الجمهورية الجزائرية الديمقراطية الشعبية

People's Democratic Republic of Algeria

Ministry of High Education and Scientific Research

University of Constantine1
Faculty of life and Natural Sciences
Department of Animal Biology

N° d'ordre : 103/Mag/2014
N° de série : 01/BA/2014

Thesis submitted for the degree of magister

Option: Biology and molecular physiology

Effect of Algerian olive oil on cardiovascular diseases and lipids status in hyperhomocysteinemia treated mice

Presented by: Fatima Zahra Sakhri

Examination board:

President: Y. Necib	**Prof. University of Constantine1**
Supervisor: S. Zerizer	**Prof. University of Constantine1**
Examiner: A. Boudah	**MC. University of Constantine1**
Examiner: A. Khedara	**MC. University of Constantine1**

Academic year : 2013 / 2014

Dedicate

Thank you, mother and father for your love, for your encouragement and for the many years of supporting me and raising me to be the woman I am today. I am grateful for all you have done for me.

I want to thank my wonderful brother .You are truly a blessing from God and I am grateful for you every day. Thank you for being my biggest support despite your little age.

My sisters are thanked for their patience, especially during the final writing of this thesis, when I was both physically and mentally in "another" place. Your existence has helped me through all the hard times in my life.

My best friends: Souad, Benyamin, Moussa, Maroua, Hakim and Yaakoub thank you for your love and encouragement which saw me through the whole time.

The rest of my large family is thanked for their interest in my project.

Acknowledgement

A special thank to my principal supervisor Pr S. Zerizer, professor at the Faculty of life and Natural Sciences at Constantine 1, for the opportunity to study and work under her. I appreciate the time she dedicated to lending me guidance and advice during the preparation and completion of this thesis.

I would like to express my special thanks to Pr Y. Necib, professor at the Faculty of life and Natural Sciences at Constantine 1, for accepting to chair the jury.

I would like to thanks Dr. A. Boudah, Doctor at the Faculty of life and Natural Sciences at Constantine 1; for examination this thesis and for his friendly guidance, advice, support and encouragement during the study years.

My sincere thanks to Dr A. Khedara, Doctor at the Faculty of life and Natural Sciences at Constantine1, for examinating this thesis.

I would also like to thanks Pr. Satta, , Professor at the Faculty of life and Natural Sciences at Constantine 1, for her friendly guidness, her support, encouragement and never-ending optimism during all my study years in university of Constantine.

I would like express my special thanks to all my teachers in both university of Constantine 1 and El hadj Lakhdar-Batna.

My friends and colleagues at the university Constantine1 are thanked for helping me during the preparation of this thesis.

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Effect of Algerian olive oil on cardiovascular diseases and lipids status in hyperhomocysteinemia treated mice.

Summary

Olive oil is one of the oldest vegetable oils and the only one that can be consumed in its raw form without prior treatment. The well-known health benefits of olive oil are linked to its fatty acid composition and the presence of minor biomolecular, such as vitamins and natural antioxidants.

Homocysteine (Hcy) is a sulphur containing amino acid in the body produced by conversion of methionine. There is a balance in the metabolism of Hcy at physiological conditions. At high level, the hyperhomocysteinemia (HHcy), considered as an independent risk factor of cardiovascular diseases.

In the present study we measured thiols amino-acid (homocysteine), lipids statuts (total cholesterol, triglycerides, HDL-c, LDL-c), and antioxidant enzyme (glutathione reduced) in mice administered with (400mg/kg) of L-methionine during 21 days. Following oral administration of L-methionine in high dose, its degradation product Hcy which is markedly elevated in plasma exerts an angiotoxic action directed to the aorta and its toxicity on the heart and liver. This is showed with the loss and degeneration of endothelium, fenestration and formation of foam cells in the media of abdominal and iliac aorta, also the alterations of the cardiac muscle and liver necrosis. However, the aortic intima of the groups of mice that had been fed with L-methionine plus olive oil (7ml/60kg) showed intact endothelium and spindle-shaped mediocytes nuclei. Nevertheless, oval-shaped mediocytes nuclei and a few foam cells were observed.

The olive oil in mice have reduced Hcy, total cholesterol, triglycerides and LDL-c however, the concentration of HDL-c and glutathione reduced were increased.

The olive oil used in this study has antioxidant effect in mice.

Our results obtained in this study showed that the Algerian olive oil can be considered as natural source in the prevention against cardiovascular disease.

Key words: HDL-c, Homocysteine, Glutathione reduced, LDL-c, Olive oil, Total cholesterol, Triglyceride.

Effet de l'huile d'olive Algérienne sur les maladies cardiovasculaires et le statut des lipides chez les souris induit par une hyperhomocystéinémie.

Résumé

L'huile d'olive est l'une des huiles végétales les plus anciens et le seul qui peut être consommé dans sa forme brute, sans traitement préalable. Les avantages bien connus d'huile d'olive pour la santé sont liées à sa composition en acides gras et en biomolécules mineur, tels que les vitamines et les antioxydants naturels.

L'homocystéine (Hcy) est un acide aminé soufré synthétisée dans le corps par la conversion de la méthionine. Il existe un équilibre dans le métabolisme de l'Hcy dans les conditions physiologiques. En cas d'excès, l'hyperhomocystéinémie (HHcy), considéré comme un facteur de risque indépendant des maladies cardio-vasculaires.

Dans cette étude, nous avons dosé l'Hcy plasmatique, les paramètres lipidiques (cholestérol total, LDL, HDL, triglycérides) et les enzymes antioxydants (glutathion réduit) chez des souris auxquelles nous avons administré (400mg/kg) de L-méthionine pendant 21 jours. Après administration orale de la dose élevée de L-méthionine, la dégradation de cette dernière produit un excès d'Hcy dans le plasma sanguin, qui est à son tour exerce une action angiotoxique sur l'aorte iliaque et abdominal et une action toxique sur le cœur et le foie. Ceci est montré à la perte et la dégénérescence de l'endothélium, la fenestration et la formation de cellules spumeuses dans les médias de l'aorte abdominale et iliaque, également l'altération du muscle cardiaque et la nécrose du foie. Cependant, l'histologie aortique des souris traitées par la L-méthionine avec huile d'olive (7ml/60kg) montre un endothélium intact et des cellules musculaires avec des noyaux fusiformes. Néanmoins, on a observé quelques noyaux ovales et quelques cellules spumeuses.

Ainsi, le traitement par l'huile d'olive diminue le taux plasmatique d'Hcy, cholestérol total, triglycéride et LDL-c cependant les niveaux des HDL-c et le glutathion réduit augmentent.

Donc, l'huile d'olive utilisé dans cette étude exerce un effet antioxydant.

Les résultats obtenus dans cette étude montrent que l'huile d'olive Algérienne peut être considérer comme une source naturelle pour prévenir et traiter les maladies cardiovasculaires.

Les mots clés : Cholestérol total, Huile d'olive, HDL-c, Homocystéine, Glutathion réduit, HDL-c, Triglycérides.

تأثير زيت الزيتون الجزائري على أمراض القلب و الأوعية الدموية و حالة الدهون في الفئران المحفزة بواسطة الهومسستين البلازمي

الملخص

يعتبر زيت الزيتون من أقدم الزيوت النباتية و الوحيد الذي يمكن استهلاكه في شكله الخام دون معالجة مسبقة . ترتبط الفوائد الصحية المعروفة لزيت الزيتون باحتوائه على أحماض دهنية و جزيات بيولوجية ثانوية مثل الفيتامينات و مضادات الأكسدة.

الهوموسستيين عبارة عن حمض أميني مكبرت، يخلق من ميتابولزم المثيونين . في الظروف الفيزيولوجية الطبيعية، يوجد دائما توازن في ميتابولزم الهوموسستيين .في حالة الارتفاع في بلازما الدم يعتبر عامل مستقل في تصلب شرايين القلب و الأوعية الدموية.

خلال هذه الدراسة تم تقدير الأحماض الأمينية الكبريتية (الهوموسستين), اللبيدات (الكولسترول الكلي, الغليسريدات الثلاثية, LDL-c, HDL-c) و الانزيمات المضادة للأكسدة (جلوثاثيون المختزل) عند الفئران المتغذية على المثيونين بمقدار (400 مل غ/كل غ) لمدة 21 يوم . خلال تناول هذه الجرعة العالية من المثيونين فإن هدم هذا الأخير أدى إلى ارتفاع الهوموسستيين في بلازما الدم و الذي أدى إلى تخريب الشريان اللأورطي بالإضافة إلى الأعضاء الأخرى (القلب و الكبد). حيث لوحظ فقدان و تقشر البطانة الداخلية، تكوين خلايا رغوية في الطبقة المتوسطة من الشريان الأورطي البطني و الحرقفي وأيضا تغيير في عضلة القلب ونخر الكبد. بينما البطانة الداخلية للأورطي لمجموعة الفئران التي تم تغذيتها بواسطة المثيونين مع زيت الزيتون (7مل/60كغ) ظهرت بشكل سليم و بدون أي تقشر على مستوى البطانة الداخلية مع ظهور أنوية مغزلية للخلايا العضلية , لكن رغم ذلك فقد لوحظ ظهور أنوية دائرية للخلايا العضلية مع بعض الخلايا الرغوية القليلة جدا.

إن العلاج بالزيت الزيتون يؤدي إلى انخفاض مستويات الهوموسستين, الكولسترول الكلي, الدهون الثلاثية و LDL-c و اتفاع HDL-c و الجلوثاثيون المختزل.

إذن، زيت الزيتون الجزائري المستخدم في هذه الدراسة لها تأثير مضاد للأكسدة عند الفئران المتغذية على المثيونين .

إن النتائج المتحصل عليها في هذه الدراسة تبين زيت الزيتون يعتبر مصدر طبيعي يمكن استخدامه في وقاية و معالجة أمراض القلب والأوعية الدموية.

الكلمات الافتتاحية : **:** الهوموسستيين ، الكولسترول الكلي، LDL-c، HDL-c، الجليسريدات الثلاثية ، الجلوثاثيون المختزل, زيت الزيتون.

Abbreviations

Abbreviations

ALP: Phosphatase Alkaline

ATP: Adenosine Triphosphate

BSA: Bovine Serum Albumin

CβS: Cysthathionine β-Synthase

CVD: Cardiovascular Diseases

DAP: Dihydroxy-Acetone Phosphate

DNA: Desoxyriboonucleic acid

DTNB: Di-Thiobis-2-NitroBenzoic acid

EDTA: Ethylene Diamine-Tetra-acetic Acid

F: Flour

G3P: Glycerol 3 Phosphate

GSH: Glutathione

GK: Glycerol Kinase

GPO: Glycerol Phosphate Oxidase

Hcy: Homocysteine

HDL-c: High Density Lipoprotein cholesterol

HHcy: Hyperhomocysteinemia

HSFC: Heart and Stroke Foundation of Canada

ICAM-1: Intercellular Adhesion Molecule 1

LDL-c: Low Density Lipoprotein cholesterol

LP: Lipoprotein lipase

M: L-Methionine

MO: L-Methionine+ Olive oil

mRNA: messenger Ribonucleic acid

MS: Methionine Synthase

MT: Methyl-Transferase

Abbreviations

MTHF: Methyl Tetra-HydroFolate

MTHFR: Methylene Tetra-HydroFolate Reductase

NF-kB : Nuclear Factor kB

NO: Nitric Oxide

OO: Olive oil

Ox LDLc: Oxidize LDL

PBS: Phosphate Buffer Saline

PLP: Pyridoxal Phosphate

POD: Peroxidase

SAM: S-Adenosyl Methionine

SAH: S-Adenosyl-Homocysteine

SHMT: Serine Hydroxy-Methyl-Transferase

SMC: Smooth muscle cell

T-CH: Total Cholesterol

TG: Triglyceride

tHcy: total Homocysteine

THF: Tetra-Hydro-Folate.

TNB: 5′-Thio-2-NitroBenzoic acid

VCAM-1: Vascular Cell Adhesion Molecule-1

VLDL: Very Low Density Lipoproteins

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Introduction

Introduction

Cardiovascular disease (CVD) with its complications; is the leading cause of death and disability in developed nations and is increasing rapidly in the developing world (Tayal *et al.*, 2011). Worldwide deaths from cardiovascular disease are projected to rise from 17.1 million in 2004 to 23.4 million in 2030 (W.H.O., 2008).

Conventional risk factors for cardiovascular disease, including hypercholesterolemia, hypertension, smoking and diabetes, account for approximately 50% of all cases. . Evidence now indicates that hyperhomocysteinemia, which occurs in approximately 5 to 7% of the general population, is an important, independent risk factor for atherosclerosis and thrombotic disease (Lawrence de Koning *et al.*, 2003).

Hyperhomocysteinemia (HHcy) defined as an elevated concentration of total homocysteine due to the disruption in Hcy metabolism, which is caused by genetic or environmental factors or a combination of both factors

Homocysteine (Hcy) is a sulphur containing amino acid, derived from methionine and normally converted to cysteine and partly remethylated to methionine via enzymatic processes involving vitamin B12 and folate as cofactors (Akanji *et al.*, 2007).

The first link between homocysteine and cardiovascular disease was made by Mc Cully in 1969. Mc Cully observed advanced arterial lesions in children with inborn errors in Hcy metabolism and proposed a hypothesis that Hcy causes vascular disease (Jakubowski, 2008). Hyperhomocysteinemia has been suggested to be associated with cardiovascular disease as it is proved to cause cytotoxicity, lipid peroxidation, increased platelet aggregation, enhanced activation of the coagulation system and stimulation of vascular smooth muscle cell proliferation (Golbahar *et al.*, 2007).

Olive oil is a component of the Mediterranean diet, containing variable amounts of triacylglycerols and small quantities of free fatty acids, glycerol, pigments, aroma compounds, sterols, tocopherols, phenols, unidentified resinous components and others. The pharmacological properties of olive oil, the olive fruit and its leaves have been recognized as important components of medicine and a healthy diet because of their phenolic content (Sayed Haris, 2010).

In the present study, we aimed to:

- Evaluate the effect of L-methionine and olive oil on the weight and diet consumption of mice.
- Evaluated the predictive of olive oil on the angiotoxic action of L-methionine and on some parameters such as lipid status (total cholesterol, LDL-cholesterol, HDL-cholesterol and triglycerides).
- Examine the atherogenic effect of L-methionine on the abdominal and iliac aorta, heart and liver.

Bibliographic

Current data on homocysteine

7- Definition of homocysteine

Homocysteine (hcy), an independent risk factor of cardiovascular disease (Wang *et al.*, 2003), (Woo *et al.,* 2006), (Marti *et al.*, 2011), (Zappacosta *et al.*, 2013), is a sulfur containing 4-carbon -amino acid; discovered in 1933 by Du Vigneaud (Pellanda, 2012). It is not a part of diet, synthesized in all cells of organism by conversion of methionine; an essential amino acid present in foods regularly consumed within the diet (Figure1).

Unlike to other intracellular thiols (cysteine and glutathione), Homoysteine is regarded as a thiol toxic for the cell (Pellanda, 2012).

The symmetrical disulfide of homocysteine was termed "homocystine", both names being chosen to indicate that each carbon chain of these compounds contained 1 -CH2- group more than those of, respectively, cysteine and cystine (Ungvari, 2000).

8- The metabolic pathway of homocysteine

Two major metabolic pathways can be identified (Figure 2). When there is an excess of methionine ingested, homocysteine follows the transsulfuration pathway, through which homocysteine is converted automatically to cysteine (Dwivedi *et al.,* 2011). However, when methionine levels are low, Hcy is mainly metabolized via a methionine-conserving pathway (Vizzardi *et al.,* 2009).

2-1 Remethylation pathway

The homocysteine is remethyleted into methionine (Zappacosta, 2013) and (Dwivedi *et al.,*2011); which receives a methyl group from methyltetrahydrofolate (MTHF);in a reaction catalyzed by methionine synthase (MS) (Perna *et al.,* 2004), which uses methylcobalamin (a biologically active form of vitamin B12) as coenzyme.

The formation of MTHF from tetrahydrofolate is catalyzed by methylene tetrahydrofolate reductase (MTHFR) (Dwivedi *et al.,* 2011).

A proportion of methionine is then activated by adenosine triphosphate (ATP) to form S-adenosyl methionine (SAM). SAM serves primarily as a universal methyl donor to a variety of acceptors (Sabroe Ebbesen, 2003).

