitis C patients. Hepatology., 41:995-1003.

Afroditi, H. (2006). Correlative study of iron accumulation in liver, myocardium, and pituitary assessed with MRI in young thalassemic patients. Pediatr Hematol. Oncol .,28 (5).

Ahmed, M .M . and Ali , S.E.(2012). Protective effect of pomegranate peel ethanol extract against ferric nitrilotriacetate induced renal oxidative damage in rats.journal of cell and mol.biol., 7 (2), pp: 35-43.

Akanji, A. O., Ohaeri, J. U., Al-Shammri, S. A., & Fatania, H. R. (2007) . Associations of blood homocysteine concentrations in Arab schizophrenic patients. Clinical Biochemistry, 40(13-14), 1026-1031.

Al-Bazii ,W.G.(2009). Protective And Therapeutic Role Of Olive Oil And Folic Acid In Some Biochemical Parameters And Histological Changes In

Male Rabbits Exposed To Methionine Over Load. PH. D .Thesis, College of veterinary medicine, University of Baghdad .

Al-Beer, A. A., Al-Shalah, H. H., & Abdul-Latif, A. R. (2013). The Role of Some B Vitamins in Methionine-Induced Hyperhomocysteinemia in Male Rabbits. Karbala Journal of Medicine, 6(1), 1539-1545.

Al-Beer, A.A,(2013).The Role of Some B Vitamins in MethionineInduced Hyperhomocysteinemia in Male Rabbits.Thesis. college of Medicine. University of Babylon.

Al-Ghreaty , H.B. A, (2011).Some changes physiological and histological caused by iron overload in male rabbits . MSC. Thesis, College of Education, University of Karbala (Arabic).

Al-Hashmy, R. A., & Khudiar, K. K. (2009). Protective role of alcoholic extract of black currant (Vitis Vinifera L.) on renal function of adult male rats exposed to methionine overload and hydrogen peroxide. Iraqi Journal of Veterinary Sciences, 23(3).

Al-Hazza, I. M. (2008). Cadmium induced hepatotoxicity and oxidative stress in rats: Protection by selenium .Reasech J. Genome., 2(4):305-309.

Al-kenany, R. K. M., Abd-Altaif, S. H., & Al-Bazii, W. G. (2019). Protective Role of Folic Acid in Oxidative Stress Induced by Methionine over Load on Lipids and Lipoproteins Levels in Female New Zealand Rabbits. Sci. J. Med. Res, 3(9), 47-50.

Al-kenany, R. K. M. (2010). Effect of methionine overload onsome hormonal and physiological parameters in female rabbits new zaland. MSc. Thesis, College of Education of Kerbala University. (Arabic)

Al-Okaily, B. N., Abdulla, L. N., & Khudair, K. K. (2015). The protective effect of pometone (pomegranate seed oil) on oxidants/antioxidant status in methio-nine overload rabbits (part III). Adv. Anim. Vet. Sci, 3(7), 377-383.

Al-Salame, H.A.(2011). Protective role of grape seeds oil (Vitis vinifera) on some physiological and hormonal changes induced by iron overload in male rabbits. MSc. Thesis, College of Education of Kerbala University. (Arabic)

Al-Shammry, Z. M. M., & Al-Okaily, B. N. (2009). Effect of alcoholic extract of black currant (Vitis vinifera L.) on hepatic function and damage induced by hydrogen peroxide and methionine overload in rats. Al-Anbar Journal of Veterinary Sciences, 2(2), 19-34.

Al-Sowyan, N. S. (2009). Efficacy and safety of folic acid during toxic hepatitis induced by acute overdose of paracetamol. International Journal of Pharmacology, 5(3), 208-14.

Al-Zubaidi, A. H. (2007). Comparative study between the prophylactics effects of aqueous extract of Black Currant (Vitis vinferia. L) Concentration and vitamin E on some biological parameters related with heart disease in oxidative stressed rats. MSc. Thesis ,College of veterinary Medicine,University of Baghdad.

Ambrosczkiemicz, J.; Klemarczyk, W.; Chelchowska, M.; Gajeewska, J, and Laskowska, T. (2006). Serum Homocysteine, folate, vitamin B12 and total antioxidant status in vegetarian children. Advances in Medical Sciences., 51(6): 51-56.

Ambrosino, P., Lupoli, R., Di Minno, A., Nardo, A., Marrone, E., Lupoli, V., ... & Di Minno, M. N. D. (2015). Cyclic supplementation of 5-MTHF is effective for the correction of hyperhomocysteinemia. Nutrition Research, 35(6), 489-495.

Anderson, E. R., & Shah, Y. M. (2013). Iron homeostasis in the liver. Comprehensive Physiology, 3(1), 315-330.

Anderson, O. S., Sant, K. E., & Dolinoy, D. C. (2012). Nutrition and epigenetics: an interplay of dietary methyl donors, one-carbon

metabolism and DNA methylation. The Journal of nutritional biochemistry, 23(8), 853-859.

Appleton, A., & Vanbergen, O. (2012). Crash Course: Metabolism and Nutrition E-Book. Elsevier Health Sciences.

Argyropoulou, M.I. ;Metafratzi, Z. ; Kiortsis, D.N. ; Bitsis, S. ;Tsatsoulis, A. and Efremidis, S. (2000). T2 Relaxation Rate as an Index of Pituitary Iron Overload in Patients with β-thalassemia Major. AJR. ,175: 1567-1569.

Au-Yeung ,K.;Woo ,C. ;Sang ,F. ;Yip ,J. ; Siow , Y. and O,K.(2004). Hyperhomocysteinemia activates nuclear factor-Kappa Bin endothelial cells via oxidative stress .Circ Res., 94:28-36.

Baggott, J. E., & Tamura, T. (2015). Homocysteine, iron and cardiovascular disease: a hypothesis. Nutrients, 7(2), 1108-1118.

Bailey, L. B., & Gregory III, J. F. (1999). Folate metabolism and requirements. The Journal of nutrition, 129(4), 779-782.

Balkan, J., Dogru-Abbasoglu, S., Cevikbas, U., AYKAÇ-TOKERI, G., & UYSAL, M. (2004). Methionine supplementation did not augment oxidative stress, atherosclerotic changes and hepatotoxicity induced by high cholesterol diet in C57BL/6J mice. Journal of nutritional science and vitaminology, 50(4), 258- 264.

Banecka-Majkutewicz, Z., Sawuła, W., Kadziński, L., Węgrzyn, A., & Banecki, B. (2012). Homocysteine, heat shock proteins, genistein and vitamins in ischemic stroke--pathogenic and therapeutic implications. Acta Biochimica Polonica, 59(4).

Barbato, J.C.; Catanescu, O.; Murray, K.; Di Bello, P.M. and Jacobsen,D.W. (2007).Targeting of metallothionein by L-homocysteine: a novel mechanism for disruption of zinc and redox homeostasis Arterioscler. Thromb. Vasc., Biol., 27:49-5.

References

Barroso, Madalena & Handy, Diane & Castro, Rita. (2017). The Link Between Hyperhomocysteinemia and Hypomethylation: Implications for Cardiovascular Disease. Journal of Inborn Errors of Metabolism and Screening. 5. 232640981769899. 10.1177/2326409817698994.

Behera, J., Bala, J., Nuru, M., Tyagi, S. C., & Tyagi, N. (2017). Homocysteine as a pathological biomarker for bone disease. Journal of cellular physiology, 232(10), 2704-2709.

Belalcázar, A. D., Ball, J. G., Frost, L. M., Valentovic, M. A., & Wilkinson, J., 4th (2014). Transsulfuration Is a Significant Source of Sulfur for Glutathione Production in Human Mammary Epithelial Cells. ISRN biochemistry, 2013, 637897.

Belcastro, V., & Striano, P. (2012). Antiepileptic drugs, hyperhomocysteinemia and B-vitamins supplementation in patients with epilepsy. Epilepsy research, 102(1-2), 1-7.

Bergen, N. E., Jaddoe, V. W. V., Timmermans, S., Hofman, A., Lindemans, J., Russcher, H., ... & Steegers, E. A. P. (2012). Homocysteine and folate concentrations in early pregnancy and the risk of adverse pregnancy outcomes: the Generation R Study. BJOG: An International Journal of Obstetrics & Gynaecology, 119(6), 739-751.

Bonkovsky, H.L.; Ponka, P. & Bacon, B.R. (1996). An update on iron metabolism: summary of the fifth international conference on disorders of iron metabolism. Hepa., 24:718-729.

Boxmeer, J. C., Macklon, N. S., Lindemans, J., Beckers, N. G., Eijkemans, M. J., Laven, J. S., ... & Steegers-Theunissen, R. P. (2009). IVF outcomes are associated with biomarkers of the homocysteine pathway in monofollicular fluid. Human reproduction, 24(5), 1059-1066.

References

Brustolin, S., Giugliani, R., & Félix, T. M. (2010). Genetics of homocysteine metabolism and associated disorders. Brazilian Journal of Medical and Biological Research, 43(1), 1-7.

Cacciapuoti, F. (2019). N-Acetyl-Cysteine supplementation lowers high homocysteine plasma levels and increases Glutathione synthesis in the trans-sulfuration pathway. Italian Journal of Medicine, 13(4), 234-240

Cagnacci, A.; Cannoletta, M.; Baldassari, F. and Volpe M. (2004). Low vitamin B12 and bone loss: A role for folate deficieny. J. Clin. Endo. Metab. , 89(9):4770-4771.

Castro, R., Rivera, I., Blom, H. J., Jakobs, C., & De Almeida, I. T. (2006). Homocysteine metabolism, hyperhomocysteinaemia and vascular disease: an overview. Journal of Inherited Metabolic Disease: Official Journal of the Society for the Study of Inborn Errors of Metabolism, 29(1), 3-20.

Celik, I.; Temur, A. and Isik, I. (2009). Hepatoprotective role and antioxidant capacity of pomegranate (punica granatum) flowers infusion against trichloroacetic acid exposed in rat. Food chem.Toxicol . , 47, pp:145-149.

Çelik, N., Vurmaz, A., & Kahraman, A. (2017). Protective effect of quercetin on homocysteine-induced oxidative stress. Nutrition, 33, 291-296.