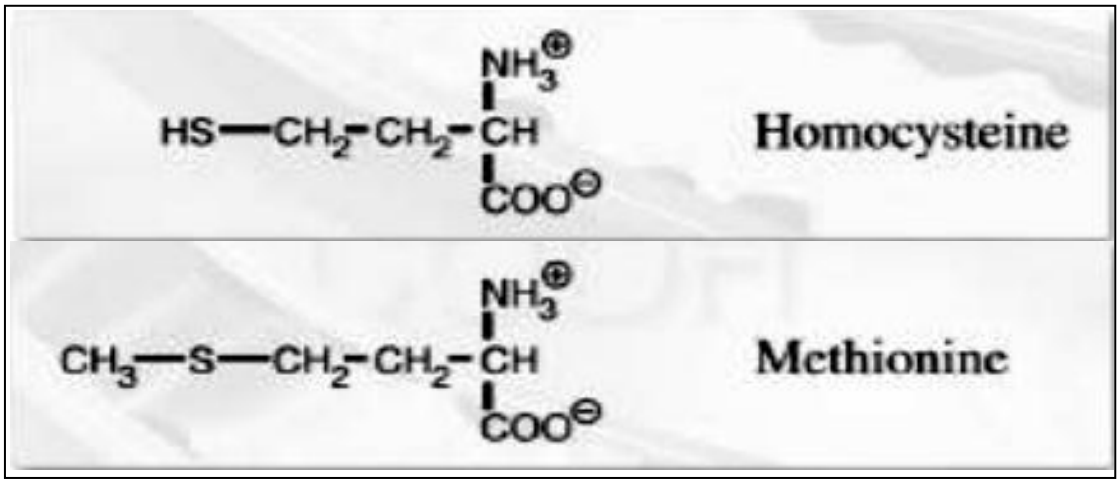

Figure 1: Structures of methionine, homocysteine (Jacobsen, 2001)

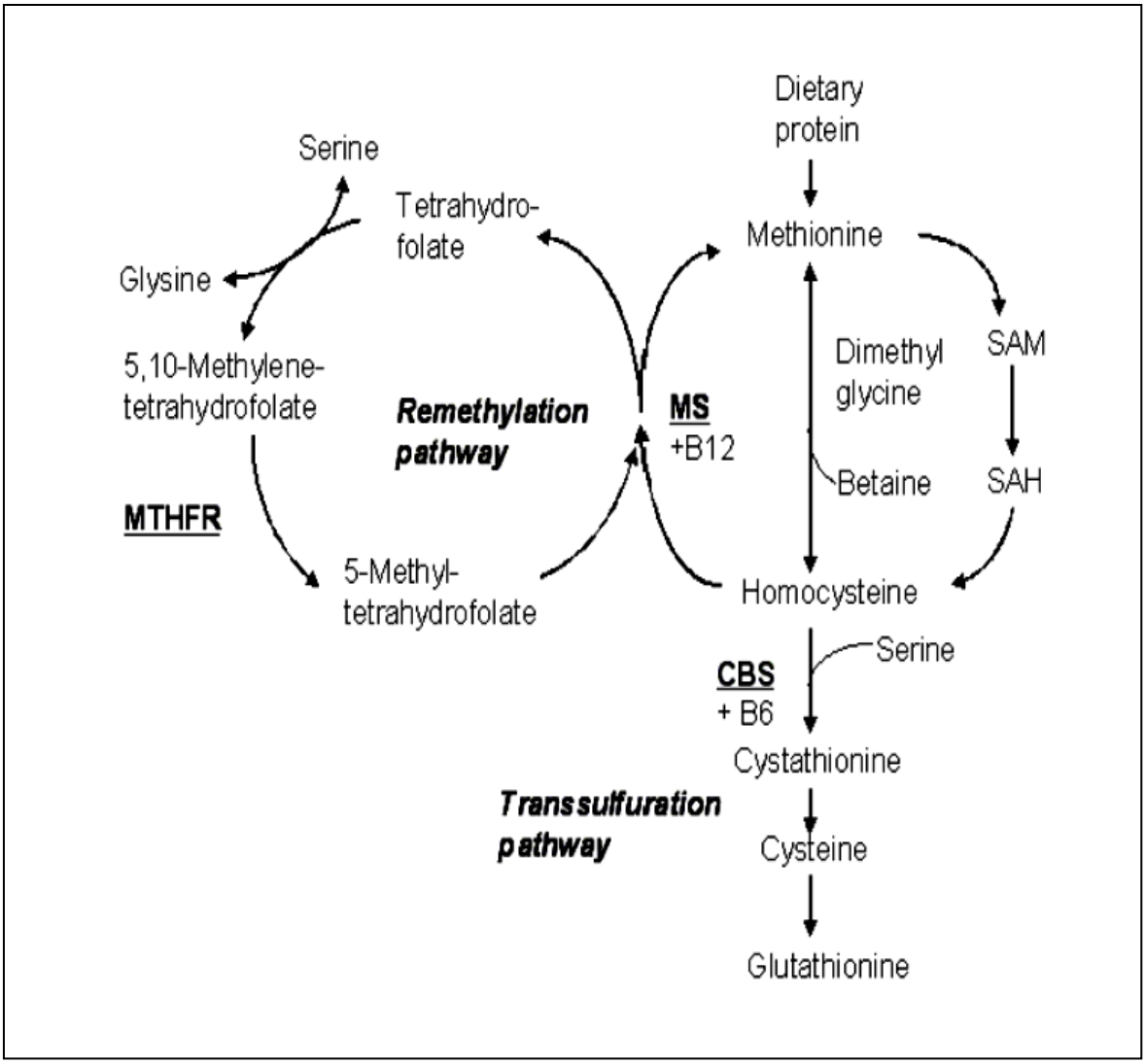

Figure 2: The metabolic pathway of homocysteine (Dwivedi *et al.*, 2011**)**

In an alternative remethylation route, which is also mainly restricted to the liver and kidney, betaine is used as the methyl donor by the enzyme betaine-homocysteine methyltransferase (Pellanda, 2012).

S-adenosylhomocysteine (SAH), the by-product of these methylation reactions, is subsequently hydrolyzed into homocysteine and Adenosine catalyzed by S-adenosyl homocysteine hydrolase, thus regenerating Hcy, which then becomes available to start a new cycle of methyl-group transfer (Ungvari, 2000).

It is important to note that this hydrolysis is a reversible reaction that favors the synthesis of SAH, and that elevated cellular concentrations of SAH are likely to proceed and accompany all forms of hyperhomocysteinemia (Sabroe Ebbesen, 2003).

2-2- Transsulfuration pathway

Condensation of serine with Hcy to form cystathionine in an irreversible reaction catalyzed by the vitamin B6–containing enzyme, cystathionine ß-synthase. Cystathionine hydrolyzed by a second vitamin B6-containing enzyme, cystathionase, to form cysteine and α-ketobutyrate by cystathionine β-synthase (CβS). Any excess of cysteine is oxidized to taurine or inorganic sulphates or eliminated from the body (Vizzardi *et al.,* 2009).

Thus, in addition to the synthesis of cysteine, this transsulfuration pathway effectively catabolizes excess Hcy, which is not required for methyl transfer (Ungvari, 2000) and (Sabroe Ebbesen, 2003).

9- Determinants and ranges of the homocysteine level

In humans, normal plasma levels of total homocysteine are with a mean level of about 10 µmol/L (Jacobsen, 2001) and (Yang *et al.*, 2013).

Only 1% to 2% occurs as the thiol form of homocysteine (Brosnan *et al.,* 2004) and (Woo *et al.,* 2006). The remaining 98% is in the form of disulfides from which 75% is bound to proteins (mainly albumin) through disulfide bonds with protein cysteines, whereas the remainder occurs in non–protein-bound forms: homocysteine, homocysteine-cysteine disulfide, and more minor amounts of other mixed disulfides (homocysteine-cysteinylglycine disulfide) (Ungvari, 2000) (figure 3).

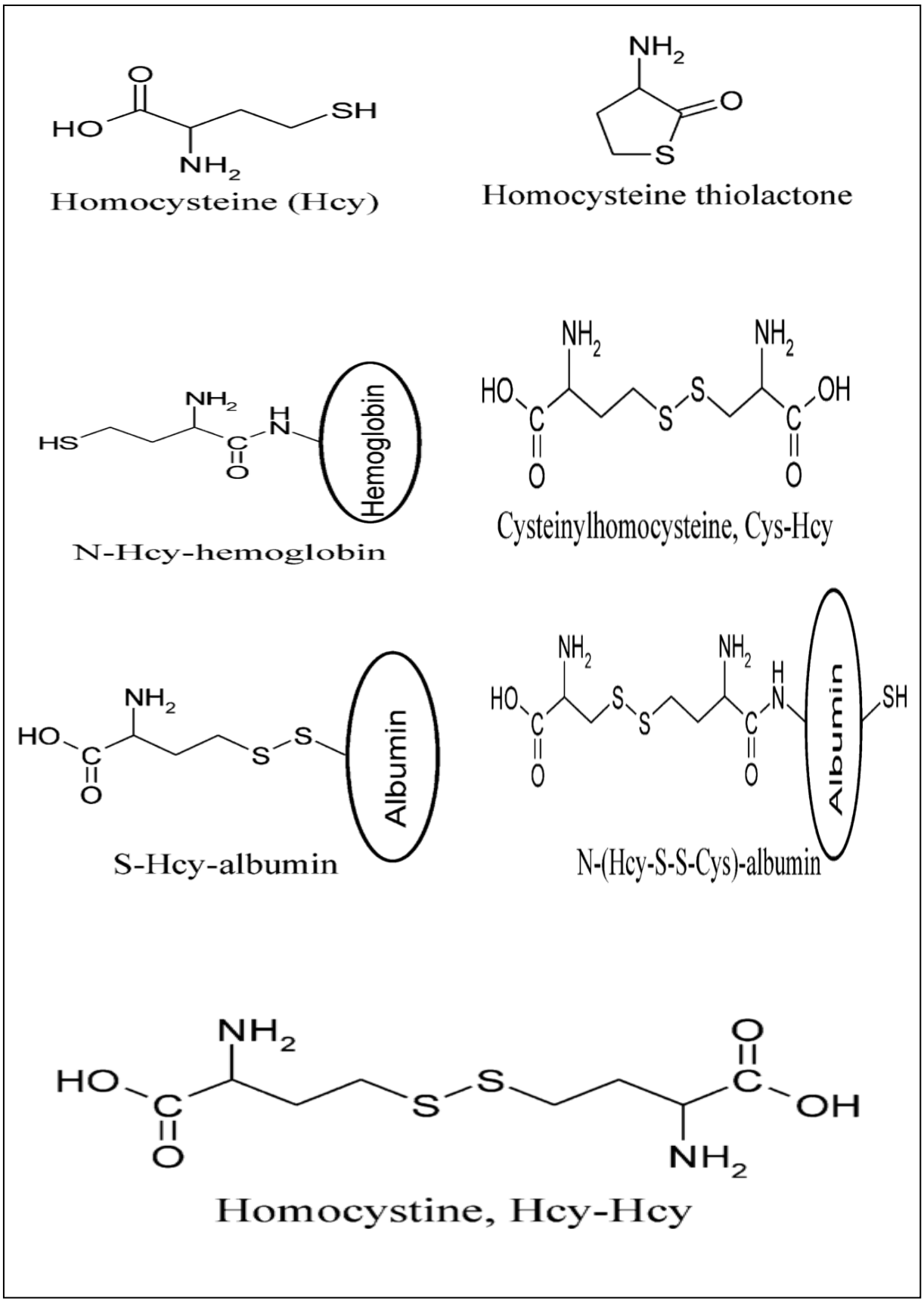

Figure 3: Molecular formula of homocysteine present in the human blood

(Perla-Kajan *et al.*, 2007)

The term "total homocysteine" (tHcy) is commonly used to describe the pool of homocysteine released by reduction of all disulfide bonds in the sample. This pool, however, does not include homocysteine bound to the protein by amide bound or homocysteine thiolactone (Perla-Kajan *et al.,* 2007) and (Akchiche, 2009).

It was long believed that Hcy was not present in plasma, urine and tissue under physiologic conditions because the small concentrations were below the detection limit of amino acid (Wilcken and Wilcken, 1976).

10- Hyperhomocysteinemia

Hyperhomocysteinemia defined as fasting homocysteine concentration and/or increase concentration in homocysteine concentration (Van Der Griend *et al.,* 1998) due to the disruption in Hcy metabolism, which is caused by genetic or environmental factors or a combination of both factors (Ueland and Refsum, 1989) and (Kang *et al.,* 1992).

It is denoted as:

- **Moderate hyperhomocysteinemia** (15–30 µmol/L)
- **Intermediate hyperhomocysteinemia** (Between 30–100 µmol/L)
- **Severe hyperhomocysteinemia** (>100 µmol/L)

(Damiens, 2000), (El Bouchti *et al.,* 2008), (Jay Blank, 2009) and (Scherer *et al.,* 2011).

4-1- Factors influencing plasma total homocysteine level

4-1-a *Age and sex*

Plasma homocysteine concentration was higher in men than in women and increased progressively with age (Prerost, 1997). This disparity might be explained by differences in muscle mass, hormonal factors, renal function capacity and vitamins status (Sabroe Ebbesen, 2003).

Part of the relationship with age in women may be explained by menopausal; since the tHcy concentration was found higher in post menopausal women compared with premenopausal women (De Bree *et al.,*2002) and (Sabroe Ebbesen, 2003).

Pregnancy has been shown to substantially lower plasma homocysteine (Jay Blank, 2009).

Maternal plasma homocysteine is inversely related to neonatal mass and gestational age after delivery, which suggests that the decrease during pregnancy might be due to fetal uptake of maternal plasma homocysteine (Malinow *et al.,* 1998) and (Ueland *et al.,* 2000). Another hypothesis is that lower plasma homocysteine levels are a physiological adaptation, which supports placental circulation (Bonnette *et al.,* 1998) and (Ueland *et al.,* 2000).

4-1-b *Dietary factors*

Several intervention studies have provided evidence for the importance of B vitamins in homocysteine metabolism (Sabroe Ebbesen, 2003).Plasma homocysteine concentration is influenced by the intake of these vitamins from dietary sources and nutritional supplements, and supplementation can at least partially normalize elevations in plasma homocysteine (Cuskelly *et al.,*2001).

There are three types of deficiency in B vitamins associated with accumulation of homocysteine (Stabler *et al.,* 1988):

- **Interruption of the transsulfuration pathway by a deficiency of vitamin B6 derivation (PLP-pyridoxal phosphate) co-enzyme**

Dietary vitamin B-6 is necessary for the formation of pyridoxal 5'phosphate (PLP) (Lamers *et al.,* 2011), which serves as a coenzyme in the transsulfuration pathway. In humans and rats studies, the average fasting Hcy levels did not differ significantly between subjects exposed to a diet deficient in vitamin B6 and those with a diet rich in vitamin B6 (El Mabchour, 2010); vitamin B-6 deficiency does not seem to have an effect on plasma homocysteine levels. However, if a vitamin B6 deficient individual takes a methionine load than has tHcy measured, it will be dramatically elevated (Jay Blank, 2009).

Vitamin B6 can alter homocysteine metabolism by reducing the activity of serine hydroxymethyltransferase (SHMT) and by suppressing homocysteine catabolism (Cuskelly *et al.,* 2001).

- **Folate deficiency responsible for a deficit methyl tetrahydrofolate (THF) which is a methyl donor**

Of the three vitamins, which are necessary for normal Hcy metabolism, depletion of folic acid is most frequently reported (Racek *et al.,* 2005). Humans cannot synthesize folate and therefore they must obtain it in their diet. Major sources of folate are green vegetables, citrus fruits, liver and whole grains (Gok *et al.,* 2004). It is used as a substrate; it is donated the

methyl group of the conversion of homocysteine to methionine. Deficiencies in folic acid can lead to moderate to severe hyperhomocysteinemia (Jay Blank, 2009)

A higher dietary folate intake is associated with a lower tHcy level in adults, independent of other dietary and lifestyle factors (De Bree *et al.*, 2002).

- **The vitamin B12 deficiency**

Vitamin B12, also known as cobalamin, is the main catalyst in the 5methyltetrahydrofolate conversion of homocysteine into methionine. Without cobalamin, the entire remethylation cycle essentially gets shut off (Jacques *et al.*, 2001). This creates an imbalance between remethylation and transsulfuration causing a metabolic folate trap in the form methyl THF, making them unusable for other metabolic reactions (Akchiche, 2009). Vitamin B12 deficiency is a common cause of moderate to severe fasting hyperhomocysteinemia (Ueland *et al.*, 2000).

-The relation between intake of vitamin B2 and tHcy concentration is scarcely investigated, and the weak inverse associations found could well be due to inadequate correction for the intake of other dietary components like methionine and alcohol (De Bree *et al.*, 2002).

4-1-c *Life style factors*

Coffee consumption is positively associated with the tHcy concentration (De Bree *et al.*, 2002) because caffeine may inhibit the conversion of hcy to cysteine by acting as a vitamin B6 antagonist (De Bree *et al.*, 2001).

Smoking is positively associated with plasma total homocysteine level (Jay Blank, 2009). The exact mechanism behind the increase in the tHcy concentration is unidentified, but smoking may induce local effects in cells exposed to cigarette smoke, influence the tHcy concentration by changing the plasma thiol redox status, or inhibit enzymes such as methionine synthase (De Bree *et al.*, 2002)

Alcohol seems to have a similar effect as coffee on plasma homocysteine levels (Jay Blank, 2009). In a recent study, it was shown that alcohol consumption could have detrimental effects on both folic acids and B12 status. This suggests that alcohol consumption

might raise plasma homocysteine levels by hindering the remethylation pathway in homocysteine metabolism (Jay Blank, 2009).

The relation between plasma homocysteine and physical activity is unclear and more research needs (Prerost, 1997) but is probably not or weakly inversely associated with the tHcy concentration because an active lifestyle is generally associated with a more healthy lifestyle, and a more healthy lifestyle with a lower tHcy concentration (De Bree *et al.*, 2002).

4-1-d *Drugs and diseases*

Plasma tHcy concentration increased rapidly in patients that were given; Antifolate, Antiepileptic, Neuroleptics, Sulfonamides, Nutalan, B6 antagonists (De Bree *et al.*, 2002).

Similar to drugs, any diseases that negatively affect the absorption or function of the vitamins crucial to homocysteine metabolism will cause plasma homocysteine to rise. Some of these conditions are kidney function, cancer and psoriasis, Crohn's diseases (Sabroe Ebbesen, 2003), renal failure, liver failure, certain leukemia, hypothyroidism (El Mabchour, 2010).

4-1-e *Genetics factors*

Of all the determinants, genetic mutations have the largest capacity to affect plasma homocysteine levels (Jay Blank, 2009). Almost 70 genetic determinants have been identified (El Mabchour, 2010).Two common polymorphisms (C677T and A1298C) in the gene encoding 5-10 MTHFR have been described (Fekih-Mrissa *et al.*, 2013). If an individual is homozygous for this genetic defect (C677T mutation of MTHFR), activity of this vital enzyme can decrease by 50 percent.

Other genetic polymorphisms have been seen in hyperhomocysteinemia, including a defect in CβS. Heterozygote for either CβS or MTHFR deficiency often have elevated homocysteine concentrations (Willems, 2003). The gene mutation or polymorphism MS 2756A / G causing its inactivation, this mutation is rare or by a genetic mutation in the gene encoding the transcobalamin (El Mabchour, 2010).

5- Hyperhomocysteinemia and cardiovascular diseases

5-1- Cardiovascular system

The cardiovascular system consists of the heart, major arteries, arterioles, capillaries, venules and veins that form a closed system of blood vessels that carry the blood (Eroschenko and Di Fiore, 2012).

5-1-a- *The heart*

The heart is the muscle organ; serves as a pomp to drive the flow of blood through the body. With every heartbeat, the normal healthy heart at rest typically pumps 70ml of blood out into the systemic circulation. At a heart rate of 70 beats per minute, approximately 5 liters of blood is ejected from the heart every minute (Steding, 2010).

The human heart lies inside the thoracic cavity, resting on the diaphragm. It is within the mediastinum in between the lungs. Its posterior border is near the vertebral column, and its anterior border is near the sternum (figure 4).

An average adult has a heart about 12 cm in length, 8 to 9 cm in breadth at the broadest part, and 6 cm in thickness and weight about 280 to 340g in male and 230 to 280g in the female. The heart continues to increase in weight and size up to an advanced period of life; this increase is more marked in men than in women (Gray, 2000).

The heart's structure has four chambers: the left and right atria and the left and right ventricle. The ventricles are larger and more muscular than the atria. Each ventricle contains two valves (Thbbell *et al.*, 2002) the mitral valve separates the left atria and ventricle and the tricuspid valve separates the right atria and ventricle (figure 4) (Steding, 2010).

Blood flow the through the heart is controlled by the valves, which open to allow blood to flow from on chamber to another and close to prevent blood from flowing back into the heart (Thbbell *et al.*, 2002).

Venous blood, low on oxygen, via the great veins (superior and inferior vena cave) enters the right atrium and then passes through the tricuspid valve into the right ventricle. The blood is pumped through the pulmonary valve into the pulmonary arteries and on through the pulmonary capillaries in the lungs where it is oxygenated and transported to the left atria, then trough the mitral valve into the left ventricular (Elkington and Gwinnutt, 2009). Finally, the left ventricle pumps the oxygen-rich blood through the aerotic valve to all parts of the body.

The wall of each atrium and ventricle consists of three layers (figure 5):

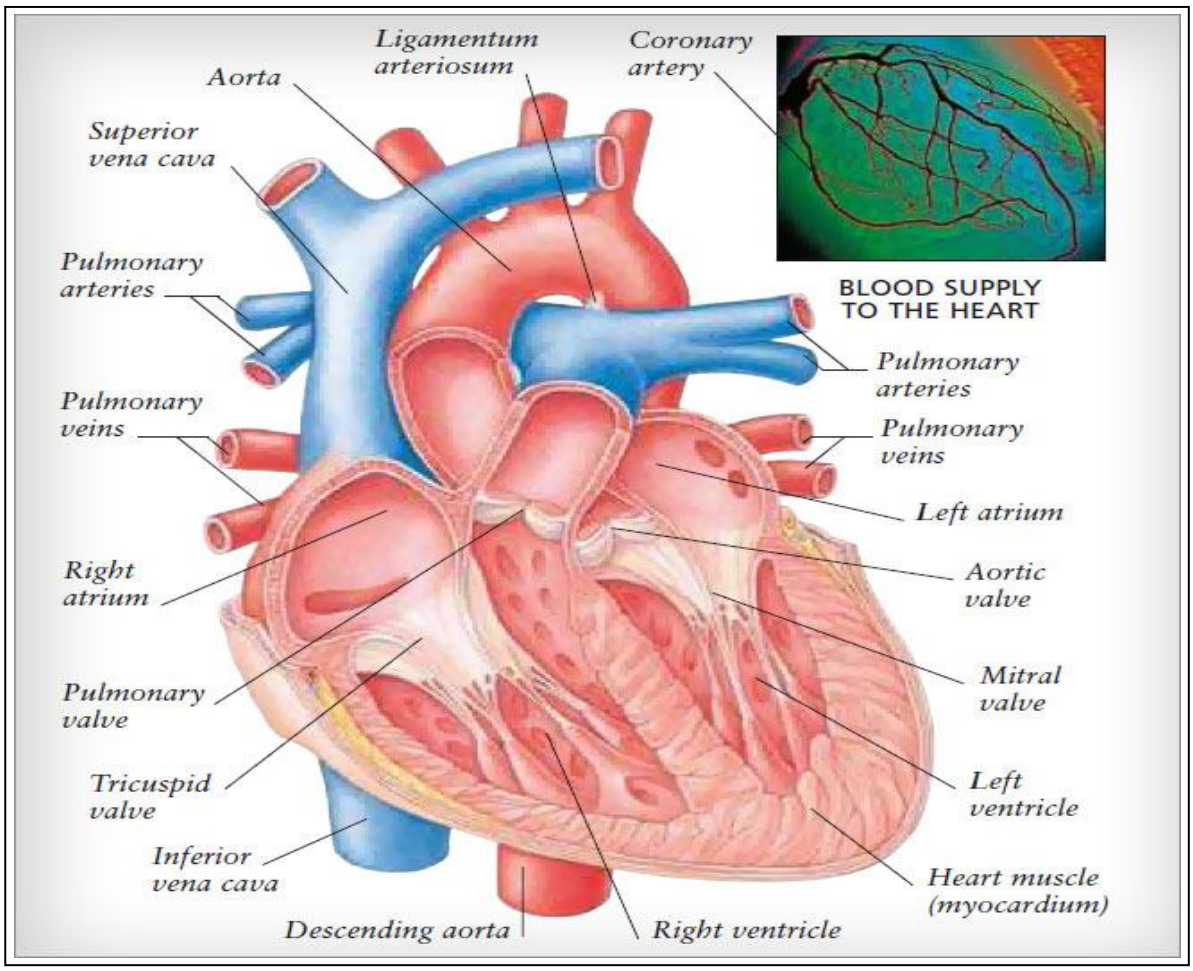

Figure 4: Structure of the heart **(**http://www.doctorcare4u.com/cardiovascularsystem.aspx**)**

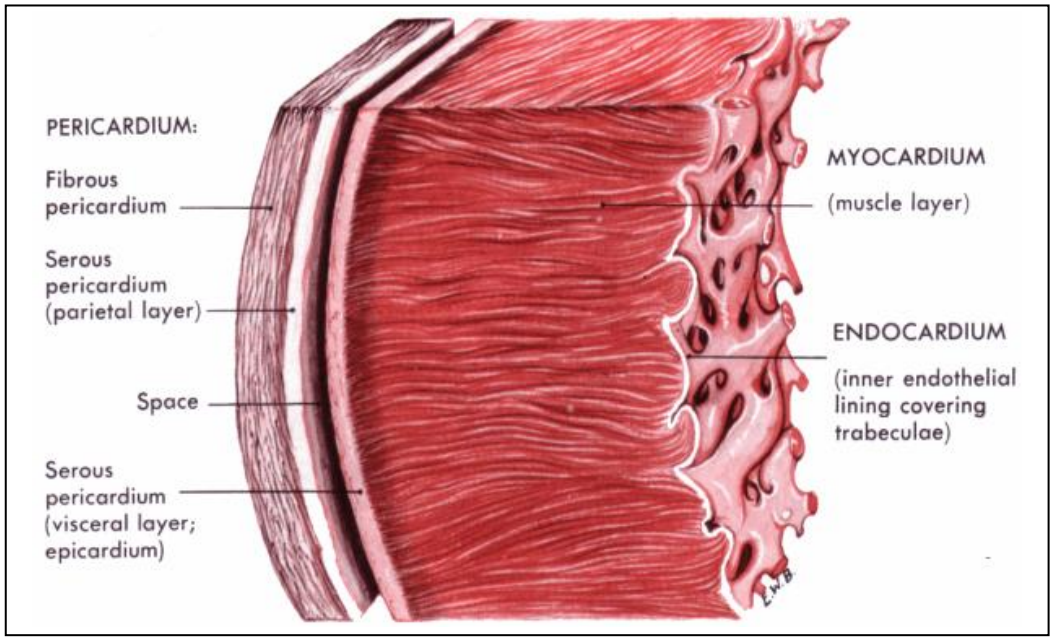

Figure 5: section of the heart wall showing the components of the outer pericardium (heart sac), muscle layer (myocardium) and inner lining (endocardium) (http://histologyolm.stevegallik.org/node/347)

-**The endocardium**: consisting of inner layer of endothelial and subendothelial connective tissue, a middle layer of connective tissue and smooth muscle cells, and a deeper layer of connective tissue also called the subendocardial layer, which is continuous with the connective tissue of the myocardium (Ross and Wojciech, 2006).

-**The myocardium** is the contractile layer of the heart wall and is formed by cardiac muscle, connective tissue, and an extensive vasculature (Henrikson and Mazurkiewicz, 1997). The thickness of this muscle in various chambers of the heart varies according to the workload of that specific chamber (Verma, 2001)

-**The epicardium:** is the visceral layer of the pericardium (Henrikson and Mazurkiewicz, 1997) consisting of a layer of mesothelial cells on the outer surface of the heart and its underlying connective tissue (Ross and Wojciech, 2006).

The heart is enclosed in a sac, the pericardial sac (cavity) composed of compact fibrocollagenous layers, an outer parietal pericardium (Verma, 2001). There is a small amount of serous fluid in the pericardial sac. This fluid lubricates the surface and permits frictionless movement of the heart within the cavity during its muscular contraction (Verma, 2001).

5-1-b *The blood vessels*

There are five basic types of blood vessels: arteries, arterioles, capillaries, venules and veins. The various types of vessels differ in their structures and functions (figure 6) (Whittemore and Cooley, 2009).

- **Arteries**: There are three types of arteries in the body:

-**Elastic arteries**: Are the largest blood vessels in the body and include the pulmonary trunk and aorta with their major branches, the brachiocephalic, common carotid, subclavian, vertebral, pulmonary and common iliac artery. The walls of these vessels are primarily composed of elastic connective fibers interspersed with circularly arranged smooth muscle cells. The elastic fibers provide great resilience and flexibility during blood flow (Eroscchenko and Di Fiore, 2012).

-**Muscular artery** (medium artery), the most numerous vessels in the body, the walls of this arteries contain greater amounts of smooth muscle fibers.

-**Arterioles** are the smallest branches of the arterial system. Their walls consist of one to five layer of smooth muscle fibers, arterioles deliver blood to the smallest blood vessels; the capillaries. Capillaries connect arterioles with the smallest veins or venules (Eroscchenko and Di Fiore, 2012).

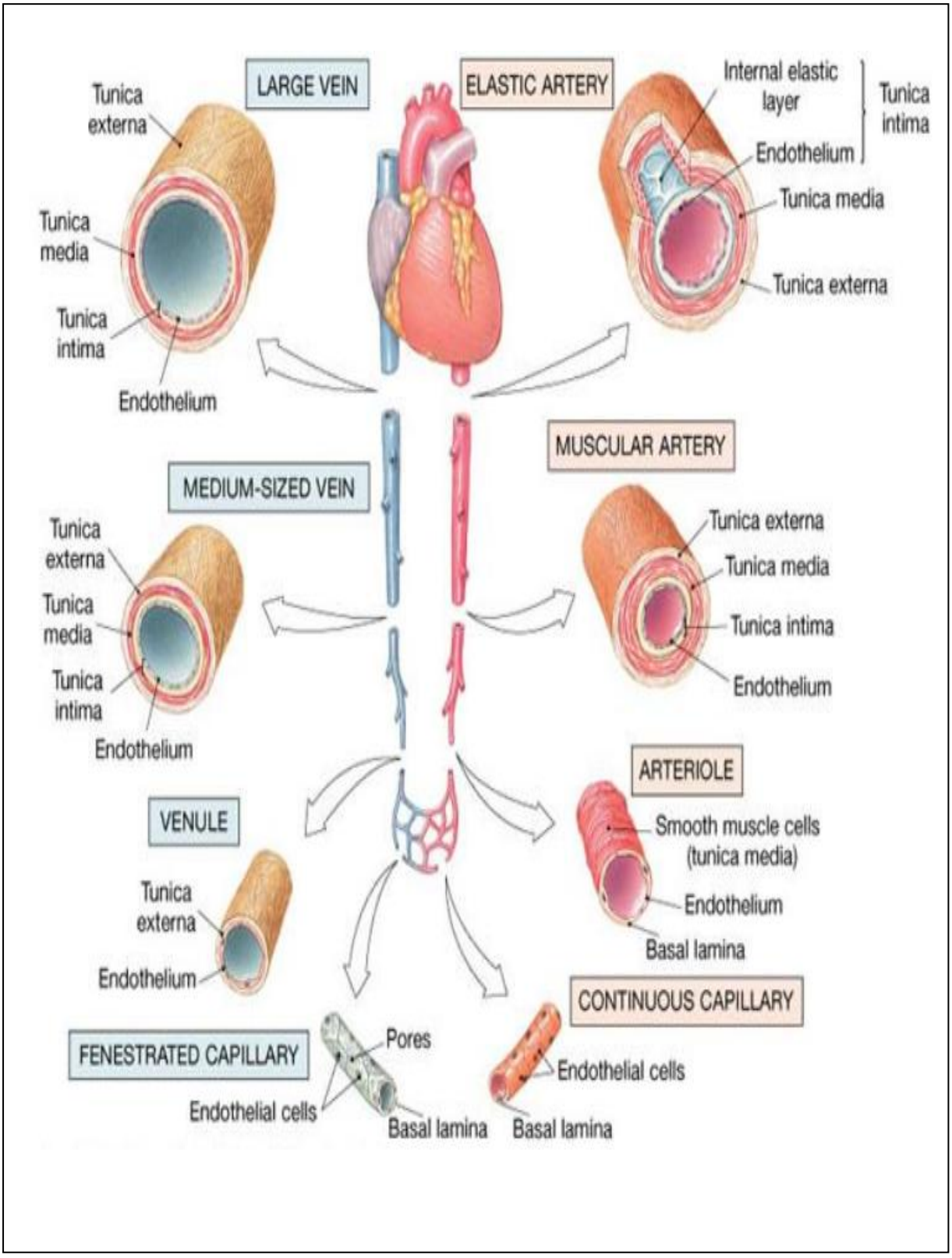

Figure 6: Blood vessels and their layers**.**

(http://www.studyblue.com/notes/note/n/chapter-14/deck/6408995)

❖ **Capillaries**

Capillaries are the smallest diameter blood vessels (Ross and Wojciech, 2006), highly branched vessels that feed the tissues and collect wastes to be carried back to the lungs, liver or kidney for elimination. After leaving the capillaries, the blood is collected into venules and then veins of increasing size, before being returned to the heart (Whittemore and Cooley, 2009).