Ciaccio, M., & Bellia, C. (2010). Hyperhomocysteinemia and cardiovascular risk: effect of vitamin supplementation in risk reduction. Current clinical pharmacology, 5(1), 30-36.

Cohen, E., Margalit, I., Shochat, T., Goldberg, E., & Krause, I. (2019). Gender differences in homocysteine concentrations, a population-based cross- sectional study. Nutrition, Metabolism and Cardiovascular Diseases, 29(1), 9-14.

Coles, E. H. (1986). Veterinary Clinical Pathology. 4th (ed)., WB Saunders Co Philadelphia, London, Toronto. 408-436.

Cornejo, P.; Varela, P.; Videla, L.A.&Fernandez, V. (2005). Chronic iron overload enhances inducible nitric oxide syntheses expression in rat liver. Nitric Oxide., 13: 54–61.

Corradini, E., Rozier, M., Meynard, D., Odhiambo, A., Lin, H. Y., Feng, Q., ... & Fleming, R. E. (2011). Iron regulation of hepcidin despite attenuated Smad1, 5, 8 signaling in mice without transferrin receptor 2 or Hfe. Gastroenterology, 141(5), 1907-1914.

Crawford, R. D. (1995) .Proposed role for a combination of citric acid and ascorbic acid in the production of dietary iron overload: a fundamental cause of disease. Biochem. Mol. Med. 54: 1–11.

Dabbagh, A. J. ; Shwaery, G. T. ; Keaney, J. F. & Frei, B. (1997).effect of iron overload and iron deficiency on atherosclerosis in the hypercholesterolemic rabbit. Arterioscle. Thrombi. Vasc. Biol., 17: 2638–2645.

Dahlhoff, C., Desmarchelier, C., Sailer, M., Fürst, R. W., Haag, A., Ulbrich, S. E., ... & Daniel, H. (2013). Hepatic methionine homeostasis is conserved in C57BL/6N mice on high-fat diet despite major changes in hepatic one-carbon metabolism. PloS one, 8(3), e57387.

D'angelo, G. (2013). Role of hepcidin in the pathophysiology and diagnosis of anemia. Blood research, 48(1), 10-15.

De-Lea, V. Marco, A.; Margante, G.; Cinani, G.; Zammerchi, E. and Setaccic, (2002). Low dose folic acid supplementation reduces plasma levels of the cardiovascular risk factors Homocysteine in postmenopausal women. Am. J. Obstet. Gynecol., 183:945-947.

Demir, S. & Öner, G. (1995). The effect of cadmium on the fragility of red blood cell.J. Aca. sci., 8(2). PP: 73-78.

Denise R. Ferrier. (2014). Lippincott’s Illustrated Reviews: Biochemistry. Sixth Edition.

Desouza, C.; Keebler, M.; Namara, D.B. and Fonseca, V. (2002). Drugs affecting Homocysteine metabolism: impact on cardiovascular risk .Drugs., 62(4):605-16.

Dhonukshe-Rutten, R. A., Pluijm, S. M., de Groot, L. C., Lips, P., Smit, J. H., & van Staveren, W. A. (2005). Homocysteine and vitamin B12 status relate to bone turnover markers, broadband ultrasound attenuation, and fractures in healthy elderly people. Journal of Bone and Mineral Research, 20(6), 921-929.

Di Pasquale, M. G. (2007). Amino acids and proteins for the athlete: The anabolic edge. CRC Press.

Diez, N.; Perez, R.; Hurrtado.and Satidrian, S. (2005).Hyperhomocysteinemia induced by dietary folate restriction causes kidney oxidative stress in rats. British .J. of Nutr .,94(2):204-210.

Dilgor; R. and Baker, D. (2008). Excess dietary L-cysteine causes lethal metabolic acidosis in chick's .J .Nutr. . 138(9):1628-1633.

Ding, R., Lin, S., & Chen, D. (2012). The association of cystathionine β synthase (CBS) T833C polymorphism and the risk of stroke: a meta-analysis. Journal of the neurological sciences, 312(1-2), 26-30.

Donovan, A., Lima, C. A., Pinkus, J. L., Pinkus, G. S., Zon, L. I., Robine, S., & Andrews, N. C. (2005). The iron exporter ferroportin/Slc40a1 is essential for iron homeostasis. Cell metabolism, 1(3), 191-200.

Dufour, D.R.; Lott,J.A,; Nolte,F.S.; Gretch,D.R. ; Koff, R.S. and Seeff, L.B. (2000). Diagnosis and monitoring of hepaticinjur: II. Recommendations for use of laboratory tests. Clin. Chem., 46: 2050-68.

Dworakowski, R., Anilkumar, N., Zhang, M., & Shah, A. M. (2006). Redox signalling involving NADPH oxidase-derived reactive oxygen species.

Ebbesen,S.and Ingersity,J.(2005).Folatedeficiency-induced huperhomocystenemia attenuates, and folic acid supplementation

restores, the functional activities of rat coagulation factors XII, X and II. J.Nutr., 135 (8):1836-1840.

Ebisch, I. M. W., Peters, W. H. M., Thomas, C. M. G., Wetzels, A. M. M., Peer, P. G. M., & Steegers-Theunissen, R. P. M. (2006). Homocysteine, glutathione and related thiols affect fertility parameters in the (sub) fertile couple. Human reproduction, 21(7), 1725-1733.

El- Demerdash, F. M.; Yousef, M.; Elaswad, F. M. (2006).Biochemical study on the role of folic acid in rabbits treated with chromium (VI). Journal of environment science and health. Part B. Pesticides, food contaminations and agicultural wastes. ISSN., 41(5): 431-745.

El-Saleh, S. C., Al-Sagair, O. A., & Al-Khalaf, M. I. (2004). Thymoquinone and Nigella sativa oil protection against methionine-induced hyperhomocysteinemia in rats. International journal of cardiology, 93(1), 19-23 .

Faraci, F. and Lentz,S. (2004). Hyperhomocysteinemia, oxidative stress and cerebral vascular dysfunction. Stroke ., 335:345.

Feng, P. N., Liang, Y. R., Lin, W. B., Yao, Z. R., Chen, D. B., Chen, P. S., & Ouyang, J. (2018). Homocysteine induced oxidative stress in human umbilical vein endothelial cells via regulating methylation of SORBS1. Eur. Rev. Med. Pharmacol. Sci, 20, 6948-6958

Feng, Q., Migas, M. C., Waheed, A., Britton, R. S., & Fleming, R. E. (2012). Ferritin upregulates hepatic expression of bone morphogenetic protein 6 and hepcidin in mice. American Journal of Physiology-Gastrointestinal and Liver Physiology, 302(12), G1397-G1404.

Ferit- Gursu, L.; Muhitin, O.; Funda, G.and Kazim,S.(2004). Effect of vitamin C and folic acid supplementation on serum paraoxonase activity and metabolites induced by heat stress in vivo. Nutrition Reaserch., 24:157-164.

Fernández-Rodríguez, C., González-Reimers, E., Quintero-Platt, G., de la Vega- Prieto, M. J., Pérez-Hernández, O., Martín-González, C., ... & Santolaria- Fernández, F. (2016). Homocysteine, liver function derangement and brain atrophy in alcoholics. Alcohol and Alcoholism, 51(6), 691-697.

Ferré, N., Gómez, F., Camps, J., Simó, J. M., Murphy, M. M., Fernández - Ballart, J., & Joven, J. (2002). Plasma homocysteine concentrations in patients with liver cirrhosis. Clinical chemistry, 48(1),183-185.

Fleming, R. E., & Ponka, P. (2012). Iron overload in human disease. New England Journal of Medicine, 366(4), 348-359.

Fuenfstuck, R.; Straube, E.;and Schildbach,O.(1997). Prevention of recurrent urinary tract infection by L-methionine. Med.Klin., 92(10):574-581.

Fufagawa, N.K. (2008).Sparing of methionine requirements: evaluation of human data takes sulfur amino acids beyond protein. J. Nutr.,136:1676S- 1681S.

Furness, D., Fenech, M., Dekker, G., Khong, T. Y., Roberts, C., & Hague, W. (2013). Folate, vitamin B12, vitamin B6 and homocysteine: impact on pregnancy outcome. Maternal & child nutrition, 9(2), 155-166.

Ganguly, P., & Alam, S. F. (2015). Role of homocysteine in the development of cardiovascular disease. Nutrition journal, 14(1), 1-10.

Ganz, T. (2005). Hepcidin—a regulator of intestinal iron absorption and iron recycling by macrophages. Best Practice & Research Clinical Haematology, 18(2), 171-182.

Ganz, T. (2006). Hepcidin and its role in regulating systemic iron metabolism. ASH Education Program Book, 2006(1), 29-35.

Ganz, T. (2011). Hepcidin and iron regulation, 10 years later. Blood, The Journal of the American Society of Hematology, 117(17), 4425-4433.

Ganz, T., & Nemeth, E. (2011). Hepcidin and disorders of iron metabolism. Annual review of medicine, 62, 347-360.

García-Tevijano, E. R., Berasain, C., Rodríguez, J. A., Corrales, F. J., Arias, R., Martín-Duce, A., & Avila, M.A.(2001) Hyperhomocysteinemia in liver cirrhosis: mechanisms and role in vascular and hepatic fibrosis. Hypertension, 38(5), 1217-1221.

Goljan, E. F. (2011). Rapid review pathology: with student consult online access. Elsevier Health Sciences.

Gorial, F. I., Aubaese, N. D., & Husaeen, N. H. (2013). Prevalence and associated factors of osteoporosis in post-menopausal Iraqi women: a cross-sectional two centers study. Int. J. Modern Biol. Med, 3(1), 41-49.

Gregg, L. V. (2000). Hematology Techniques And Concepts for Veterinary Techniques. 1st (ed). Iowa State Univ. Press. 97-100.

Gursu, M. F., Onderci, M., Gulcu, F., & Sahin, K. (2004). Effects of vitamin C and folic acid supplementation on serum paraoxonase activity and metabolites induced by heat stress in vivo. Nutrition Research, 24(2), 157-164.

Halliwell, B. (2001). Albumin important extracellular antioxidant. Pharmacol., 37:569-571.

Hameed, B. K. (2019). The Effect of Iron Overload on Histological Picture in Testis of Mature Male Rats. Tikrit Journal for Agricultural Sciences18 (2),170-175.