❖ **Veins**

The veins return blood to the heart. The superior vein cave is the large vein that brings blood from the upper part of the body to the heart and the inferior vein cave brings blood from the abdomen and lungs to the heart (Eurell, 2004).

5-1-c *Histology of blood vessels*

The wall of arteries and veins are composed of three layers called tunics and the various type of them are distinguished from each other on the basis of the thickness of the vascular wall and differences in the composition of the layer.

The three layer of the vascular wall from the lumen out ward are:

❖ **The intima**, or **tunica interna**, the innermost layer of the vessel. It consists of:

- A single layer of squamaus epithelial cells, the endothelium
- The basal lamina of the endothelial cells
-The subendothelial layer consisting of loose connective tissue (Ross and Wojciech, 2006)

The ability of the endothelial monolayer to repair itself and maintain function has a significant role in the development of atherosclerotic plaque. Beneath the intima lies, a single layer of elastic fibers forming a matrix called the internal elastic lamina (Hall and Bassiouny, 2012).

❖ **The tunica media** (middle layer), is composed of smooth muscle and connective tissue and it is located between the tunica interna and tunica externa. A condensation of elastic fibers, the external membrane, is presented in some vessels as an outer layer of the tunica media (Eurell, 2004).

❖ **The tunica adventitia** is the outermost layer in the wall of the blood vessels (Henrikson and Mazurkiewicz, 1997). It is composed primarily of longitudinally arranged collagenous tissue and a few elastic fibers. These connective tissue elements gradually merge with the loose connective tissue surrounding the vessels. The tunica adventitia ranges from relatively thin in most of the arterial system to quit thick in the venules and veins, where it is the major component of the vessel wall (Ross and Wojciech, 2006).

5-2- Cardiovascular diseases and Atherosclerosis

The heart and stroke foundation of Canada (HSFS) (2003) indicated that cardiovascular diseases were defined as all diseases of the circulatory system (Dedkhard, 2006). The vast majority of cardiovascular disease is attributed to a disease of arterial blood vessels, known as "Atherosclerosis" (Gerdes, 2005).

The word Atherosclerosis derived from the Greek, athéré (gruel) and skléros (hard) and reflect quit well the macroscopic morphology of an Atherosclerosis lesion, yellow-white thickening a long arteries walls lead to occlusion of the vessels, thus interrupting blood flow and oxygen supply to vital organs such as the heart and brain.

In the beginning of the 20th century, atherosclerotic lesion demonstrating that is not a recent disease (Tupin, 2004). Today, cardiovascular diseases are the leading cause of mortality worldwide and cause an estimated 30% of all death (Sjöberg, 2008)

5-2-a- *Pathophysiology of Atherosclerosis*

Atherosclerosis is a chronic, immune-inflammatory, fibro proliferative disease of large and medium sized arteries (Singh *et al.,* 2012) throughout the body. Atheromatous lesions, or atheromas are asymmetrical focal thickening of the innermost layer of the intima and narrowing of the artery. The formation of a lesion is a slow process and can begin already in the childhood. Fatty streaks are prevalent even among young people and may progress to atherosclerotic lesion or eventually disappear (Kangas-Kontio, 2011).

There are three stages in the life history of an atheroma: initiation, progression and complication (figure 7 and 8) (Singh *et al.,* 2012).

- **Initiation**

The recruitment of mononuclear leucocytes to the intima characterizes initiation of the atherosclerotic lesion (Singh *et al.,* 2012). Modified lipids, such as oxidized LDL particles promote increased expression of adhesion molecules, especially vascular cell adhesion molecule-1(VCAM-1), intercellular adhesion molecule 1(ICAM-1) and P-selectin, on the surface of endothelial cells (Troseid, 2010). The adhesion molecules participate in the recruitment of monocytes and T cells to the vessel wall (Sjöberg, 2008). Monocytes differentiate into macrophages in the sub endothelial space, and begin to phagocytose the

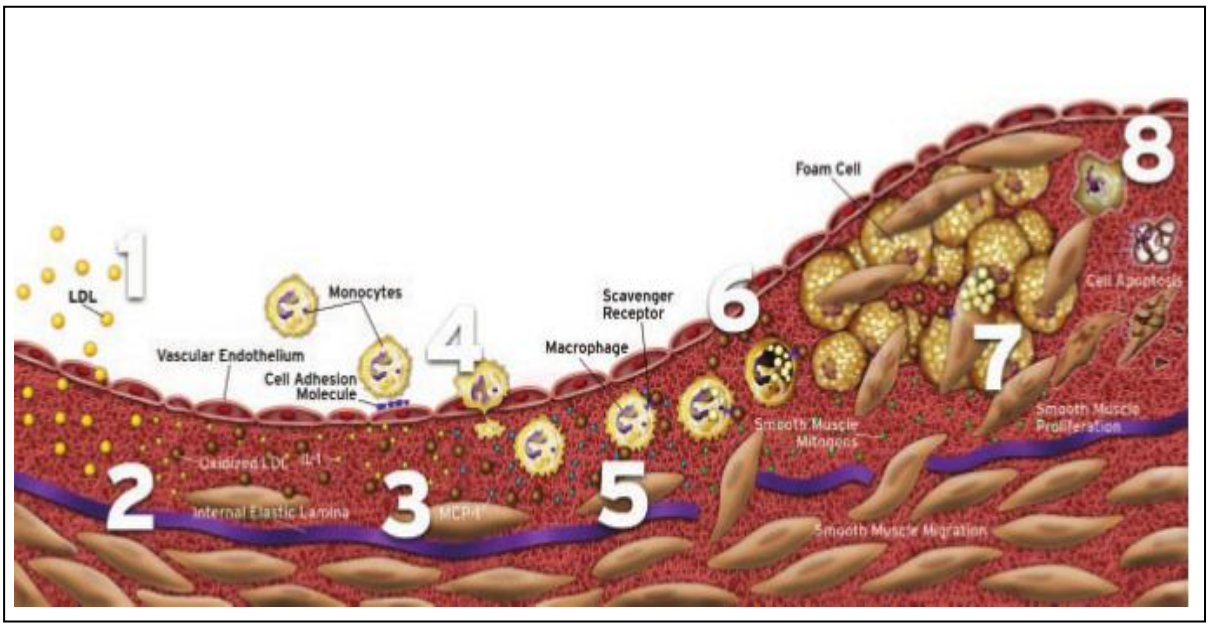

Figure 7: The stages of development of an atherosclerotic plaque. First LDL moves into the subendothelium and is oxidized by macrophage and SMCs (1and 2). Release of growth factors and cytokines attracts additional monocytes (3 and 4). Foam cell accumulation and SMC proliferation result in growth of the plaque (6, 7, and 8) (Faxon *et al.*, 2004).

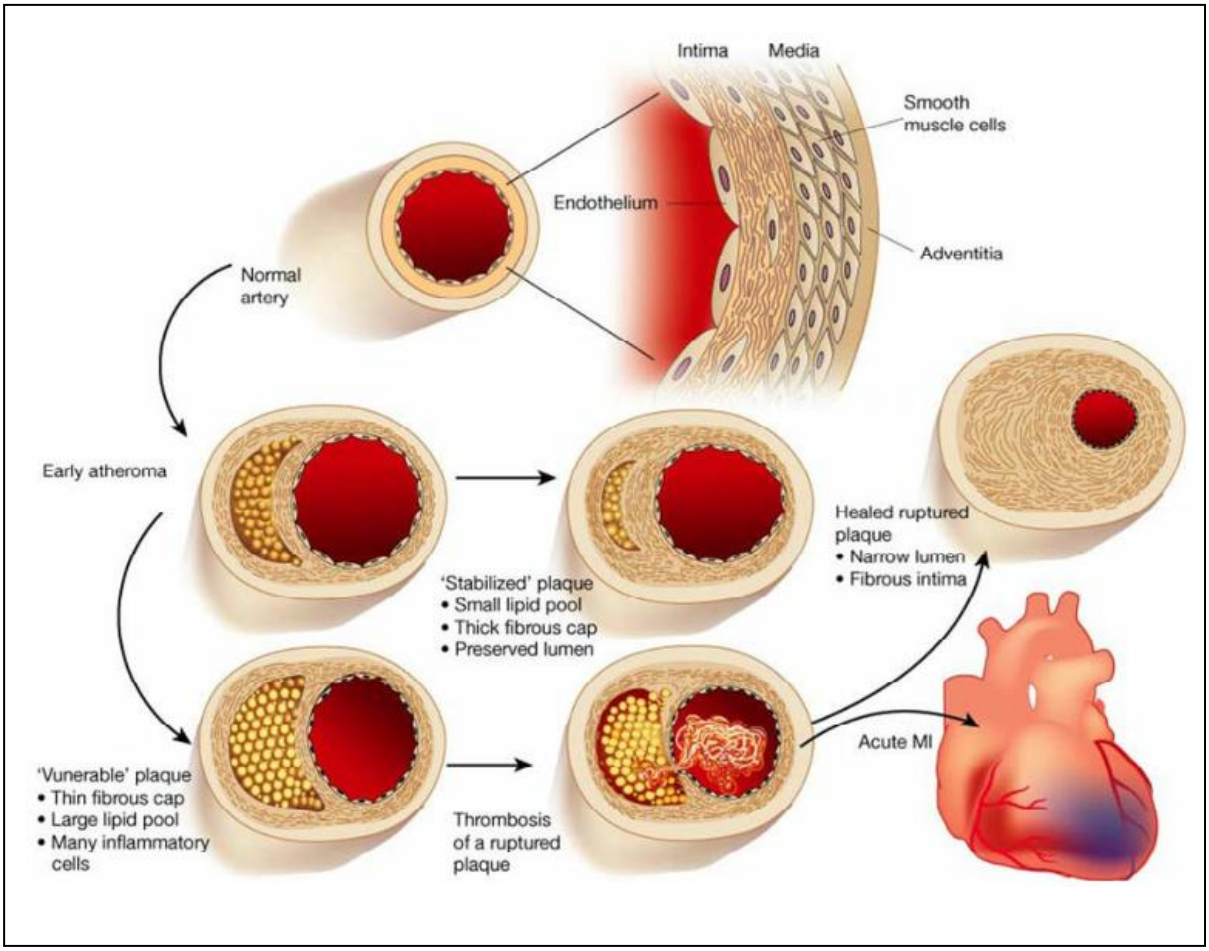

Figure 8: Schematic of the life history of an atheroma (Gerdes, 2005).

modified LDL particles via scavenger receptor, resulting in foam cell formation (Sjöberg, 2008). Accumulation of foam cells is the hallmark of early and asymptomatic atheromatous precursor, the fatty streak.

❖ **Progression**

Cytokines, chemokines and growth factors secreted by macrophages stimulates smooth muscle cells (SMC) from the media to alter their cytoskeleton, produce proteins and enzymes and migrate into the intimal space, where they proliferate and secret extracellular matrix components that form a fibrous layer over the developing atheroma.

Plaque macrophages and SMCs can die in advancing lesion, some by apoptosis. Extracellular lipid derived from dead and during cells accumulate in the central of the lesion often denoted the lipid or necrotic core. Necrotic core are surrounded by a fibrous cap of varying thickness (Faxon *et al.*, 2004).

Although the lesion grows in the intima, the lumen diameter remains constant due to remodulation of artery's external boundaries. As the lumen narrows, however, the lesion becomes more fibrous due to of the abundance of synthetic, collagen-producing SMC (Sjöberg, 2008).

Very advanced lesion with abundant SMCs and few macrophages can lead to clinical atherosclerosis by flow limiting stenosis (Sjöberg, 2008).

❖ **Complications**

The last stage of atherosclerosis occurs when the fibrous plaque break open exposing its prothrombotic content to blood and activating the extrinsic coagulation cascade (Tupin, 2004). There by causing thrombus formation.

The stability of the advanced atherosclerotic plaque depends on its cellular and extracellular content. Plaques with small lipid cores, thick fibrous cap, few inflammatory cells and a preponderance of smooth muscle cells are typically stable. Whereas those with large lipid cores, thin fibrous cap, numerous macrophages and relatively few/smooth muscle cells are most likely to rupture (Dufton, 2012)

Nonetheless, the progression from fatty streak to advanced, complex lesion likely does not occur inevitably and continuously overtime. Clinical observation rather suggest that many

human lesions develop discontinuously, featuring "bursts" of growth and progression of atheroma. Therefore, comprehensive understanding of the mechanisms that cause plaque progression and destabilization is crucial for the future treatment of the disease and prevention of acute clinical events (Sjöberg, 2008).

5-3- Possible mechanism attributed by homocysteine in atherosclerosis

The hypothesis that Hcy is a atherosclerotic was proposed by Kilmer Mc Cully was back in 1969, during a post mortem study of two small children who had very high blood Hcy levels and had extensive atherosclerotic lesion in most of their blood vessels. Mc Cully hence, hypothesized that raised levels of Hcy could be a risk factor of atherosclerosis (Mc Cully, 1969).

Recent epidemiological studies support the concept that increased Hcy concentrations are associated with cardiovascular disease but data from randomized controlled trials have shown that there is no clinical benefit to lowering plasma Hcy concentrations with folic acid and other B vitamins. This lack of effect of Hcy lowering strongly suggests that homocysteine is not an instigator but merely indicator of CVD (Vizzardi *et al.,* 2009).

Various theories have been propounded for explaining the mechanisms by which hyperhomocysteinemia may be contributes to atherosclerosis. Some of them are:

- Auto-oxidation of homocysteine causes endothelial cell desquamation, smooth muscle cell proliferation and intimal thickening. High concentrations of homocysteine are also toxic to endothelial cells *in vitro*. Homocysteine inhibits DNA synthesis in vascular endothelial cells and causes growth arrest at the G1 phase of cell cycle (Tayal *et al.*, 2011).

- Homocysteine can oxidize LDL (oxLDLc) as well as combined with LDL to form thiolated LDL (Tayal et al., 2011). OxLDL promotes the differentiation of monocytes into tissue macrophages by enhancing the release of macrophage colony stimulating factor from endothelial cells, and inhibits the motility of resident macrophages. Unlike native LDL, oxLDL is immunogenic, and it is also cytotoxic to various cell types, including endothelial cells, resulting in loss of endothelial integrity (Dwivedi *et al.,* 2011).

- Homocysteine has ability to inhibit the expression of antioxidant enzymes such as glutathione peroxidase, superoxide dismutase, which might potentate the toxic effects of free radicals (Baydas *et al.*, 2005) and (Antoniades *et al.*, 2009).

Hyperhomocysteinemia and cardiovascular diseases

❖ Homocysteine reduces bioavailability of nitric oxide (NO) (Raposo *et al.*,2004) and causes deterioration of the elastic structure of the arterial wall through alteration in metalloproteinase activity. It may also increase vascular rigidity by augmenting breakdown of elastin in vascular cells (Tayal *et al.,* 2011). Clinical studies have shown that patients with hypertension have a blunted arterial vasodilatory response to infusion of endothelium dependent vasodilators and that inhibition of NO raises blood pressure (Dwivedi *et al.,* 2011).

❖ DNA hypomethylation is induced by increase in homocysteine levels. Thus global or selective DNA methylation may contribute to alterations in gene expression and vascular changes during hyperhomocysteinemia. Homocysteine at high concentration also increases the transcription and activity of tissue factor. Homocysteine elicits a DNA damage response in neurons that promotes apoptosis and hypersensitivity to excitotoxins (Tayal *et al.,* 2011).

6-Olive oil

6-1- Characteristics of olive oil

The olive is the fruit of the olive tree (Olea europaea) and belongs to the family Oleaceae (Owen *et al,* 2000). Its cultivation is wide spread throughout the Mediterranean region and is important for the rural economy, local heritage and the environment (Kandylisa *et al.,* 2011).

At national level, the current growing area of Algeria is 207 822ha, with 20.5 million trees planted, a little over 16 million in production. This particular area is distributed on the East and Central areas of the country (Kerboua, 2003) (figure 9).

Olive oil is the edible oil obtained solely from the olive fruit. It has a density of 0.92 and a caloric intake 9kcl/g. The oil keeps better if stored cool and protected from light. It is best to consume within two years after its manufacture (Al-Azzawie and Alhamdani, 2006).

6-2- Compositions of olive oil

Olive oil can be classified into two fractions from a quantitative point of view. The major fraction constitutes 98–99% of the oil and is mainly composed of saponifiable glyceridic compounds (figure 10) as triglycerides (Perona *et al.,* 2006). Oleic acid makes up 70% to 80%, of the fatty acids (Huang and Sumpio, 2008) in triacylglycerol. Whereas linoleic acid, the major essential fatty acid and the most abundant polyunsaturated acid in our diet, is present in concentrations between 3% and 21% (Perona *et al.,* 2006).

The minor components (figure 11) are present in about 2% of oil weight (Huang and Sumpio,2008) and (Ruiz-Canela and Martínez-González, 2011) even where present in small quantities, this fraction is very important from a nutritional and analytical point of view to check the authenticity of the oil and its stability. It comprises unsaponifiable compounds, phenolics and waxes (Perona *et al.,* 2006).

The most abundant lipohilic phenolic compound is α-tocopherol and there are also several hydrophilic phenolic compounds such as oleuropein and hydroxytirosol (Ruiz-Canela and Martínez-González, 2011).

6-3- Pharmacological effects of olive oil

6-3-a- *Anti-oxidant properties*

Olive oil is rich monounsaturated fatty acids and antioxidant compounds. The concentration of antioxidants olive oil is influenced by the olive oil extraction procedures (Fitó *et al.,* 2005).

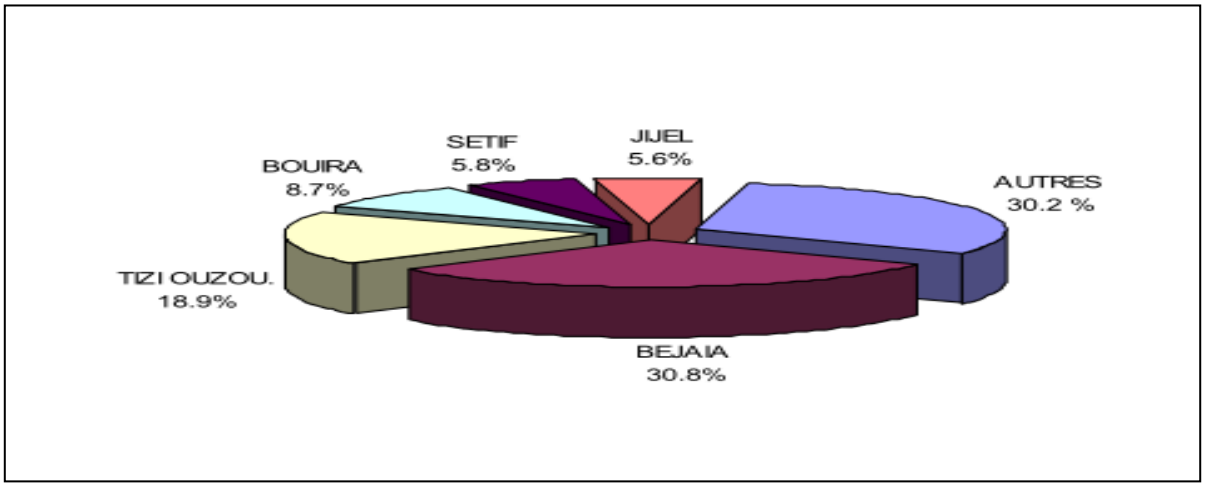

Figure 9: repartition of Olivier in Algerian (Benabid, 2009).

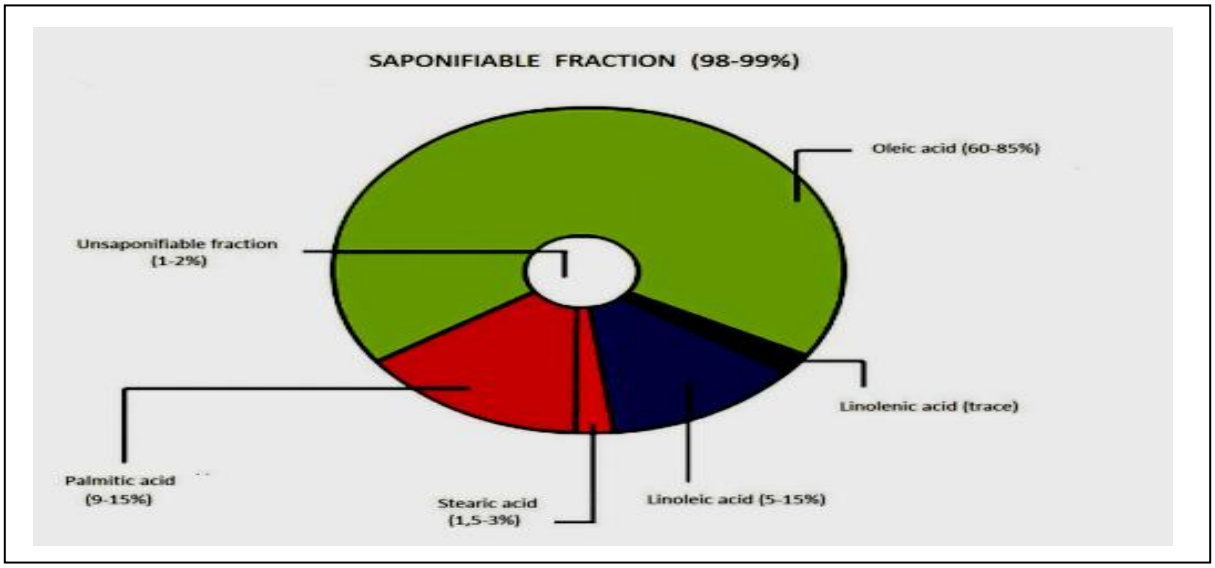

Figure 10: Chemical compositions of unsaponifiable and saponifiable fraction of olive oil. (www.parkinelmer.com)

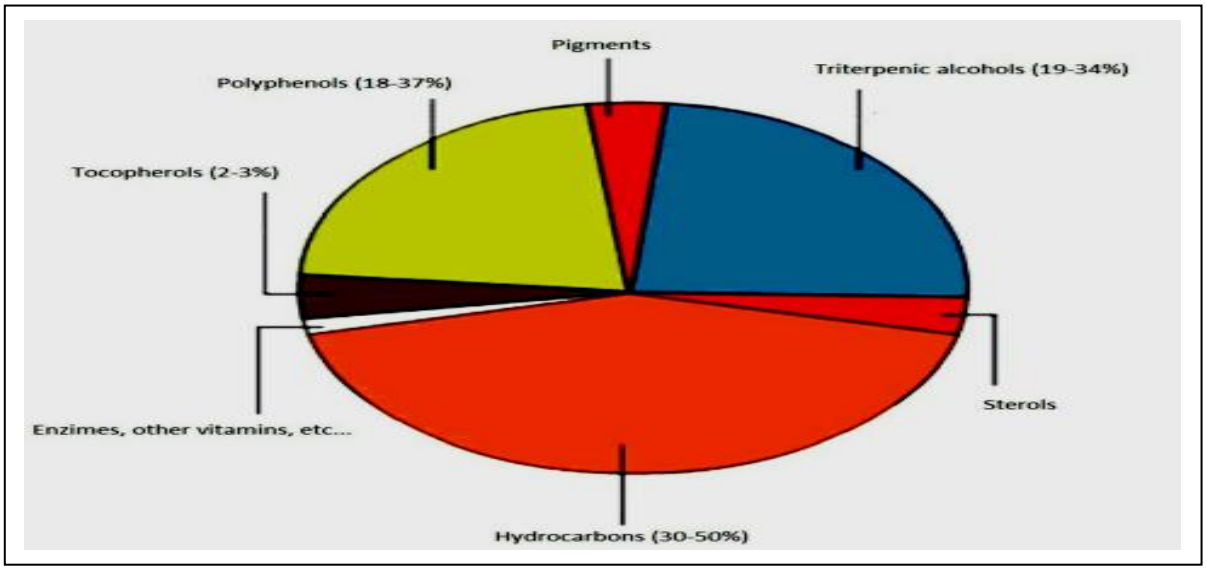

Figure 11: Chemical compositions of unsaponifiable fraction of olive oil. (www.parkinelmer.com)

Olive oil is able to prevent the endothelial dysfunction by decreasing the expression of cell adhesion molecules, and increasing (NO) production and inducible NO synthesis by quenching vascular endothelium intracellular free radicals (Fitó *et al.*, 2007).

6-3-b- ***Anti inflammatory activities***

Oleic acid was able to reduce the inflammatory effects of saturated fatty acids in human aortic endothelial cells by reducing the incorporation of stearic acid into phospholipids and by the reduction of NFKB activation. In other studies, it reverses the *in vitro* inhibitory effects of the inflammatory cytokine TNFα on insulin production in the rat pancreatic B cells line INS-1 and decreased membrane expression of VCAM1 and NFKB activation in endothelial cells (Urpi-Sarda *et al.*, 2012).

6-3-c- ***Anti thrombotic***

Olive oil phenolic compounds inhibited platelet-induced aggregation and have been reported to enhance the mRNA transcription of the antioxidant enzyme glutathione peroxidase (Fitó *et al.*, 2007).

6-3-d- ***Anti tumor***

In general most case-control and cohort studies have shown that oleic acid and Olive oil is associated with a reduction in the risk of cancer (mainly breast, colorectal and prostate cancer), at variance from diets rich in total fat and in linoleic acid or saturated fatty acids, which have been related to an increased cancer risk (Lopez-Miranda *et al.*, 2010).

Material & Methods

Materiel and methods

1-Material

1-1-Animals

The investigation was performed on 28 males mice of the species Mus musculus (2.5-3 months), weighing between (25-35g). All animals were obtained from central pharmacy Algeria and housed in plastic cages with free access to water and diets every day at temperature room 25°c.

Table 1: Composition of diet taken by the mice during 21 days (ONAB):

Composition	Amount in g / kg	Percentage%
Corn	620	62
Soja	260	26
Phosphate	16	1,6
Limestone	9	0,9
Cellulose	10	1
Minerals	10	1
Vitamins	10	1

1-2 -Blood samples:

After each experiment animal were fasted overnight and the blood was obtained from sinus venipuncture and collected into EDTA tubes by using glass capillaries.

1-3 Organs samples

Animal were sacrificed and samples were obtained from the aorta (abdominal and iliac aorta), liver and heart.

1-4 Chemical products

Chemical Products used in our study are:

L-methionine, chloroform, Phosphate Buffer Saline (PBS), formalin 10%, Bouin's solution, dithiobis-2-nitrobenzoic acid (DTNB), sulfo-salcylic acid (0.01M), Bovine Serum Albumin

(BSA), , Coomassie Brilliant Blue, *orthophosphoric acid* (85%), Tris, Ethylene Di-amine Tetra Acetic acid (EDTA, 0.02M), different concentrations of ethanol (50%, 70%, 95% and 96%), Hcl, NaoH, Nacl, butanol, xylene, paraffin and glycerin.

1-5 Equipments:

Precision Weighing Balances (readability 0.01g) to determine the weight of the mice, Precision Weighing Balances (readability 0.0001g) to determine the quantity of methionine, Heating magnetic stirrer, PH meter, Centrifuge, Spectrophotometer , Oven, Microtome and Photo microscope connected to computer.

2- Methods

2-1 Biochemical analysis

After acclimatization to the laboratory conditions for 1 week, the twenty-eight mice were divided into four groups of similar mean body weights and fed for 21 days with control and experimental diets (table 2).

The dosage of L- methionine (400mg/kg/day) was calculated relatively with body weight, and given with white bread (0.1g/mice), however the olive oil (7ml/60kg/day) was given by oral administration by micropipette and animal were kept in cages at room temperature. The weights and diet consumption of mice were taken daily throughout the experiment at the same time.

Table 2: Traitement of mice

Experimental Group	Substance administered	Number of animal	Duration of the experiment	Daily dose
F	Flour	7	21 days	0.1g/mice
M	L-methionine			400mg/kg
MO	L-methionine + olive oil			400mg/kg+ 7ml/60kg
OO	olive oil			0.1g/mice+7ml/60kg

At the end of the study, mice were fasted overnight, and the blood samples were collected from the retro orbital vein into EDTA tubes by using glass capillaries. They were centrifuged immediately, and the plasma was stored at -20°C until biochemical analysis.

Plasma Hcy and lipids status determination were performed in the analytical laboratory medical IBN SINA, Constantine.

2-1-a *Plasma Hcy determination*

Hcy levels were measured by competitive solid phase chemiluminescance immunoassay (IMMULITE).

Hcy involved a preliminary manual sample pretreatment step .Hcy in the plasma sample is released from its binding proteins and converted to SAH by an off-line 30 minute incubation at 37°c in the presence of SAH hydrolase and Dithiothreitol. The treatment sample and alkaline phosphate –labeled-anti-SAH antibody are simultaneously introduced into a test unit containing an SAH coated polystyrene bead. During a30 minute incubation, the converted SAH from the sample completes with the immobilized SAH for binding the alkaline phosphatase labeled-anti SAH antibody conjugated. Unbound enzyme conjugated is removed by centrifugal wash. The substrate is added and the procedure continues as described for the typical immunoassays.

2-1-b *Lipids determination*

Total cholesterol, HDL-c, LDL-c and triglyceride concentrations were assessed using colorimetric automatic procedures (*Auto-analyzer type Integra 400).*

❖ **Clinical significant of total cholesterol**

Cholesterol is an unsaturated alcohol of the steroid family of compounds and found in blood, bile, and brain tissue. It is synthesized in many types of tissue, but particularly in the liver and intestinal wall. It serves as a precursor to bile acids, adrenal and gonadal steroid hormones and vitamin D (Cox and Garcia-Palmieri, 1990).

Epidemiological studies have shown a positive relationship between total cholesterol concentrations and mortality from coronary heart disease (Obeid and Herrmenn, 2009).

The series of reactions involved in the assay system are as follows:

$$\text{Cholesterol esters} + H_2O \xrightarrow{\text{Cholesterol Oxidase}} \text{Cholesterol} + \text{fatty acids}$$

$$\text{Cholesterol} + O_2 \xrightarrow{\text{Cholesterol Oxidase}} \text{Cholest-4-ene-3-one} + H_2O_2$$

$$2\,H_2O_2 + \text{4-AAP} + \text{Phenol} \xrightarrow{\text{Peroxidase}} \text{Quinoneimine dye} + 4\,H_2O$$

The intensity of the color produced is directly proportional to cholesterol concentration. It is determined by measuring the increase in absorbance at 500 – 550 nm (Young, 2001).