Handy, D. and Zhang, L (2006). Homocysteine and oxidative stress: Time for reality chec. Arterioscl. Thromb. Vasc. , 20:1182-1184.

Hayden, M. and Tyage, H. (2004). Homocysteine and reactive oxygen species in metabolic syndrome, type 2 diabetes mellitus, and atheroscleropat The pleiotropic effects of folate supplementation. Nutr. J. ,3: 4.

Hemanth Kumar, B., Dinesh Kumar, B., & Diwan, P. V. (2017). Hesperidin, a citrus flavonoid, protects against l-methionine-induced hyperhomocysteinemia byabrogation of oxidative stress, endothelial

dysfunction and neurotoxicity in Wistar rats. Pharmaceutical Biology, 55(1), 146-155.

Hentze, M. W., Muckenthaler, M. U., Galy, B., & Camaschella, C. (2010). Two to tango: regulation of Mammalian iron metabolism. Cell, 142(1), 24- 38.

Herlin, G.; Sullivan, P.; Csako, G. (2001). Relationships among plasma, Cysteine , and albumin concentration : potential utility of assessing the cysteine / Homocysteine ratio . Clin chem., 47(11):1121-1124.

Hilton, K. B., & Lambert, L. A. (2008). Molecular evolution and characterization of hepcidin gene products in vertebrates. Gene, 415(1-2), 40-48.

Ho, C. L., Quay, T. A., Devlin, A. M., & Lamers, Y. (2016). Prevalence and predictors of low vitamin B6 status in healthy young adult women in metro vancouver. nutrients, 8(9), 538.

Hoeler, D. and Hooge,D.M.(2003). Relative effectiveness of methionine sources in turkeys-scientific and new commercial data. International Journal of poultry .Science., 2(5):361-366.

Horigan, G., McNulty, H., Ward, M., Strain, J. J., Purvis, J., & Scott, J. M. (2010). Riboflavin lowers blood pressure in cardiovascular disease patients homozygous for the 677C→ T polymorphism in MTHFR. Journal of hypertension, 28(3), 478-486.

Hosseini, E., Nafisi, S., & Zare, S. (2011, December). The effects of electromagnetic fields on plasma levels of corticosterone, free-T3, free-T4 malonyldialdehyde in white male rabbit with normal diet and hyperchlostrol diet. In Veterinary Research Forum (Vol. 2, No. 4, pp. 222-225). Faculty of Veterinary Medicine, Urmia University.

Hsu, C. C., Cheng, C. H., Hsu, C. L., Lee, W. J., Huang, S. C., & Huang, Y. C. (2015). Role of vitamin B6 status on antioxidant defenses, glutathione,

and related enzyme activities in mice with homocysteine-induced oxidative stress. Food & Nutrition Research, 59(1), 25702.

Huang, R. F. S., Hsu, Y. C., Lin, H. L., & Yang, F. L. (2001). Folate depletion and elevated plasma homocysteine promote oxidative stress in rat livers. The Journal of nutrition, 131(1), 33-38.

Huliberg, B.; Berglund, M.; Anderson, A. and Frank, A. (2005). Elevated plasma Homocysteine in alcoholic in plasma Homocysteine with parallel increase in plasma S- Adenosyl. Alcoholic. Clin. Exp. Res., 17:786-689.

Humadi, E. H. (2016). Treatment of hyperhomocysteinemia and pregnancy outcome in patients with recurrent miscarriage. Mustansiriya Medical Journal, 15(2), 9-13.

Ientile, R., Curro, M., Ferlazzo, N., Condello, S., Caccamo, D., & Pisani, F. (2010). Homocysteine, vitamin determinants and neurological diseases. Frontiers in Bioscience (Scholar Edition), 2, 359-372.

Ilyin, G., Courselaud, B., Troadec, M. B., Pigeon, C., Alizadeh, M., Leroyer, P., ... & Loréal, O. (2003). Comparative analysis of mouse hepcidin 1 and 2 genes: evidence for different patterns of expression and co-inducibility during iron overload. *FEBS letters*, *542*(1-3), 22-26.

Inagi, R. and Miyata, T.(1999). Oxidation, proteins damage with carbonyl and lipids in uremia "Carbonyl stress" .Blood purify. , 17(2-3):95-98.

Jain, N. C. (1986). Schalm's Veterinary Hematology. 4th. (ed). Lea and Fibiger Philadelphia.

Jassim, A. M.A ,(2008).Effects of Desferal and green tea plant on some biochemical variants in rabbits induced diabetes mellitus and iron overload. PhD thesis, college of Science, University of Babylon.

Jawad, Alaa Hussein. (2005). A study of some biochemical variables in thalassemia patients (Mediterranean anemia). PhD thesis, College of Education, University of Baghdad.

John, A. B., & Steven, D. A. (1978). Microsomal lipid peroxidation in methods in enzymology. New York: Academic, (Vol. 52, pp. 302- 310).

Jolin, WJ.; Aafje, S.; Gertjan, S.; Kok, FJ.; Struys, EA.; Jakobs, C. and Hendriks,FJ.(2005). Kinetics of homocysteine metabolism after moderate alcohol consumption .Alcohol Clin. Exp Res ., 29:739-45.

Jordao Júnior, A. A., Domenici, F. A., Lataro, R. C., Portari, G. V., & Vannucchi, H. (2009). Effect of methionine load on homocysteine levels, lipid peroxidation and DNA damage in rats receiving ethanol. Brazilian Journal of Pharmaceutical Sciences, 45(4), 709-714.

Kaneko,J.J. ;Harvey,J.W. & Bruss,M.L.(1997). Clinical biochemistry of domestic animals. 5th (ed). Academic press. London. PP:932.

Karamouzis , I. ; Sarafidis , P.A. ; Karamouzis , M. ; Iliadisd , S. ;Haidich , A.B. ; Sioulis, A.; Triantos, A.; Vavatsi-Christaki, N. and Grekas, D.M. (2008) .Increase in oxidative stress but not in antioxidant capacity with advancing stages of chronic kidney disease. Am. J. Nephrol., 28:397-404.

Keech, A. Simes, r. and Barter, P. (2005). Study investigators, effects long-term fenofibrate therapy on cardiovascular events in 9795 people with type 2diabetes mellitus (the field study) randomized controlled trail. Lancet., 366:1849-1861.

Kerkeni, M.; Addad, F. and Xhauffert, M. (2006). Hyperhomocysteinemia, methylenete trahydrofolate reductase polymorphism and risk of coronary artery disease. Ann. Clin. Bio., 43:200-6.

Khan, M.F.;Wu, X.; Alcock, N.W. (1999). Iron exacerbates aniline- associated splenic toxicity. J. Toxicol. Environ. Health., ***57***: 173-184.

Khudiar, K.K. (2000). The role of aqueous extraction of olive (Allium Sativum) in ameliorating the effects of experimentally induced atherosclerosis in rats . Ph. D. Thesis, College of Veterinary Medicine, University of Baghdad.

Kim, S. Y., Kim, H., & Min, H. (2015). Effects of excessive dietary methionine on oxidative stress and dyslipidemia in chronic ethanol-treated rats. Nutrition Research and Practice, 9(2), 144-149.

Kim, T.; Knoops- Varela, A. ; Alessandrom and Wija, A . (2004) Mediterranean diet, life style factors and 10 years mortality in Elderly European men and women: the HALE project .JAMA. , 292:1433-1439.

Kitamoto, N. ; Ishihashi, M. ; Usui, M. ; Inoue, S. ; Haisa,K. ; Zhao, Q. ; Nishida, K. ; Takeshita, A. (2004). Protein –I is an essential inflammatory mediater in angiotensin II, Progression of establisher atherosclerosis in hypercholesteremia in mice. Atherosol. Throm. Vas. Biol., 24:534-539.

Komatsu, M., & Tsukamoto, I. (1998). Effect of folic acid on thymidylate synthase and thymidine kinase in regenerating rat liver after partial hepatectomy. Biochimica et Biophysica Acta (BBA)-General Subjects, 1379(2), 289-296.

Kovacevic, Z. and Richardson, R. (2006). The metastasis suppressor , Ndrg-1: a newally in the fight against cancer. Carcinogenesis. , 27(12):2355-2366.

Ku, P. K.; Miller,,E. R. & Ullrey,D. E. (1983). Effect of parenteral iron on serum electrolytes of the baby pig. Anim. Sci., 57: 638-644.

Kumar, A., Palfrey, H. A., Pathak, R., Kadowitz, P. J., Gettys, T. W., & Murthy, S. N. (2017). The metabolism and significance of homocysteine in nutrition and health. Nutrition & metabolism, 14(1), 1-12.

Lee, HJ., Choi, JS., Lee , HJ., Kim, WH., Park, SI., Song, J. (2015). Effect of excess iron on oxidative stress and gluconeogenesis through hepcidin during mitochondrial dysfunction. J. Nutr. Biochem. 26(12), 1414-1423

Lewis, A.and Baley, H. (1995). Amino acid bioavailability of nutrients. J. Agric .Food Chem. , 35-65.

Lieu, P.; Heiskala, M.; Peterson, P. & Yang, Y. (2001). The roles of iron in health and disease. Mol. Aspect Med., 22:1-87.

Liu, H. H., Shih, T. S., Huang, H. R., Huang, S. C., Lee, L. H., & Huang, Y. C. (2013). Plasma homocysteine is associated with increased oxidative stress and antioxidant enzyme activity in welders. The Scientific World Journal, 2013.

Lu, S., Seravalli, J., & Harrison-Findik, D. (2015). Inductively coupled mass spectrometry analysis of biometals in conditional Hamp1 and Hamp1 and Hamp2 transgenic mouse models. Transgenic research, 24(4), 765-773.

Luna, L.G. (1968). Manual of Histological Staining Methods of the Armed institutes of Pathology . 3rded .Mc Grow-Hill Book Company. New York.

Mahachoklertwattana, P.; Sirikulchayanonta, V.; Chuansumrit, A. ; Karnsombat, P.; Choubtum, L.; Sriphrapradang, A.; Domrongkitchaiporn, S.; Sirisriro, R. and Rajatanavin, R. (2003). Bone Histomorphometry in Children and Adolescents with β thalassemia Disease: Iron Associated Focal Osteomalacia .J. Clin . Endocrinol Metab.,88(8):3966-3972.