Calculate the cholesterol concentration by using the following formulae:

$$\text{Cholesterol Concentration (mmol/l)} = \frac{\text{Absorbance of Specimen}}{\text{Absorbance of Standard}} \text{ (Cholesterol standard)*200 mg/dl}$$

❖ **Clinical significant of triglyceride**

Triglyceride (TG) is water insoluble lipids, synthesized in the intestinal mucosa by the esterification of glycerol and free fatty acids. They represent a concentration source of metabolic energy.

Triglyceride are transported in the blood as core constituents of all lipoproteins, but the greatest concentration of these molecule is carried in the TG-rich chylomicrom and very low density lipoproteins (VLDL) (Rifai *et al.*, 2001).

The triglycerides are determined after enzymatic hydrolysis with lipases. The indicator is a quinoneimine formed from hydrogen peroxide, 4-aminophenazone and 4-chlorophenol under the catalytic influence of peroxidase (Young, 2001).

$$\text{Triglycerides (TG)} \xrightarrow{\text{Lipoprotein Pipase (LP)}} \text{Glycerol} + \text{fatty acids}$$

$$\text{Glycerol} + ATP \xrightarrow{\text{Glycerol Kinase (GK)}} \text{Glycerol-3-phosphate (G3P)} + ADP$$

$$G3P + O_2 \xrightarrow{\text{Glycerol Phosphate Oxidase (GPO)}} \text{Dihydroxyacetone phosphate (DAP)} + 2\,H_2O_2$$

$$2\,H_2O_2 + \text{4-AAP} + \text{4-Chlorophenol} \xrightarrow{\text{Peroxidase (POD)}} \text{Quinoneimine dye} + 4\,H_2O$$

Calculate the triglycerides concentration by using the following formulae:

$$\frac{\text{Absorbance of Specimen}}{\text{Absorbance of Standard}} * 200 \text{ (Standard concentration)} = \text{mg/ml} * 0.0114 \text{ mmol/l.}$$

❖ **Clinical significant of HDL-c**

High density lipoprotein cholesterol (HDL-c) also known as "good" cholesterol, molecules consisting of cholesterol and protein that carry cholesterol from cells back to the liver (Obeid and Herrmenn, 2009).

HDL-c was determined with enzymatic procedure after lipoproteins were precipitate by phosphotungstate in the presence of magnesium ions. After centrifugation, the HDL cholesterol in the supernatant is determined by the same technique as the total enzymatic cholesterol, and the calculation as shown below:

$$\text{HDL-c Concentration} = \frac{\text{Absorbance of Specimen}}{\text{Absorbance of Standard}} \text{(Standard concentration)} * 200 \text{ mg/dl}$$

❖ **Clinical significant of low density lipoprotein cholesterol**

Low density lipoprotein cholesterol (LDL-c) particle carry cholesterol from the cell back to the tissue. LDL-c is known as bad cholesterol because high levels are thought to increase the risk of heart disease (Zerizer, 2006).

LDL-c concentration was obtained by direct calculation according to Friedwald formula:

$$\text{LDL} = \text{total cholesterol} - \text{HDL} - \text{triglycerides} / 5$$

When the level of TG is greater than 3.4 g / l (3.75 mmol / L), LDL cholesterol cannot be calculated by this formula, it should be assayed by a direct enzymatic method.

2-2 Determination of oxidative stress parameters

❖ Preparation of homogenate

The weight of 0,5g of the liver was homogenized in 2ml of TBS solution. Then the homogenates were centrifuged at 9000 g for15 min at 4˚C after that the supernatant was used for determination of glutathione reduced (GSH) (Kehili, 2014).

Glutathione assay

Glutathione (GSH) is a water-soluble tripeptide (γ-glutamyl-cysteinylglycine) produced naturally by the liver. Due to the thiol function of cysteine, glutathione is an important compound in maintaining the redox balance of the cell. It maintains in the proper redox state the thiol groups of soluble and structural proteins, and participates in the detoxification of hydroxyperoxides.

In addition to detoxification, GSH plays a role in other cellular reactions, including, the glyoxalase system, reduction of ribonucleotides to deoxyribonucleotides, regulation of protein and gene expression via thiol: disulfide exchange reactions (Townsend *et al.,* 2003)

The concentrations of the GSH are proportioned by the method of Weckbecker and Cory as shown below , The spectrophotometric reader assay method for GSH involves oxidation of GSH by the sulfhydryl reagent 5,5′-dithio-bis2-nitrobenzoic acid (DTNB) to form the yellow derivative 5′-thio-2-nitrobenzoic acid (TNB), measurable at 412 nm.

Liver homogenate sample 0.8ml was deproteinized with 0.2ml 5-sulfosalicylic acid solution (0.25%) and was allowed stand on ice for 10 min. Following centrifugation at 1000 tours/mn) during 5minutes to remove the precipitated protein. 0.5 ml supernatant was mixed with 1 ml Tris/EDTA buffer (pH 9.6) and 0.025 ml DTNB-reagent (0.01M 5,5'-dithiobis-2-nitrobenzoic acid) and left at room temperature for 5 min. Then the absorption at 412nm was readed on a spectrophotometer by comparing to the blank reaction.

Glutathione concentration was obtained by direct calculation the following formulae:

$$\text{GSH (uM GSH/mg of protein)} = \frac{\text{OD} * 1 * 1.525}{13100 * 0.8 * 0.5.\text{Mg protein}} * 10^5$$

- OD: optical density
- 1: total volume of solutions in the deproteinisation (0.8ml homogenate+ 0.2ml 5-sulfosalicylic acid)
- 1.525: total volume of the solutions used in the assay of GSH (0.5ml supernatant+ 1 ml Tris/EDTA+ 0.025 ml DTNB)
- 13100: absorbance coefficient at Groupment—SH to 412nm
- 0.8: volume of homogenat sample
- 0.5: volume of supernatant

❖ **Protein determination**

Protein concentration was measured by the method of Bradford (1976), using bovine serum albumin as standard. The procedure is based on the formation of a blue complex between the comaissie bruillant blue G-250 dye, and proteins in solution. The amount of absorption is proportional to the protein present.

Liver homogenate sample 0.1ml was mixed with 5ml Bradford reagent and was allowed stand for 5min. Then the absorption at 595 nm was readied on a spectrophotometer by comparing to the blank reaction.

The protein concentration of a test sample is determined by comparison to that of a standard series of bovine serum albumin to reproducibly exhibit a linear absorbance profile in this assay (Figure 01 annex).

2-3 Histological analysis

After the blood samples collection, the animals were sacrificed, and samples for light microscopic investigations were obtained from aorta, heart and liver. For histological investigations the aorta was divided into 4 sections (arch, thoracic, abdominal, and iliac).

The samples were rinced of all adherent tissues in phosphate buffered saline (pH 7.4) then the samples were:

- Fixed in formol 10%.
- Place the different parts of the aorta in the Bouin solution for 5 min for color.
- Dehydrate in ascending percentage of ethanol (50%, 70% and 95% for 1h and 30min for each concentration) then in butanol for three days.
- Cleared in xylene for 10min at two exchanges

- Embedded in paraffin at 60 °C for 1h and 30 min at three exchange
- Embed tissue into paraffin blocks
- Tissues are microtomed at 5 µm thick
- The sections obtained are placed in a water bath (50°c) to melt the excess paraffin wax then dried on a 50°C hot plate overnight.
- Deparaffinization in xylene for two hours.
- Staining sections with hematoxylin-eosin (annex).
- After drying on a 50°c hot plate, the section was mounted by xylene then placed between a slide and coverslip.
- Reading is performed by a photo microscope connected to computer.

3- Statistical analysis

The body weights, diet consumption, biochemical analysis (Hcy and lipids) and Glutathione assay are presented as means ± s.e. The comparison between groups was performed by one-way ANOVA. When the ANOVA revealed a significant difference between treatments ($p<0.05$), multiple comparisons were made by using the Tukey test. Pearson's correlation analysis was used to evaluate the correlation between the plasma homocysteine and other biochemical parameters.

The comparison or correlation is considered, according to the probability (P) as:

- No significant if $P > 0.05$.
- Significant (*) if $P < 0.05$.
- Highly significant (**) if $P < 0.01$.
- Very highly significant (***) $P < 0.001$.

All analyses related to the case–control study were performed using the Statistical Package for the Social Sciences SPSS version20.

Results

Results

1-Animal investigations

1-1 Experiment 1

The objective of this experiment is to evaluate the effect of L-methionine on the diet and weight of the animals.

1-1-a *Diet variation*

The diet taken from the mice during the first and second week in the group (F) were (106.03 g ±12.23) and (109.09g ±7.78) respectively and (109.15g ±6.36) in the third week.

The diet is increased but not significantly between the three week in group (F) (P>0,05) **(Figure12, Table 01 appendix)**.

In-group (M), the consumption of food from the first to the third week (101.51g ± 31.18, 137.78g ±8.71 and 146.57 ± 28.40 g, respectively) indicates that there is a highly significant difference ($p < 0.001$) between the three weeks **(Figure 12, tableau 02appendix)**.

The Tukey test, indicates that the food consumption increased highly and significantly (P = 0.007) between the first and third week and increased significantly (P=0.037) between the first and third week

1-1-b *The weight variation*

The weight increased highly and significantly between the three weeks in group (F) (31.61± 0.66, 32.23± 0.90, and 34.06± 0.36). **(Figure 13, tableau 01 appendix)**.

The Tukey test, indicates that the weight increased highly and significantly (P = 0.00) between the first and third week and between the second and third week.

The body weight from the mice during the three weeks in the group (M) were (32.63 ±0.63) (32.36 ±0.22) and (33.28 ±0.62) respectively, the weight of this mice were increased significantly p=0.01 **(Figure 13, tableau 02 appendix)**.

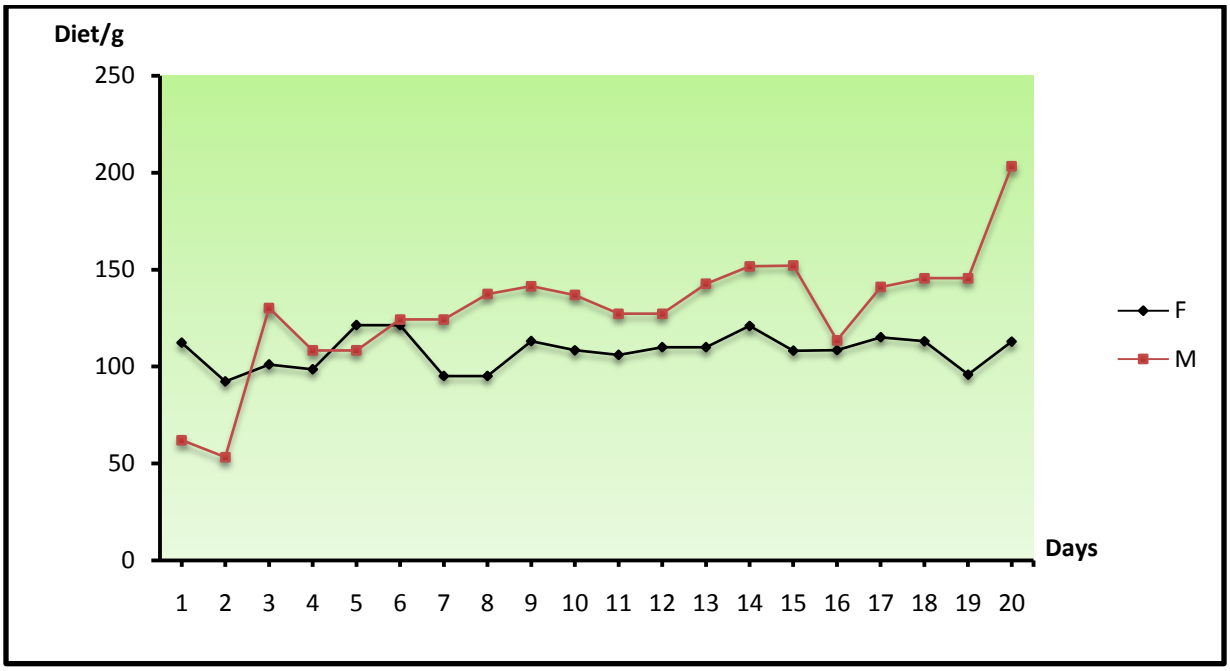

Figure 12: The effect of L-methionine intake on the diet in mice during 21 days.

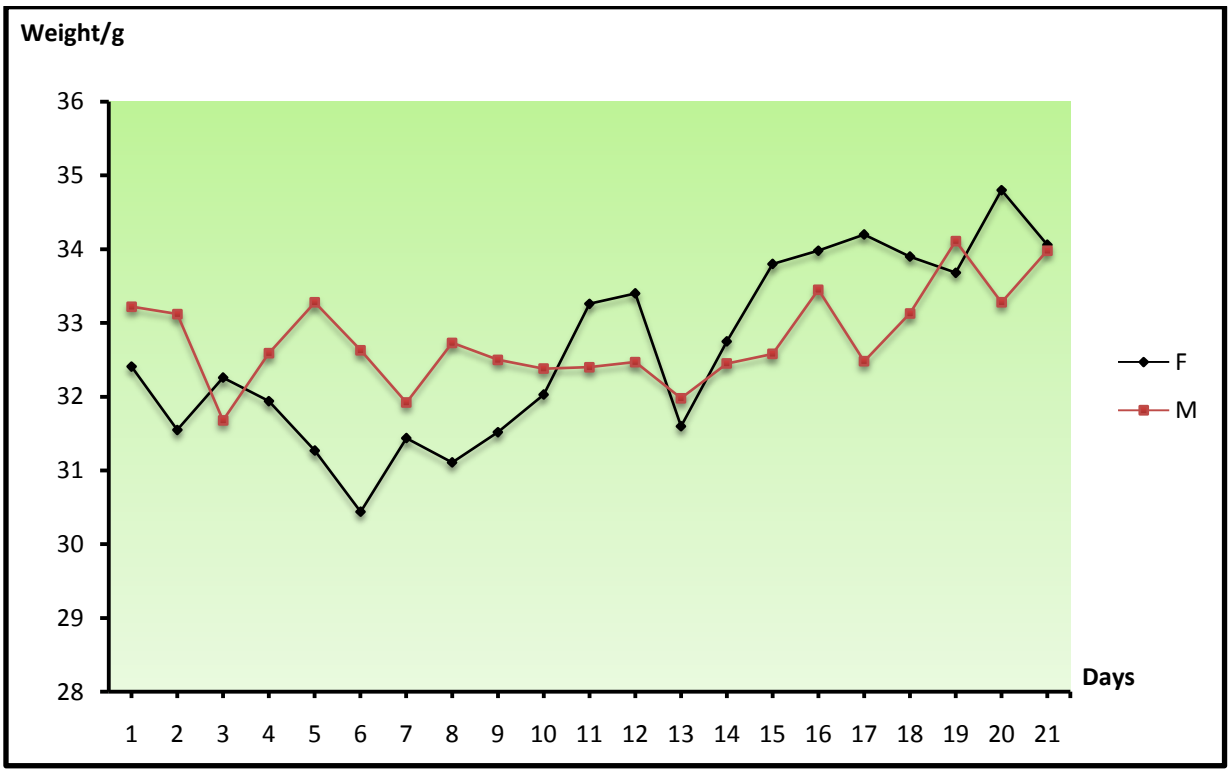

Figure 13: The effect of L-methionine intake on the weight in mice during 21 days.

The Tukey test revealed a significant increased (p = 0.025) of mice weight between second and last week.

1-2 Experiment 2

The objective of this experiment is to evaluate the effect of olive oil on the diet and weight of the animals.

1-2-a *Diet variation*

The diet taken from the mice in the group (MO) during three week were (113.24g ±28.81, 108.00g ±17.24 and 80.62g ±11.65) respectively **(figure 14, table 3 appendix)**.

The diet is decreased significantly (P= 0.02).

The Tukey test, indicates that the diet consumption decreased significantly (P = 0.026) between the first and third week but not significantly (P>0.05) between first week and second week and between the second and the third week.

In-group (OO), the consumption of food from the first to the third week were (82.95 ±31.34, 111.64 ±13.21 and 97.25 ±12.06g respectively) indicated that there was no significant difference (P>0.05) in the consumption of the diet between the three weeks **(figure 14, table 4 appendix)**.

1-2-b *The weight variation*

The weight change in the group (MO) from the first to the third week (30.87 ± 0.96 g, 31.32 ± 0.74 g and 32.00 ± 0.34 g) respectively, these results indicates that the mice weight is increased significant (p = 0.03) in this three weeks **(Figure 15, table 03 appendix)**.

The Tukey test revealed a significant increased (p = 0.025) of mice weight between first week and third week.

In group (OO), The body weight from the mice during the three weeks were (31.32 ± 1.05 g, 31.39 ± 0.46 g and 32.08 ± 0.76 g) respectively. The weight of this mice was increased but not significantly P>0.05.

(Figure 15, table 04 appendix).

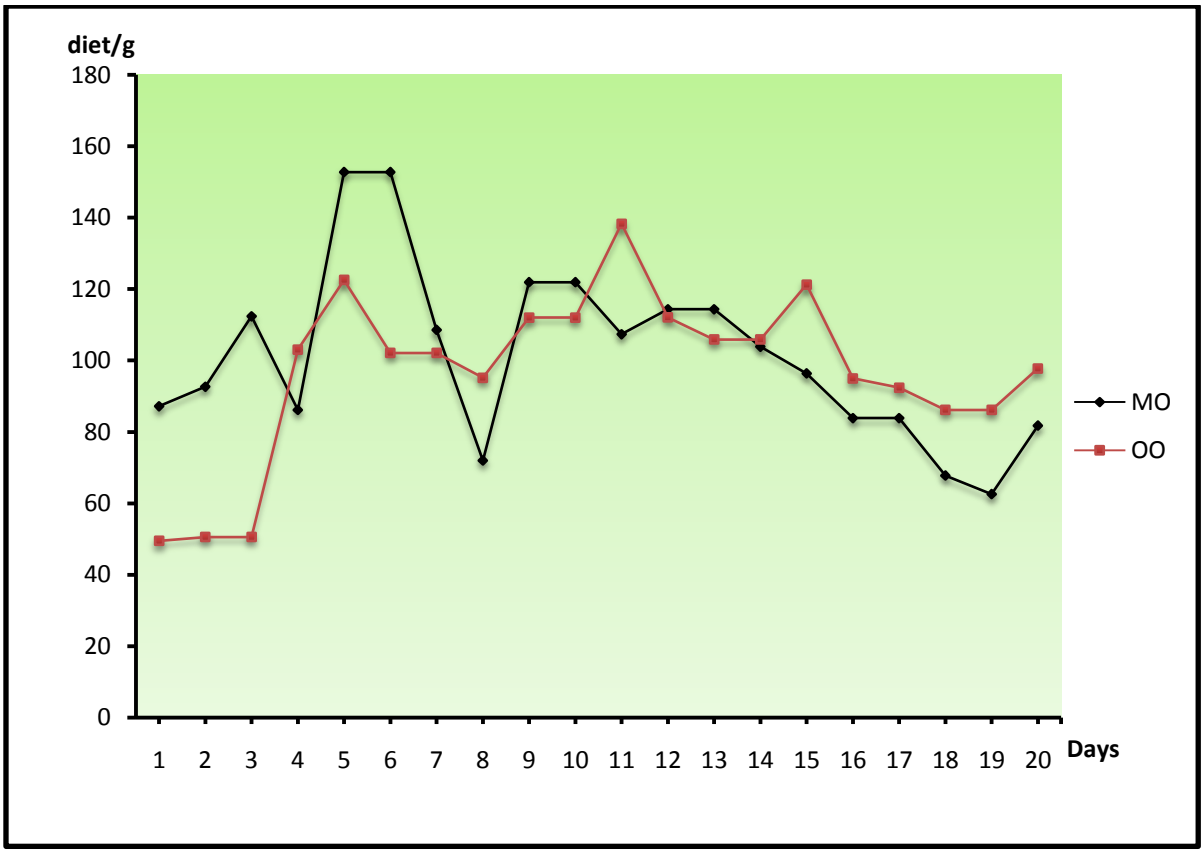

Figure 14: The effect of L-methionine and olive oil intake on the diet in mice during 21 days

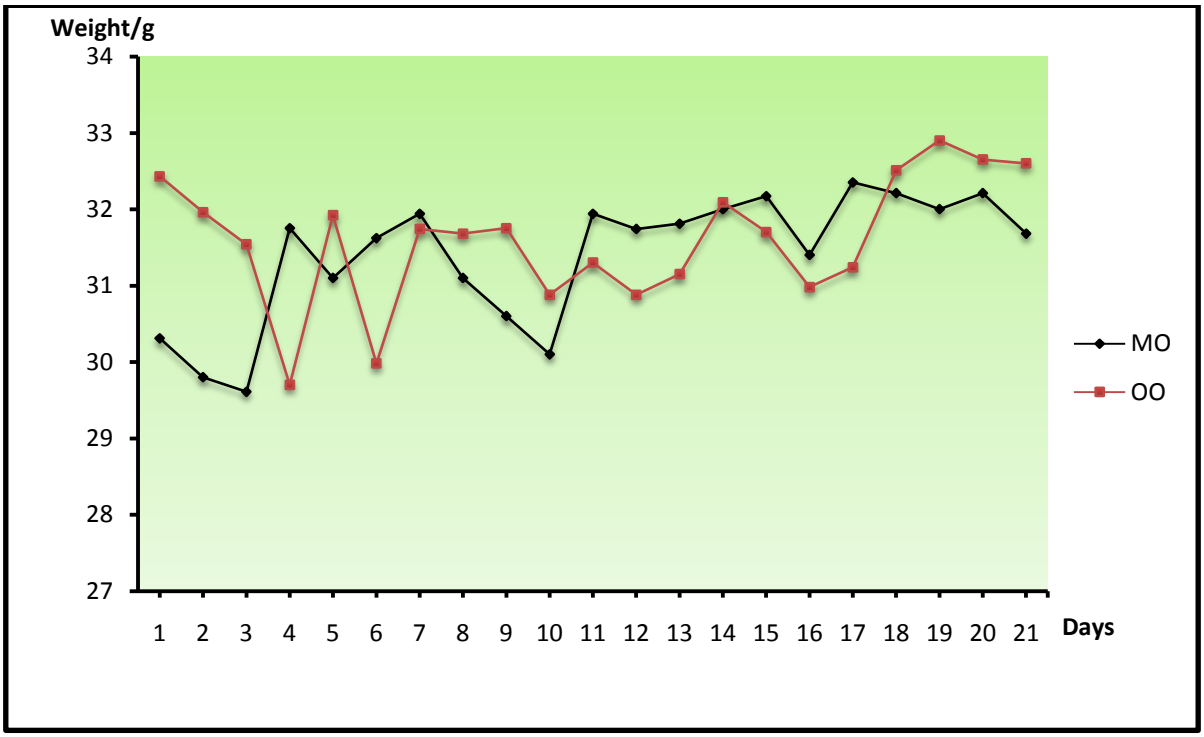

Figure 15: The effect of L-methionine and olive oil intake on the weight in mice during 21 days.

2-Biochemical results

2-1 Homocysteine concentration

The concentration of homocysteine were (6.59 ± 2.35 µmol/l) in F group, (12.89 ±4.37 µmol/l) in M group, (9.35 ± 3.63) in MO group and (6.45 ±2.54 µmol/l) in OO group; these values were statistically different significantly (P=0.026) between groups**.**

The Tukey test revealed a significant difference between the group (M and F) P = 0.04 and the group (M and OO) P = 0.03.

(Figure 16)

2-2 Lipids status

2-2-a *T-cholesterol*

The results of the determination of total cholesterol in the F group (0.90 ±0.26 g / l), M group (1.33 ± 0.34 g / l), MO group (0.94 ± 0.17 g / l), OO group(0.78± 0.26 g / l) show that there is a significant difference between groups (P = 0.028).

The Tukey test revealed a significant difference between the groups (M) and (MO) (P = 0.024).

T-cholesterol levels were decreased but not significantly in OO group when it is compared with the other groups.

(Figure 17).

2-2-b *Triglyceride*

The results of the determination of TG in F group (0.98±0.50 g/l), in M group (1.05± 0.44g/l), in MO group (0.94±0.43 g/l) and (0.83± 0.19g/l) in OO group; show that there is a difference between groups but not significantly (P> 0.05).

TG levels were increased in M group and decreased in OO group but not significantly compared to other groups.

(Figure 18).

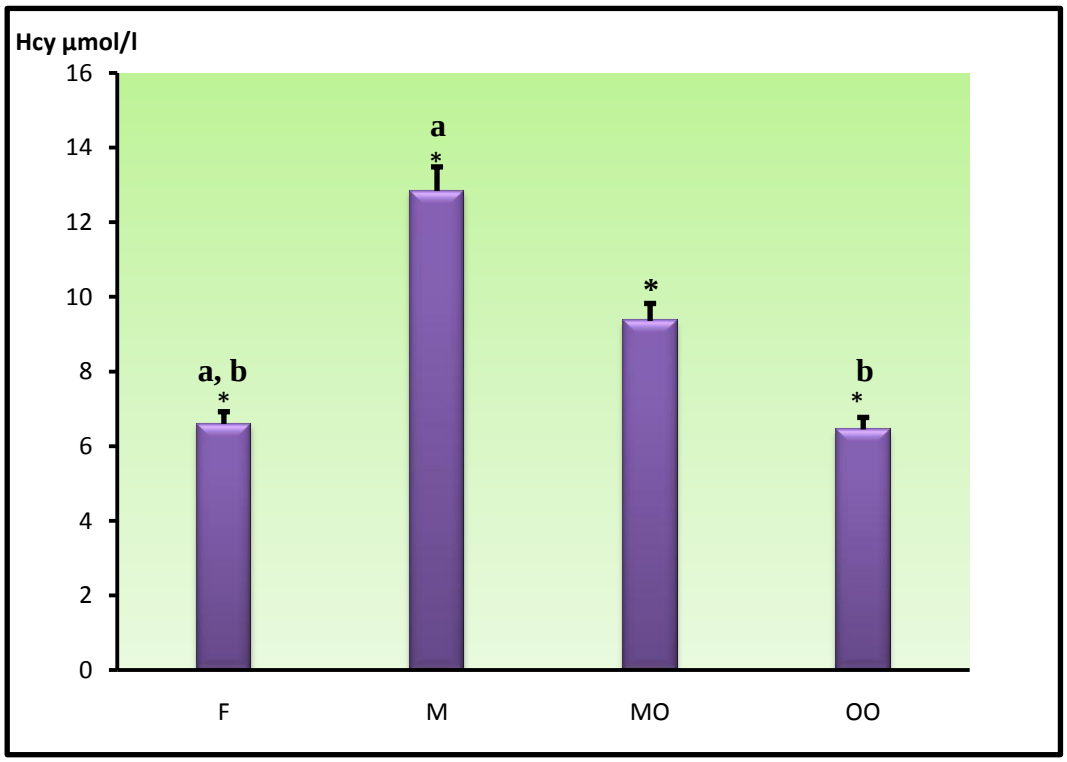

Figure 16: The interaction of L-methionine and olive oil on Hcy in mice 21 days of application

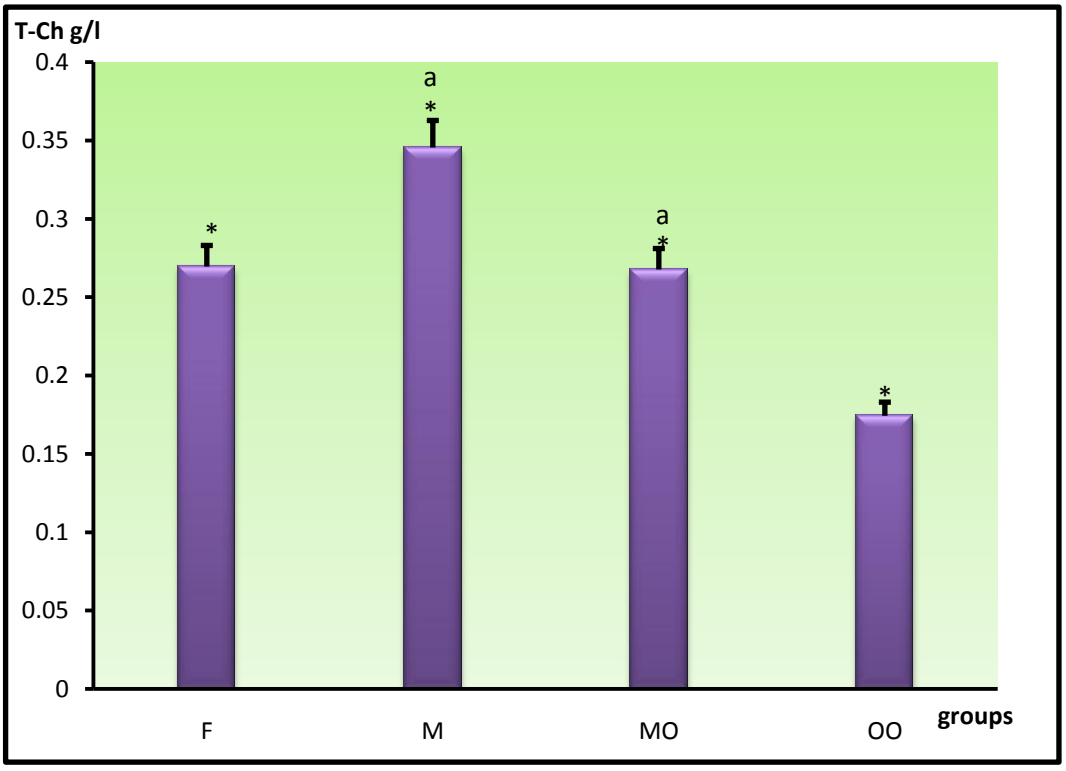

Figure 17: The interaction of L-methionine and olive oil on the lipid –T-Ch in mice 21 days of application

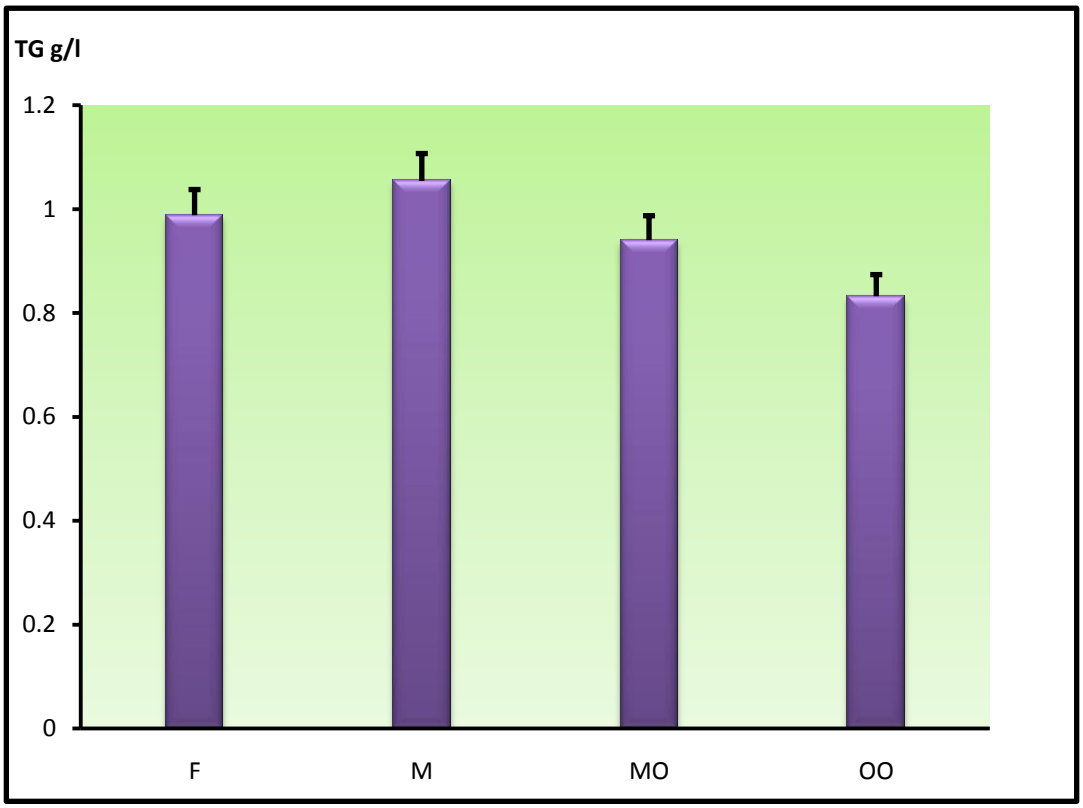

Figure 18: The interaction of L-methionine and olive oil on the enzyme TG in mice 21 days of application

2-2-c *HDL-c*

The results of the determination of HDL-c in F group (0.90 ±0.31g / l), M group (0.34 ± 0.18 g / l), MO group (0.92 ± 0.23 g / l), OO group(0.56± 0.29 g / l) show that there is a highly and significantly difference between groups (P = 0.008).