Mahmood, L. (2014). The metabolic processes of folic acid and Vitamin B12 deficiency. Journal of Health Research and Reviews, 1(1), 5.

Majors, A., Ehrhart, L. A., & Pezacka, E. H. (1997). Homocysteine as a risk factor for vascular disease: enhanced collagen production and accumulation by smooth muscle cells. Arteriosclerosis, thrombosis, and vascular biology, 17(10), 2074-2081.

Majors, A.; Sengupta, S.; Willard, B.; Kiter, M.; Pyteritz, R. and Jaocobsen D. (2002). Homocysteinenemia , Bids to human plasma fibronectin and

ihibits its interaction with fibrin . Athero. Thro Vas. Biol., 22:1354-1359.

Mangiagalli, A., Samuele, A., Armentero, M. T., Bazzini, E., Nappi, G., & Blandini, F. (2004). Effects of homocysteine on apoptosis-related proteins and anti-oxidant systems in isolated human lymphocytes. European journal of biochemistry, 271(9), 1671-1676.

Mario, M.; Colell, A.; Morales, A.; Poneda, C.; Vorela, I.; Carcia, C. and Ferandeze, C. (2006). Acidic sphyngomylinase down regulates the liver specific methionine adenosyltransferase IA, contributing to tumor necrosis factor- induced lethal hepatitis. J. Clin. Invest., 113(6):895-904.

Marry, Ward (2001). Homocysteine folate and cardiovascular disease . Inter. J. Vit., 71:3.

Marsillach, J., Ferre, N., Camps, J., Riu, F., Rull, A., & Joven, J. (2008). Moderately high folic acid supplementation exacerbates experimentally induced liver fibrosis in rats. Experimental Biology and Medicine, 233(1), 38-47.

Martella, B. M., Veiga, G. R. L., Alves, B. C., Azzalis, L. A., Junqueira, V. B., Gehrkei, F. S., ... & Bacci, M. R. (2018). The importance of homocysteine levels in the prognosis of patients with chronic renal disease and in hemodialysis patients. Jornal Brasileiro de Patologia e Medicina Laboratorial, 54(3), 170-176.

Masaratana, P., Patel, N., Latunde-Dada, G. O., Vaulont, S., Simpson, R. J., & McKie, A. T. (2013). Regulation of iron metabolism in Hamp−/− mice in response to iron-deficient diet. European journal of nutrition, 52(1), 135-143.

Matte, C., Stefanello, F. M., Mackedanz, V., Pederzolli, C. D., Lamers, M. L., Dutra-Filho, C. S., ... & Wyse, A. T. (2009). Homocysteine induces

oxidative stress, inflammatory infiltration, fibrosis and reduces glycogen/glycoprotein content in liver of rats. International Journal of Developmental Neuroscience, 27(4), 337-344.

McLachlan, S., Page, K. E., Lee, S. M., Loguinov, A., Valore, E., Hui, S. T., ... & Ganz, T. (2017). Hamp1 mRNA and plasma hepcidin levels are influenced by sex and strain but do not predict tissue iron levels in inbred mice. American Journal of Physiology-Gastrointestinal and Liver Physiology, 313(5), G511-G523.

McLean, E., de Benoist, B., & Allen, L. H. (2008). Review of the magnitude of folate and vitamin B12 deficiencies worldwide. Food and nutrition bulletin, 29(2_suppl1), S38-S51.

Micovic, Z., Stamenkovic, A., Nikolic, T., Stojanovic, M., Scepanovic, L., Hadzibegovic, A., ... & Jakovljevic, B. (2016). The effects of subchronic methionine overload administered alone or simultaneously with L-cysteine or N-acetyl-L-cysteine on body weight, homocysteine levels and biochemical parameters in the blood of male wistar rats. Serbian Journal of Experimental and Clinical Research, 17(3), 215-224.

Mimic-Oka, J.; Simic , T. ; Pljesa , M. ; Stupar, N. and Turkovic , S. (2001) . Oxidative modifications of plasma proteins in deferent stages of chronic renal failure. Sci. Med.Bio., 8(5):1-5.

Minich, D. M., & Brown, B. I. (2019). A review of dietary (Phyto) nutrients for glutathione support. Nutrients, 11(9), 2073.

Miranda, M. A., St Pierre, C. L., Macias-Velasco, J. F., Nguyen, H. A., Schmidt, H., Agnello, L. T., ... & Lawson, H. A. (2019).Dietary iron interacts with genetic background to influence glucose homeostasis. Nutrition & metabolism, 16(1), 13.

Mohammad, I. I., & Al-Doski, F. S. (2012). Assessment of Liver Functions in Thalassaemia. Tikret Journal of Pharmaceutical Sciences, 8(1), 87-95.

Mori, N.H. and Hirayama, K. (2000). Long term consumption of a methionine supplemented diet increase iron and lipid peroxide levels in rat liver. J Nutr., 130:2349-2355.

Muñoz, M., Villar, I., & García-Erce, J. A. (2009). An update on iron physiology. World journal of gastroenterology: WJG, 15(37), 4617.

National Center for Biotechnology Information. (accessed on Nov. 2, 2019). PubChem Database. 5,10-Methylenetetrahydrofolate, CID=135398652,https://pubchem.ncbi.nlm.nih.gov/compound/5_10-Methylenetetrahydrofolate.

Nelen, W. L., Blom, H. J., Steegers, E. A., den Heijer, M., & Eskes, T. K. (2000). Hyperhomocysteinemia and recurrent early pregnancy loss: a meta-analysis. Fertility and sterility, 74(6), 1196-1199.

Nemeth, E., & Ganz, T. (2009). The role of hepcidin in iron metabolism. Acta haematologica, 122(2-3), 78-86.

Neves, J. V., Ramos, M. F., Moreira, A. C., Silva, T., Gomes, M. S., & Rodrigues, P. N. (2017). Hamp1 but not Hamp2 regulates ferroportin in fish with two functionally distinct hepcidin types. Scientific reports, 7(1), 1-14.

Nevmerzhytska, N. M., Orzheshkovskyi, V. V., Dzevulska, I. V., & Savosko, S.I. (2019). Mechanisms of Toxic Effects of Homocysteine on the Nervous System. Neurophysiology, 1-9.

Ni, G., Qin, J., Fang, Z., Chen, Y., Chen, Z., Zhou, J., & Zhou, L. (2014). Increased homocysteine levels in valproate-treated patients with epilepsy: a meta-analysis. BMJ open, 4(7).

Niesen, S. ; Tuzcu, E. and Schonenhangen, P.(2004). Effect of intensive compared with moderate lipid lowering therapy on progression of coronary atherosclerosis. A randomized controlled trial. JAMA., 291:1071-1080.

Norri, M.; Jacopues, P. and Rosenberg, H. (2001). Hyperhomocysteinemia associated with poor recall in the third national health and nutrition examination. Survey. Am. J. Clin. Nut. ,73:927-933.

Okura, T., Miyoshi, K. I., Irita, J., Enomoto, D., Nagao, T., Kukida, M., ... & Higaki, J. (2014). Hyperhomocysteinemia is one of the risk factors associated with cerebrovascular stiffness in hypertensive patients, especially elderly males. Scientific reports, 4(1), 1-5.

Olynyk, J.; Cullen,D. ; Aquilia,S. ; Rossi,E. & Powell, L. (1995). A long term study of the interaction of iron and alcohol in an animal model of iron overload. J. Hep., 22: 671-676.

Osunkalu, V. O., Makwe, C. C., Akinsola, O. J., Adewumi, A., & Akomolafe, O. F. (2015). Preliminary report on plasma homocysteine and hormonal variations in infertile women in Lagos, Nigeria. Journal of Clinical Sciences, 12(1), 14.

Özgüner , M.& Sayın , N.(2002).Histological changes in rat liver after chronic iron-sorbitol overload, j. An. Med. sch.,24 (2): 49-54.

Panigua, A.; Wiltshire, R. Gent, L.; Piotto, C.; Hirte L and Couper J. (2007). Folic acid dose improve endothelial function in obese children and aldoescents. Diabetes Care., 30(8):2122-2127.

Pastore, A.;DE-Angelis, S.;Casciani, S.; Ruggia, R.;Di Giovamberardino, G.; Cortese, C.; Fernandew; L:m Diego; G: and Coronado; J. (2006). Concentrated red grape juice exerts antioxidant, hyolipidemic and anti-inflammatory effect in both hem dialysis patient with known MTHFR genotypes. Clin. Chem., 52(1):145-148.

Patterson ,C. and Marschall, S.R. (2006). Principle of molecular medicine. In Human press ed. Totowa, NJ .pp:657. ISBN 1, 58829-20209.

Peña, A. S., Wiltshire, E., Gent, R., Piotto, L., Hirte, C., & Couper, J. (2007). Folic acid does not improve endothelial function in obese children and adolescents. Diabetes Care, 30(8), 2122- 2127.

Perna, A. F., & Ingrosso, D. (2016). Atherosclerosis determinants in renal disease: how much is homocysteine involved?. Nephrology Dialysis Transplantation, 31(6), 860-863.

Petrak ,D; Myslivcva, P; Man, R; Cmejla , J; Vylor, M; Elleder, V ;Vulp , C(2007). Proteomic analysis of hepatic iron overload in mice suggests deregulation of urea cycle impairment of fatty acid oxidation and changes in the methylation cycle. Am. J. physiol. Gastro. Intest. Physiol. liver physiol., 292(6):490-498.

Petras, M., Tatarkova, Z., Kovalska, M., Mokra, D., Dobrota, D., Lehotsky, J., & Drgova, A. (2014). Hyperhomocysteinemia as a risk factor for the neuronal system disorders. J Physiol Pharmacol, 65(1), 15-23.

Philippe, M. A., Ruddell, R. G., & Ramm, G. A. (2007). Role of iron in hepatic fibrosis: one piece in the puzzle. World journal of gastroenterology: WJG, 13(35), 4746.

Pietrangelo and Antonello . (2010). "Hereditary Haemochromatosis :Pathogenesis , Diagnosis , and Treatment " Gastroenterology ,139,(2), pp:393-408.

Prasnicka, A., Lastuvkova, H., Faradonbeh, F. A., Cermanova, J., Hroch, M., Mokry, J., ... & Nachtigal, P. (2019). Iron overload reduces synthesis and elimination of bile acids in rat liver. Scientific reports, 9(1), 1-12 .

Qureshi, S. B., Ahmad, M., Qureshi, P. M. A., Memon, A., & Qazi, R. A. (2010). Hyperhomocysteinaemia, vascular related pregnancy complications and the response to vitamin supplementation in pregnant women of Pakistan. JPMA. The Journal of the Pakistan Medical Association, 60(9), 741.

Rajagopal, S., Fitzgerald, A. A., Deep, S. N., Paul, S., & Poddar, R. (2019). Role of GluN2A NMDA receptor in homocysteine-induced

prostaglandin E2 release from neurons. Journal of neurochemistry, 150(1), 44-55.

Richard A. Harvey. (2011). Lippincott's Illustrated Reviews: Biochemistry. Fifth Edition.

Rishi, G., & Subramaniam, V. N. (2017). The liver in regulation of iron homeostasis. American Journal of Physiology-Gastrointestinal and Liver Physiology, 313(3), G157-G165.

Rondal, J. (2002). Orchestrating the unfolded protein response in health and disease . J. Clin. Invest., 110: 1389-1398.

Sahi , A; Pan ,X ; Paul, R; Malladi ,P ; Kahli, R and Whitington, F.(2006). Roles of phosphotidylinsitol 3- kinase and osteopontin in steatosis and aminotransferase roles release by hepatocytes treated with methionine –choline deficient medium. Am. J .physiol. Gastro. Intest .physiol.liver physiol., 291(1)55-62.

Salman, A. D. (2014). The Protective role of Pomegranate seed oil (Pometon) on kidney (functional and structural) damage induced by Methionine overload in adult female Rabbits. The Iraqi Journal of Veterinary Medicine (ISSN-P: 1609-5693 ISSN-E: 2410- 7409), 38(2), 66-73.

Sanchez-Roman, I., Gómez, A., Pérez, I., Sanchez, C., Suarez, H., Naudí, A., ... & Barja, G. (2012). Effects of aging and methionine restriction applied at old age on ROS generation and oxidative damage in rat liver mitochondria. Biogerontology, 13(4), 399-411.

Sangkhae, V., & Nemeth, E. (2017). Regulation of the iron homeostatic hormone hepcidin. Advances in Nutrition, 8(1), 126-136.

Sans, A.; Caro, P.; Ayala, V.; Portero- Otin, M and pamplana, R. (2006) . Methionine restriction decrease mitochondrial oxygen radical generation and leak as well as oxidative damage to mitochondrial DNA and proteins FASEB .J., 20(8):1064-1073.

Santilli, F., Davi, G., & Patrono, C. (2016). Homocysteine, methylenetetrahydrofolate reductase, folate status and atherothrombosis: A mechanistic and clinical perspective. Vascular pharmacology, 78, 1-9.

Schroecksnadel , K.;Frick , B.; Winkler, C.; Leblhube, F.; Wirleitner, B.and Fuchs,D.(2006). Hyperhomocystenemia and immune activation. Clin. Chem .Lab. Med., 41(11):1438-1443.

Schroecksnadel, K., Frick, B., Wirleitner, B., Winkler, C., Schennach, H., & Fuchs, D. (2004). Moderate hyperhomocysteinemia and immune activation. Current pharmaceutical biotechnology, 5(1), 107-118.

Schwab,U.Torronen,A.;Meririnne,E.;Saarinen,M.;Alfthan,G.Aro,and Usitupa, M.(2006).orally administrated Betains has an acute and dose dependent effect on serum betaine and plasma Homocysteine concentration in healthy humans .J. Nutr.,136-:34-38.

Schwartzj, K.A.; Fisher, J.I. & Adams, E.T.(1993). Morphological investigations of the guinea pig model of iron overload. Toxic Pathol.,(21):311-20.

Scott,D.J.; Maclntosh,G.; Lowe,G.D.; Rumley,A.; Mc Mahon,A.D.; Langhorne,P.; Tait,R.C.; O'Reilly,D.S.; Spilg,E.G.; Mac Donald,J.B.; and Westendorp,R.G.(2005). Randomized controlled trial of Homocysteine-lowering vitamin treatment in elderly patients with vascular disease. Am J Clin Nutr ., 82:1320-6.

Sedlak J & Lindsay RH (1968). Estimation of total, protein-bound and nonprotein sulfhydryl groups in tissues with Ellman’s reagent. Analytical Biochemistry, 25: 192-205.

Selhub, J., Jacques, P. F., Wilson, P. W., Rush, D., & Rosenberg, I. H. (1993). Vitamin status and intake as primary determinants of homocysteinemia in an elderly population. Jama, 270(22), 2693-2698.

Seshadri, N., & Robinson, K. (2000). Homocysteine, B vitamins, and coronary artery disease. Medical Clinics of North America, 84(1), 215-237.

Sharma, P.; Senthilkumar, R.D.; Brahmachari, V.; Sundaramoorthy, E.; Mahajan, A.; Sharma, A.; and Sengupta, S.(2006). Mining literature for a comprehensive pathway analysis: a case study for relative of Homocysteine related genes for genetic and epigenetic studies. Lipids and Health Disease., 5(1): 1186-1476.

Shoob, H. Reoger, D. and Sargen,J .(2001). Dietary methionine is involved in the etiology of neural tube defect –related pregnaneles in Gumans . Journal on nutrition., 131: 2653.

Shunhe ,Y. and Nobuhiro ,S.(2006). Fatty liver and oxidative stress . J.Kantansui., 52(6):853-858.

Sidho P.;Gary, M. and Dhawan , K. (2004). Protective role of zinc induce hepatotoxicity in rats .Chem .Biol. Inter., 150:199-209.

Silva, M., Silva, M. E., de Paula, H., Carneiro, C. M., & Pedrosa, M. L. (2008). Iron overload alters glucose homeostasis, causes liver steatosis, and increases serum triacylglycerols in rats. Nutrition research, 28(6), 391-398.

Sirdah, M. M., Yassin, M. M., Shekhi, S. E., & Lubbad, A. M. (2014). Homocysteine and vitamin B 12 status and iron deficiency anemia in female university students from Gaza Strip, Palestine. Revista brasileira de hematologia e hemoterapia, 36(3), 208-212.

Škovierová, H., Vidomanová, E., Mahmood, S., Sopková, J., Drgová, A., Červeňová, T., ... & Lehotský, J. (2016). The molecular and cellular effect of homocysteine metabolism imbalance on human health. International journal of molecular sciences, 17(10), 1733.

Smith, A. D., Refsum, H., Bottiglieri, T., Fenech, M., Hooshmand, B., McCaddon, A., ... & Obeid, R. (2018). Homocysteine and dementia: an

international consensus statement. Journal of Alzheimer's Disease, 62(2), 561-570.

Smith, A. D., Smith, S. M., De Jager, C. A., Whitbread, P., Johnston, C., Agacinski, G., ... & Refsum, H. (2010). Homocysteine-lowering by B vitamins slows the rate of accelerated brain atrophy in mild cognitive impairment: a randomized controlled trial. PloS one, 5(9), e12244.

Solini , A.; Sanline, E.and Ferrannim , G.(2006). Effect of solvent lesion folic acid supplementation on insulin sensitivity and inflammatory markers in over weight subjects . Int. J., 30(8):1197-1202.

Stadman , E. (1998). Reactive oxygen mediated protein oxidation in aging and disease .Drug/Metab. Rev., 30:225-243.

Stal, P.; Johansson, I.&Ingelman-Sundberg , M. (1996). Hepatotoxicity induced by iron overload and alcohol. Studies on the role of chelatable iron, cytochromes P450 2E1 and lipid peroxidation. J. Hepatol., 25(4): 538-46.

Stark,G.(2005).Functional consequences of oxidative membrane damage .J.Member Biol ., 205:1-16.

Steel, R.and Torries, J. (1980). Principles and Procedures Statistics a biometrical Approach .2nd edition .Mc.Jan. 29(1):44-48.

Stipanuk, M. H., & Ueki, I. (2011). Dealing with methionine/ homocysteine sulfur: cysteine metabolism to taurine and inorganic sulfur. Journal of inherited metabolic disease, 34(1), 17–32.

Stipanuk, M.H.(2004). Role of the liver in regulation of body cysteine and turine level .Neur. Chem. Res. Jan., 29(1):105-110.

Stopper, H.; Treutlein,A.-T.; Bahner ,U.; Schupp,N.; Schmid ,U.and Brink, A.(2008). Reduction of the genomic damage level in haemodialysis patients by folic acid and vitamin B12 supplementatio Nephrol .Dial. Transplant.,1:2541.

Sukayar, I. (2005). A biochemical study of liver function test in female used low dose combination oral contraceptive. MSc. Thesis university of Kufa, College of Medicine.

Suvarna , S.K. ; Lyaton , C. and Bancroft , J. D. (2013) . Bancroft ,s Theory and practice of histological technique . Seven ed. Elsevier Limited, China. Xiv- 604.

Suzuki, Y.; Uehara, R.; Tajima, C.; Noguchi, A.; Ide, M.; Ichikawa, Y.; an Mizushima, Y.(1999). Elevation of serum hepatic aminotransferase during treatment of rheumatoid arthritis with low dose methotrexate. Risk factors and response to folic acid. Scand. J .Rheumatol., 28:273-81.

Takea, S. A., & Mhsen, S. N. (2019). Turmeric Oil Effect on the Reproductive Efficacy of Female Albino Rats Treated with Iron Overload. Tikrit Journal for Agricultural Sciences, 18(4), 124-130.

Tehlivets, O., Malanovic, N., Visram, M., Pavkov-Keller, T., & Keller, W. (2013). S-adenosyl-L-homocysteine hydrolase and methylation disorders: yeast as a model system. Biochimica et biophysica acta, 1832(1), 204–215. doi:10.1016/j.bbadis.2012.09.007.

Tiikkinen, N. ; Hakkinen, A. ; Karsheninnikova, E. ; Nyman, T. Makimattia , S. and Ki- jarvinen, H. (2004). Effect of rasiglitazone and metformin on liver fat content hepatic insulin resistance clearance and gene expression adipose tissue in patients with type 2 diabetes , Diabetes., 53: 2169-2176.

Tinelli, C., Di Pino, A., Ficulle, E., Marcelli, S., & Feligioni, M. (2019). Hyperhomocysteinemia as a Risk Factor and Potential Nutraceutical Target for Certain Pathologies. Frontiers in nutrition, 6, 49. doi:10.3389/fnut.2019.00049.

Tounyz, R.and Schiffrin, E. (2008). Reactive oxygen species and hypertension antioxidants and redox signaling . Eng. J . Med., 10(6):1041-1044.

Truksa, J., Gelbart, T., Peng, H., Beutler, E., Beutler, B., & Lee, P. (2009). Suppression of the hepcidin-encoding gene Hamp permits iron overload in mice lacking both hemojuvelin and matriptase-2/TMPRSS6. British journal of haematology, 147(4), 571-581.

Tsai, J. C., Perrella, M. A., Yoshizumi, M., Hsieh, C. M., Haber, E., Schlegel, R., & Lee, M. E. (1994). Promotion of vascular smooth muscle cell growth by homocysteine: a link to atherosclerosis. Proceedings of the National Academy of Sciences, 91(14), 6369-6373.

Tug, N. ; Celik , H. ; Cikim , G. ; Ozcelik, O and Ayar, A.(2006). The correlation between plasma Homocysteine and malondiadehyde levels in preeclampsia . Neuroendocrinology Letters., 24(6):445-448.

Turbino-Ribeiro, S. M., Silva, M. E., Chianca Jr, D. A., de Paula, H., Cardoso, L. M., Colombari, E., & Pedrosa, M. L. (2003). Iron overload in hypercholesterolemic rats affects iron homeostasis and serum lipids but not blood pressure. The Journal of nutrition, 133(1), 15-20.

Umran, M. A., Ibraheem, S. R., & Abed, R. M. (2013). Evaluation the Effects of Three Types of Pomegranate Fruit Extracts on Some Biochemical Markers and Cytogenetic Parameters in Mice Administrated With Iron Dextran to Iron Overload. Iraqi Journal of Science, 54(4), 862-871.

Ungvari, Z.; Csiszar, A.; Edward, J. G.; Kaminski, P.M.; Wolin, M.S.; Kaley, G. and Koller , A.(2003). Increase superoxide production in coronary arteries in Hyperhomocysteinemia: role of tumor necrosis factor – alpha , NADPH oxidase , and inducible nitric oxide synthase . Arte. Thro. Vas. Biol., 23:418-424.

Valentin, B.A.; Blue, J.T.; Shelley, S.M.;and Cooper, B.J.(1990). Increase serum alanine amino transferasenactivity muscle necrosis in the dog. J Vet Intern Med .,4:140-143.

Ventura, P.; Panini, R.; Verlato, C.;Scarrpeta, G. and Salvioli, G.(2000) .Peroxidation induces and total antioxidant capacity in plasma during

hyperhomocysteinemia induced by methionine oral loading . Metabolisem .,49:225-228.

Verhoef, P.;Pasman ,W.;Van Vliet, T.; Urget,R.and Katan,M.(2007). Contribution of Cafeine to the Homocysteine –rasing effect of coffee : a randomized cotrolled trial in humans .Am.J.Clin.Nutr.,76(6):1244-1248.

Vijayakumar, A., Kim, E. K., Kim, H., Choi, Y. J., Huh, K. B., & Chang, N. (2017). Effects of folic acid supplementation on serum homocysteine levels, lipid profiles, and vascular parameter in post-menopausal Korean women with type 2 diabetes mellitus. Nutrition research and practice, 11(4), 327- 333.

Villa, P. ; Perri, C. ; Suriano,R. ; Cueinelli, S. ; Panunzi, S. ; Ranieri, M. ; Mele, C. and Lanzona, A.(2004). Folic acid supplementation in healthy menopausal women. Effect on Homocysteineand clycolipid metabolism. J. of clin. Endocrinol. and Metabolism. , 90(8):4622-4629.

Vollset, S. E., Refsum, H., Irgens, L. M., Emblem, B. M., Tverdal, A., Gjessing, H. K., ... & Ueland, P. M. (2000). Plasma total homocysteine, pregnancy complications, and adverse pregnancy outcomes: the Hordaland Homocysteine study. The American journal of clinical nutrition, 71(4), 962-968.

Wang, J., & Pantopoulos, K. (2011). Regulation of cellular iron metabolism. Biochemical Journal, 434(3), 365-381.

Waly, M. I., Al-Farsi, Y. M., Al-Sharbati, M. M., Ali, A., Al-Khalili, M. M., . Essa, M. M., ... & Al-Shafaee, M. S. (2011). Low serum levels of glutathione, homocysteine and total antioxidant capacity are associated with increased risk of autism in Oman.

Weinberg, E. D. (1990). Cellular iron metabolism in health and disease. Drug Metab. Rev. 22: 531–579.

Weiss, N. ; Zhang , Y. ; Heydrick, S. ; Biert, C. ;and Lascalze, J.(2001). Over expression of cellular glutathione rescua Homocysteine induced endothelial dysfunction . Pro. Natl. Acad. Sci. USA. , 98:12503-12508.

Westphal, S.;Radding ,A.;Luley,C.and Dierkes,J.(2003). Antihypertensive treatment and Homocysteine concentrations .Metabolism ., 52(3):261-263.

Wijekoon, E. P., Hall, B., Ratnam, S., Brosnan, M. E., Zeisel, S. H., & Brosnan, J. T. (2005). Homocysteine metabolism in ZDF (type 2) diabetic rats. Diabetes, 54(11), 3245-3251.

Winterbourn, C. C. (1995). Toxicity of iron and hydrogen peroxide: the Fenton reaction. Toxicology letters, 82, 969-974.

Willemetz, A., Lenoir, A., Deschemin, J. C., Lopez-Otin, C., Ramsay, A. J., Vaulont, S., & Nicolas, G. (2014). Matriptase-2 is essential for hepcidin repression during fetal life and postnatal development in mice to maintain iron homeostasis. Blood, The Journal of the American Society of Hematology, 124(3), 441-444.

Wilson, C. P., McNulty, H., Scott, J. M., Strain, J. J., & Ward, M. (2010). Postgraduate Symposium The MTHFR C677T polymorphism, B-vitamins and blood pressure: Conference on 'Over-and undernutrition: challenges and approaches'. Proceedings of the Nutrition Society, 69(1), 156-165.

Winkels , R. M. ; Brouwer , I.A. ;Siebelink , E. ;Katan , M.B. and Verhoef , P. (2007). Bioavailability of food folate is 80% of that of folic acid Am. J. Clin .Nutr ., 85:465-473.

Wolff, S.P. (2006). Diabetes mellitus and free radicals. Free radicals, transition metals and oxidative stress in the etiology of diabetes mellitus and complications. Neph.. Dial Transplant., 21:2686-2690.

Wong, C. & Richardson, D.R.(2003).B-Thalassemia : emergence of new and improved iron chelators for treatment..Int. J. Bioch. cell Biol.,35:1144-1149.

Woo,C.W. (2007). Role of Hyperhomocysteinemia in liver injury and abnormal lipid metabolism (protective effect of folic acid supplementation) . ph .D, Thesis . University of Manitoba.

Xie,M.;Hou,S. Huang,Wand Fan, H.(2007). Effect of excess methionine and methionine hydroxyl analogue on growth performance and plasma homocysteine of growing pekin Duks. Poult. Sci., 86(9):1995-1999.

Yamamoto, K., Isa, Y., Nakagawa, T., & Hayakawa, T. (2012). Folic acid fortification ameliorates hyperhomocysteinemia caused by a vitamin B6-deficient diet supplemented with L-methionine. Bioscience, biotechnology, and biochemistry, 76(10), 1861-1865**.**

Yamauchi, A., Kamiyoshi, A., Sakurai, T., Miyazaki, H., Hirano, E., Lim, H. S., ... & Shindo, T. (2019). Development of a mouse iron overload-induced liver injury model and evaluation of the beneficial effects of placenta extract on iron metabolism. Heliyon, 5(5), e01637.

Yang, F., Haile, D. J., Wang, X., Dailey, L. A., Stonehuerner, J. G., & Ghio, A. J. (2005). Apical location of ferroportin 1 in airway epithelia and its role in iron detoxification in the lung. American Journal of Physiology-Lung Cellular and Molecular Physiology, 289(1), L14-L23.

Yang, F., Wang, X., Haile, D. J., Piantadosi, C. A., & Ghio, A. J. (2002). Iron increases expression of iron-export protein MTP1 in lung cells. American Journal of Physiology-Lung Cellular and Molecular Physiology, 283(5), L932-L939.

Yao, Y., Gao, L. J., Zhou, Y., Zhao, J. H., Lv, Q., Dong, J. Z., & Shang, M. S. (2017). Effect of advanced age on plasma homocysteine levels and its association with ischemic stroke in non-valvular atrial fibrillation. Journal of geriatric cardiology: JGC, 14(12), 743.

Yong, Z.Z and Zou , A.P. (2003). Homocysteine enhance TIMP -1 expression and collproliferation associated with NADH oxidase in rat mesengial cells. Kidney. Int., 63:1012-1020.

Young, D.S.(2000).Effect of drug on clinical laboratory tests 5th (ed) AACC press .

Zhang, D. L., Hughes, R. M., Ollivierre-Wilson, H., Ghosh, M. C., & Rouault, T. A. (2009). A ferroportin transcript that lacks an iron-responsive element enables duodenal and erythroid precursor cells to evade translational repression. Cell metabolism, 9(5), 461-473.

Zidan, R. A., & Elnegris, H. M. (2015). Effect of homocysteine on the histological structure of femur in young male albino rats and the possible protective role of folic acid. Journal of Histology & Histopathology, 2(1), 16.

Appendix

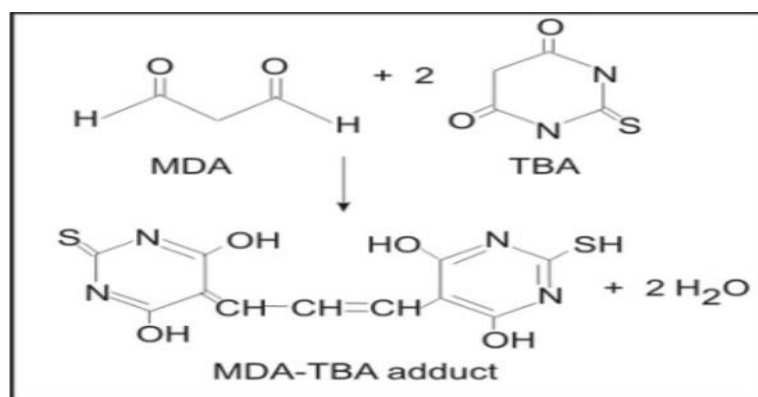
Appendix

Appendix I

Determination of serum concentration of malondaidehyde(MDA)

Principle:

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



Stock TCA – TBA – HCl Reagent:

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

Procedure:

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

Calculation:

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

MDA concentration = χ / 0.0624 nmol / ml.

Appendix II

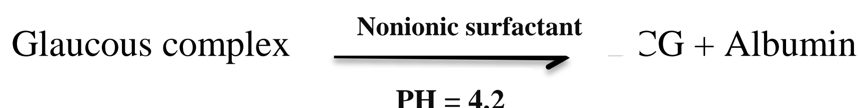
Determination of serum Reduced Glutathione concentration (GSH)

Reduced glutathione was measured following the method of Sedlak and Lindsay (1968). First, 3.0 mL precipitating solution containing metaphosphoric acid, Na2EDTA and NaCl was added to 2.0 mL of the sample. The mixture was centrifuged at 4500 × g for 10 min. 1.0 mL of supernatant was added to 4.0 mL of 0.3 M Na2HPO4 solution and 0.5 mM DTNB (5,5 –dithiobis-2-nitrobenzoic acid) was then added to this solution. Reduced glutathione was measured as the difference in the absorbance values of samples in the presence and the absence of DTNB at 412 nm. GSH value was calculated as nmol GSH/mg protein in the tissues and mmol GSH/g Hb in whole blood using the reduced glutathione as a reference (hemoglobin levels were estimated in whole blood using the Drabkins' solution).

appendix III

Serum albumin concentration

According to mindray company used the Bromcresol green (BCG) method and the reaction principle is



At a sightly acid pH (pH=4.2), serum albumin combines with bromcresol green to produce a glacous complex . the absorbency increase is directly proportional to the concentration of albumin.

Appendix IV

Serum globulin concentration

The total globulin concentration was estimated indirectly by measuring of albumin in serum and then subtract it form the result of total protein.

Globulin concentration(g/dl)= Serum Total protein – Serum albumin concentration.

According to mindray company used the Biuret method and the reaction principle is:

Cu^{+2} + protein $\xrightarrow{PH>12}$ copper-protein complex (blue-violet colour)

At an alkaline solution (pH >12) copper ions combine with protein to produce a blue-violet colour complex . the absorbency increase is directly proportional to the concentration of protein.

appendix V

Serum determination of Alanine Aminotransferase (ALT) , Aspartate Aminotransferase (AST) and Alkaline phosphatase (ALP)

The mindray apparatus was used to examine the enzymes, and the method for examining the enzymes was carried out according to the instructions of the manufacturer producing the device

According to mindray method for ALT and AST by UV-assay according to International Federation of clinical chemistry and Laboratory Medicine (IFCC) without pyridoxal phosphate activation .

And for ALP by International Federation of clinical chemistry and Laboratory Medicine (IFCC) modified method.

appendix VI

Calculate the percentage of saturation of the iron carrier Transferrin

The percentage of transferrin was calculated based on a mathematical equation . Before the calculation was performed, the blood iron level and level total iron banding capacity (TIBC) were measured.

The percentage of saturation of the iron transporter (transferrin) was estimated according to the following formula (Young, 2000).

$$\text{Saturation \%} = \frac{\text{Iron concentration in serum X100}}{\text{TIBC}}$$

Appendix VII

Measuring the level of the hormone Hepcidine in the serum

In the serum, the level of the hormone hepsidine was measured using a Kit Specific to measure the hormone level in mice Depending on the method of using Kit According to the instructions of the producing company.

The Protocol:

1.prepare all reagents before starting assay procedure . it is recommended that all standards and samples be added in duplicate to the microelisa stripplate.

2.add standard : set standard wells, testing sample wells. Add standard 50µl to standard well.

3.add sample: add testing sample 10µl then add sample well: blank well doesnot add anyting.

4. add 100µl of HRP-conjugate reagent to each well ,cover with an adhesive strip and incubate for 60 minutes at 37°C.

5. aspirate each well and wash, repeating the process four times for atotal of five washes. Wash by filling each well with wash solution(400µl) using a squirt bottle, manifold dispenser or autowasher . complete removal of liquid at each step is essential to good performance. After the last wash,remove any remaining wash solution by aspirating or decanting. Invert the plate and blot it against clean paper towels.

6. add chromogen solution A 50µl and chromogen B 50µl to each well
Gently mix and incubate for 15 minutes at 37°C . protect from light.

7. add 50µl stop solution to each well. The color in the well should change from blue to yellow . if color in the wells is green or the color change dose not appear uniform, gently tap the plate to ensure thorough mixing.

8. read the optical density (O.D.) at 450nm using a microtiter plate reader within 15 minutes.

appendix VIII

Hemocycline measurement (Hb)

Hemoclopane was measured using the Sahli method, and the basis of this method is the transformation of hemoglobin into acid hematin, the result of the reaction of 1% added hydrochloric acid, and after dilution of this mixture with distilled water it is compared with the standard color of the apparatus and the value is calculated in grams / 100 ml of blood (Jain, 1986).

appendix IX

Compact Cell Size (PCV)

I used the Microhaematocrit method using Capillary tubes and used the special device for the size of the compacted cells Microhaematocrit centrifuge, then read the result with a special ruler PCV reader, and calculated the result in percentage (%) (Coles, 1986; Jain, 1986) .

appendix X

Total RNA extraction

Total RNA were extracted from rat tissues (liver) by using (TRIzol® reagent kit) and done according to company instructions as following steps:

1.Tissue was homogenized by grinding in liquid nitrogen, and the tissue powder was transferred into DEPC – treated Eppendorff tube contains 1 ml of TRIzol® reagent. The tubes were shaken vigorously for 30 seconds.

2. Chloroform (200 µl) was added to each Eppendorff tube and shaken vigorously for 15 seconds.
3. The mixture was incubated on ice for 5 minutes.
4. Spined at 12,000 rpm , 4C° , for 15 minutes.
5. Supernatant was transferre to a new Eppendorff tube, and isopropanol (500 µl) was added.
6. Spined at 12,000 rpm , 4C° for 10 minutes.
7. Supernatant was discarded .
8. Adding 80% Ethanol (1 ml) and Vortex .

9.Spined at 12,000 rpm , 4C° for 5 minutes.

10.Supernatant was discarded and the pellet dried.

11.RNase free water (30µl) was added to the sample with vortexing until dissolving.

.12. The extracted RNA sample was kept at -20

Appendix XI

DNase inactivation (DNase I) Treatment

the extracted total RNA were treated with DNase I enzyme to remove the trace amounts of genomic DNA from the eluted total RNA by using samples (DNase I enzyme) and done according to method described by promega company, USA instructions as follow:

1.Total RNA (30µl) and 10 × reaction buffer with MgCl2 (4µl) and DNase I, RNase-free (2µl) and DEPC treated water (4µl) were added to Eppendorff tube.

2. The mixture was incubated at 37C° for 30 minutes

3. 1µl EDTA was added and incubated at 65C° for 10 minutes.

4. A volume of DNase Inactivation reagent equal to 20% of RNA sample was added to each RNA sample. The tubes vortexed to mix the DNase Inactivation Reagent with RNA sample.

5. All RNA samples left at room temperature for 2 minutes with flicking the tubes once or twice during this period to resuspend the DNase inactivation reagent.

6. The tubes were centrifuged at (12,000 rpm) for 1 minute to allow the DNase inactivation reagent separated from RNA sample solution, then, the RNA solutions transferred to new eppendorff tube.

Appendix XII

Assessing RNA yield and quality

There are three quality controls were performed on isolated RNA. First one is to determine the quantity of RNA (ng/µL) that has been isolated by used Nanodrop UV/VIS spectrophotometer (OPTIZEN POP. MECASYS Korea), the second is the purity of RNA by reading the absorbance in spectrophotometer at 260 nm and 280 nm in same Nanodrop machine, and the third is the integrity of the RNA by prepared gel electrophoresis, as follow:

1. After opening up the Nanodrop software, chosen the appropriate application (Nucleic acid, RNA).

2. A dry Chem-wipe was taken and cleaned the measurement pedestals several times. Then carefully pipet 1µl of ddH2O onto the surface of the lower measurement pedestal.

3. The sampling arm was lowered and clicking OK to initialized the Nanodrop, then cleaning off the pedestals and 1µl of the appropriate blanking solution was added as blanck solution which is same elution buffer of RNA samples.
4. After that, the pedestals are cleaned and pipet 1µl of RNA sample for measurement.
5. The purity of RNA, also determined by reading the absorbance in Nanodrop spectrophotometer at 260 nm and 280 nm, so the RNA has its absorption maximum at 260 nm and the ratio of absorbance at 260 nm and 280 nm is used to assess the purity of DNA and RNA. A ratio of ~1.8 is generally accepted as "pure" for DNA; a ratio of ~2.0 is generally accepted as "pure" for RNA. If the ratio is appreciably lower in either case, it may indicate the presence of protein, phenol or other contaminants that absorb strongly at or near 280 nm.
6. After that, the integrity of the RNA was determined by prepared gel electrophoresis as following:
   A. 1% agarose gel was prepared in 0.5X TBE buffer and heating by using (hot magnetic stirrer) for 2 minutes until disappear all crystals in agarose solution .
   B. After cooling, (3 µL) of Ethidium bromide (3%) was added to the solution, then the gel was poured in the tray and left until solidifying. Then transferred into electrophoresis machine which containing same 0.5X TBE buffer.
7. The RNA samples were prepared by mixing 3µl of RNA sample with 1µl of loading dye.
8. Then, all amount was transferred into agarose gel wells, then running the electrophoresis power at 100 Volt for 1 hours, then the RNA bands were visualized by U.V light.

Appendix XIII

Reverse transcriptase (cDNA synthesis) using PCR technique

Synthesis and amplification of cDNA were performed with the master amplification reaction (ABM, Canada), RT-qPCR is performed in a reaction solution of 20µL volume by mixing the following materials:

1. of forward (F) primer 1.0 µL
2. of reverse (R) primer 1.0 µL
3. 10µL of qPCR Master Mix Buffer (2X), (concentration: 1X).
4. 0.4µL of RT Mix Buffer (50X), (concentration:1X) .

5. 5.6 free H2O.
6. 2.0µL of extracted RNA template.

The generated solution is placed in Real time PCR Cycler for thermal reaction to measure the Cycle Threshold (CT) value. The following cycling protocol is performed :

Table (1) Master amplification reaction Component, Concentration and volume

No.	Component	Concentration	Volume
1.	Nuclease-free water up to 20 µl	-	5.6 µl
2.	Bright Green qPCR Master Mix	1X	10 µl
3.	Forward Primer (6 µM)	300NM	1 µl
4.	Reverse Primer (6 µM)	300NM	1µl
5.	qRT PCR enzyme Mix (50X)	1X	0.4 µl
6.	Total RNA	2pg - 0.2 µg 0.01pg - 2µg	2 µl

Thermal cycling reaction was performed using Sure cycler. Optimization of PCR reaction was accomplished for adopting which temperature yielded the chosen band product. The PCR reaction was carried out as shown in table (3)

Table (2) PCR program and cycling protocol

NO.	Step	Temperature	Duration	Cycle
1.	cDNA synthesis	42° C	15 min	**1**
2.	Pre – denaturation	95° C	10 min	**1**
3.	Denaturation	95° C	15 sec	**40**
4.	Annealing	60° C	60 sec	**40**
5.	Melt curve	**According to the instrument guidelines**		

RT-PCR is used for quantification of the levels of gene expression. The measured CT values during thermal reaction are recorded to compute the following measurements (Abtan, 2017) .

Appendix XIV
Data analysis of qRT-PCR

The data results of q RT-PCR for target and housekeeping gene were analyzed by the relative quantification gene expression levels (fold change) Livak method that described by (Livak and Schmittgen, 2001). The relative quantification method, quantities obtained from q RT-PCR experiment must be normalized in such a way that the data become biologically meaningful. In this method, one of the experimental samples is the calibrator such as

(Control samples) each of the normalized target values (CT values) is divided by the calibrator normalized target value to generate the relative expression levels. After that, the ΔCT Method with a Reference Gene was used as following equations:

ΔCT (calibrator) = CT (target, calibrator) – CT (ref, calibrator)

Ratio (target / reference) = $2^{\text{CT (reference)} - \text{CT (target)}}$

Table (3) CT values required for relative quantification with reference gene as the normalizer.

	Test	**Calibrator (cal)**
Target gene	CT(target, test)	CT(target, cal)
Reference gene	CT(ref, test)	CT(ref, cal)

First, normalize the CT of the reference (ref) gene to that of the target gene, for calibrator sample:

Second, normalize the CT of the reference (ref) gene to that of the target gene, for the test sample:

ΔCT (Test) = CT (target, test) – CT (ref, test)

ΔΔCT= ΔCT (test) – ΔCT (calibrator)

Fold change = $2^{-\Delta\Delta CT}$

Ratio (reference/target) = $2^{\text{CT (reference)} - \text{CT (target)}}$

So, the relative expression was divided by the expression value of a chosen calibrator for each expression ratio of test sample.

الخلاصة

أجريت هذه الدراسة للتحقق من الدور الوقائي لحمض الفوليك في تلف الكلى والكبد الناجم عن زيادة الميثيونين والحديد في ذكور الفئران.

تم تقسيم مائة وخمسين (150) فأرًا إلى خمس مجموعات (30 / مجموعة) ، تم تجريع المجموعة الأولى بماء لمدة ستة اسابيع (G1) ، وتم تجريع الفئران في المجموعة الثانية(G2) عن طريق الفم بجرعة 100 مجم / كجم B .W من الميثيونين ، بينما تم تجريع حيوانات المجموعة الثالثة(G3) بـ 0.07 ملجم / كجم من وزن الجسم من حمض الفوليك ، تم تجريع الفئران في المجموعة الرابعة (G4) عن طريق الفم باستخدام 0.07 ملجم / كجم من وزن الجسم من حمض الفوليك بالإضافة إلى الميثيونين ، وتم تجريع حيوانات المجموعة الأخيرة (G5) بـ 100 مجم / كجم من وزن الجسم حديد .

كما وقد تم جمع عينات الدم عن طريق ثقب القلب في الاسبوع السادس من التجربة لقياس:
أ- قياس مستوى تركيز Glutathion (GSH) و المالونديالديهايد Malondialdehyde MDA
ب- فعالية الانزيمات الناقلة للامين ، تركيز الألبومين والكلوبيولين ، تركيز الحديد الحر ، خازن الحديد وناقل الحديد ، ومستوى هرمون الهيبسدين ، بالاضافة الى دراسة التغيرات النسجية للكبد و الكلية ، و قد أخذت عينة من نسيج الكبد لقياس التعبير الجيني.

أظهرت النتائج وجود ارتفاع معنوي ($p < 0.01$) في تركيز MDA في G2 و G5 مقارنة مع مجموعة السيطرة وانخفاضه المعنوي ($p < 0.01$) في G3 و G4 ، و كذلك انخفاض معنوي ($p < 0.01$) في مصل GSH في G2 و G5 ، ارتفاع معنوي ($p < 0.01$) في GSH في G3 و G4 ، وانخفاض معنوي ($p < 0.01$) في تركيز الألبومين والكلوبيولين في G2و G5، أظهرت ارتفاع معنوي ($p < 0.01$) في نشاط AST ، ALP و ALT في G2, G5 و انخفاضه في G3 بالمقارنة مع مجموعة السيطرة ، وكذلك ارتفاع معنوي ($P < 0.01$) في مصل الحديد والفيريتين في G2،G5 وأظهرت انخفاضًا معنويًا ($p < 0.01$) في ناقل الحديد في G2 و كذلك ارتفاعا معنوي ($P < 0.01$) في مستوى هرمون الهيبسيدين و تركيز البيليروبين في G2 و G5.

كما وقد أظهرت نتائجنا انخفاضًا معنويًا ($p < 0.01$) في RBC و Hb و PCV في G2 و G5 بالمقارنة مع مجموعة السيطرة ، في دراستنا وجد ان التعبير الجيني لجين (HAMP) يرتفع في المجموعة التي تم تجريعها الحديد G5 بالمقارنة مع المجموعات الأخرى ، ووجدت الدراسة الحالية انخفاضًا في التعبير الجيني في مجموعة الميثيونين G2 ومجموعة حمض الفوليك G3 بالمقارنة مع المجموعة السيطرة ، والنتائج اظهرت كذلك ان الفئران التي جرعت بفرط ميثيونين وحمض الفوليك معا G4 لمدة ستة أسابيع لايوجد فرق في التعبير الجيني بالمقارنة مع مجموعة السيطرة ، أظهرت النتائج أن هناك تأثيراً ضاراً على أنسجة الكبد والكلى في المجموعة المعرضة لفرط الميثيونين حيث أظهر تمدداً في الوريد المركزي و ارتشاحا للخلايا الالتهابية و انحلالا في الخلايا البطانية للوريد المركزي وتضخم خلايا كوبفر وكذلك تضخما في خلايا الكبد ، اختفاء ترتيب الحبال الكبدية ، وضمورا في الكبيبات ، وضمور النبيب

البعيد ، و التغيير في شكل الخلايا المبطنة للنبيب حيث يكون شكلها الطبيعي هو متوازي المستطيلات ، ولكن في حالة زيادة حمل الميثيونين يصبح الشكل مسطحًا ، أما بالنسبة للنبيب القريب فلا يوجد تغيير ملحوظ ، لكننا نلاحظ أن لون السيتوبلازم أصبح أزرقا ، دليل على تلف أنسجة الكلى حيث أصبحت قاعدية ، و وجد تجمع مادة الهيالين في نسيج الكلية ، ووجود احتقان في الشريان و ارتشاحا الخلايا الالتهابية وحيدة النواة وتضخم الغلالة الباطنة ، يستنتج من الدراسة الحالية ان لحمض الفوليك دورا مهما في التقليل من الاثر الضار في ذكور الفئران المعرضة لفرط اعطاء الميثيونين ،كما و أظهرت المقاطع النسجية لكبد الحيوانات المعالجة بـ (ميثيونين وحمض الفوليك) ارتشاحا للخلايا الالتهابية وحيدة النواة ولكن أقل من الارتشاح الذي حصل في كبد الحيوانات في المجموعة الثانية وانخفاض انحلال الخلايا الكبدية وتنكس خلايا كوبفر ، وقد لوحظ أن أنسجة الكلى عادت إلى طبيعتها ولوحظ شفاء الكلى ، حيث ارتشحت الخلايا الالتهابية وحيدة النواة ولوحظ ضمور كبيبي ، ولكن بمعدل أقل من حالة الضمور في مجموعة الميثيونين فقط G2 وكان سيتوبلازم الخلايا في النبيب القريب وردي (حامضي) حيث بدأت تعود إلى حالتها الطبيعي ،اما في حالة تجريع 0.07 ملجم / كجم من وزن الجسم من حمض الفوليك ظهر عدم وجود تجمع لمادة الهيالين في النبيبات كما في حالة التجريع في الميثيونين .

و هكذا نستنتج من الدراسة الحالية الدور الوقائي لحمض الفوليك على تأثيرات فرط الميثيونين على الكبد والكلى في ذكور الفئران .

جمهورية العراق

وزارة التعليم العالي والبحث العلمي

جامعة كربلاء/ كلية الطب البيطري

فرع الفسلجة والكيمياء الحياتية والادوية

التأثير الوقائي لحمض الفوليك على الضرر الناجم عن الحمل الزائد للميثيونين على المعايير الفسلجية و النسجية و الجزيئية في ذكور الفئران

رسالة مقدمة الى

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

من قبل

تقى صبار راهي

بكالوريوس طب وجراحة بيطرية

كلية الطب البيطري / جامعة كربلاء 2017

بإشراف

أ.د. وفاق جبوري البازي	**ا.م.د.علي كاظم الجراح**
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1442 هـ **2020 م**