HDL-c concentration was decreased significantly in M group compared with the rest of groups.

The Tukey test revealed a significant difference between the groups (F and M) P = 0.018 and the group (M and MO) P = 0.014.

(Figure 19)

2-2-d *LDL-c*

The results of the determination of LDL-c in F group (0.28±0.17g / l), M group (0.84±0.55g/ l), MO group (0.32±0.10 g / l), OO group (0.30±0.06 g / l) show that there is a highly and significantly difference between groups (P = 0.007).

LDL-c concentration was increased significantly in M group compared with the rest of groups.

The Tukey test revealed a significant difference between the groups (F and M) P= 0.014, the groups (M and MO) P=0.024 and the groups (M and OO) P= 0.018.

(Figure 20)

2-3 Glutathione reduced

The concentration of glutathione in the control group (6.49±4.31), the M group (1.22±0.51), the MO group (7.44±2.39) and in the OO group was (9.46±5.62)

The concentration of glutathione was decreased significantly P=0.01 between groups.

The Tukey test revealed a highly and significantly difference between the groups (F) and (M) (p = 0.007).

(Figure 21)

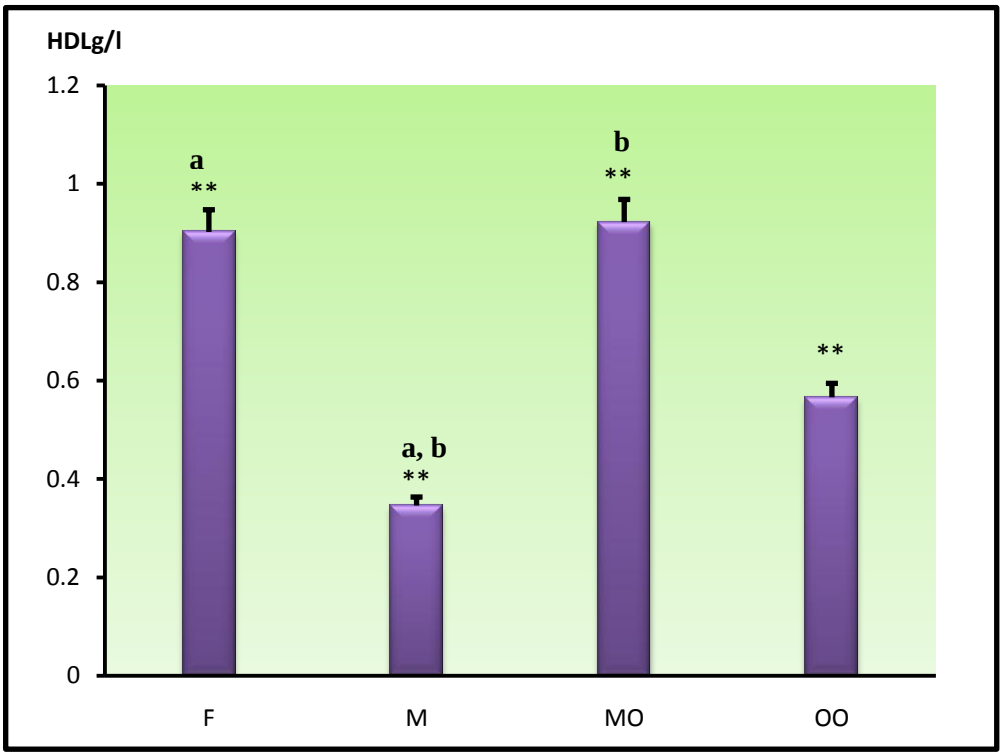

Figure 19: The interaction of L-methionine and olive oil on the HDL-c in mice 21 days of application

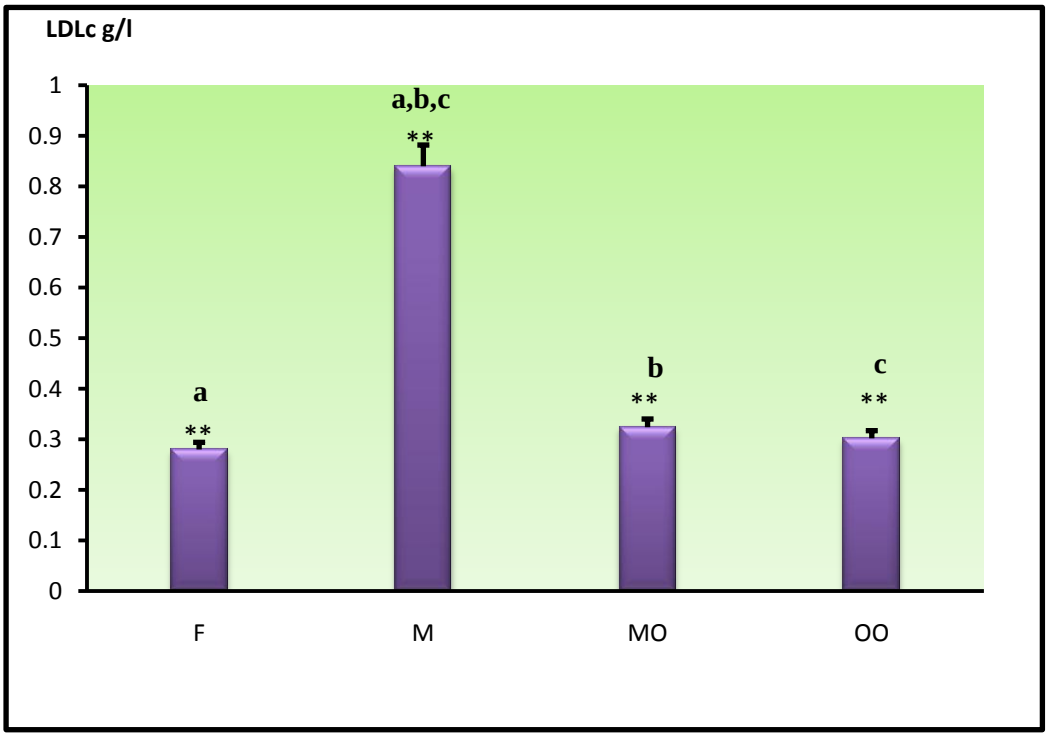

Figure 20: The interaction of L-methionine and olive oil on the LDL-c in mice 21 days of application

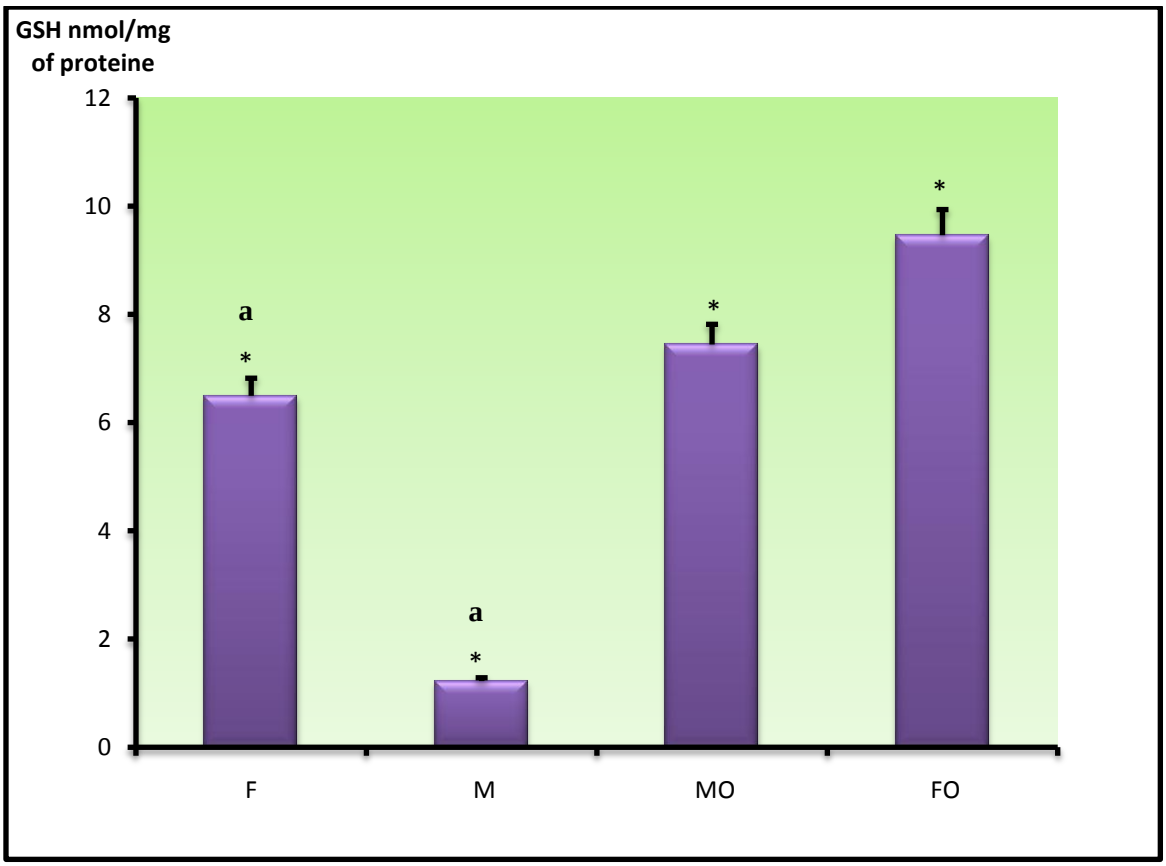

Figure 21: The interaction of L-methionine and olive oil on the glutathione reduced in mice 21 days of application

2-4 Correlation between Hcy and other biochemical parameters

The various correlations were obtained in these experiments are shown in Table (03) and **Figures (22, 23, 24, 25 and26**)

Table 3: Significant correlation between Hcy and other biochemical parameter

Group	Biochemical parameter	correlation	Coefficient (r)	Probability (p)
F	Hcy and LDL	positive	0.937**	0.007
M	Hcy and LDL	positive	0.987**	0.002
MO	Hcy and LDL	positive	0.937**	0.019
OO	Hcy and LDL	positive	0.913*	0.031
OO	HDL-c and TG	Positive	0.906*	0.034

* Correlation is significant at the 0.05 level.

** Correlation is significant at the 0.01 level

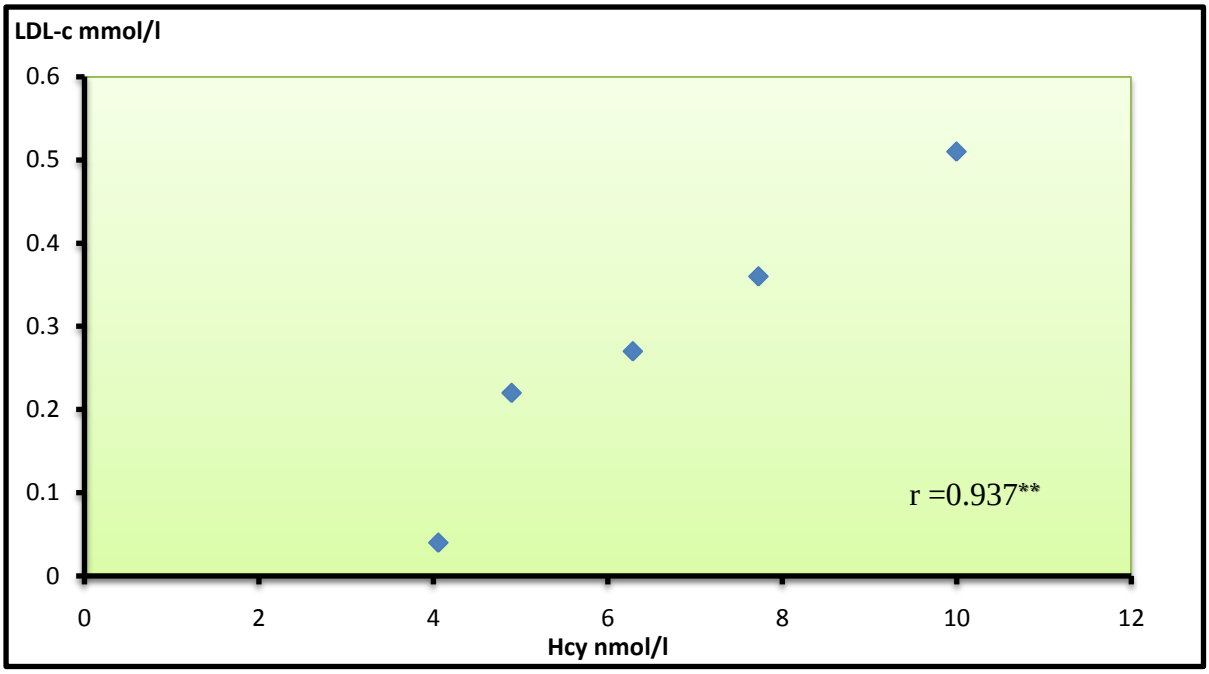

Figure 22: Correlation between lipoprotein LDL-c and homocysteine in F group

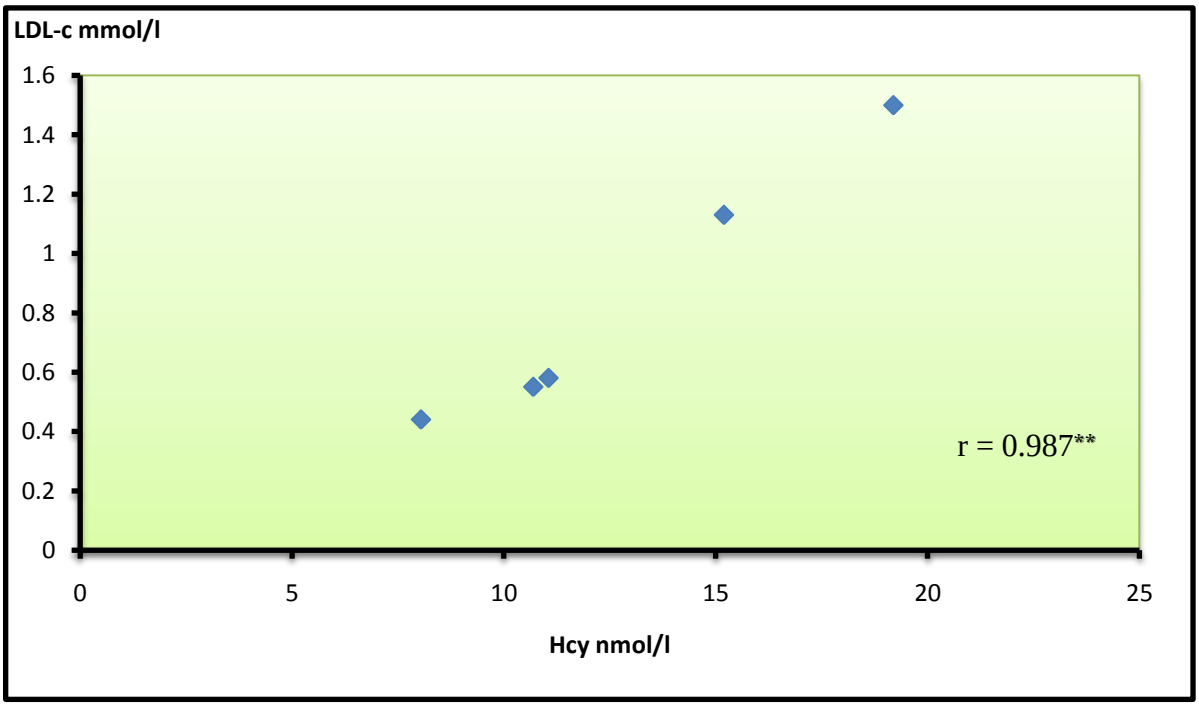

Figure 23: Correlation between lipoprotein LDL-c and homocysteine in M group

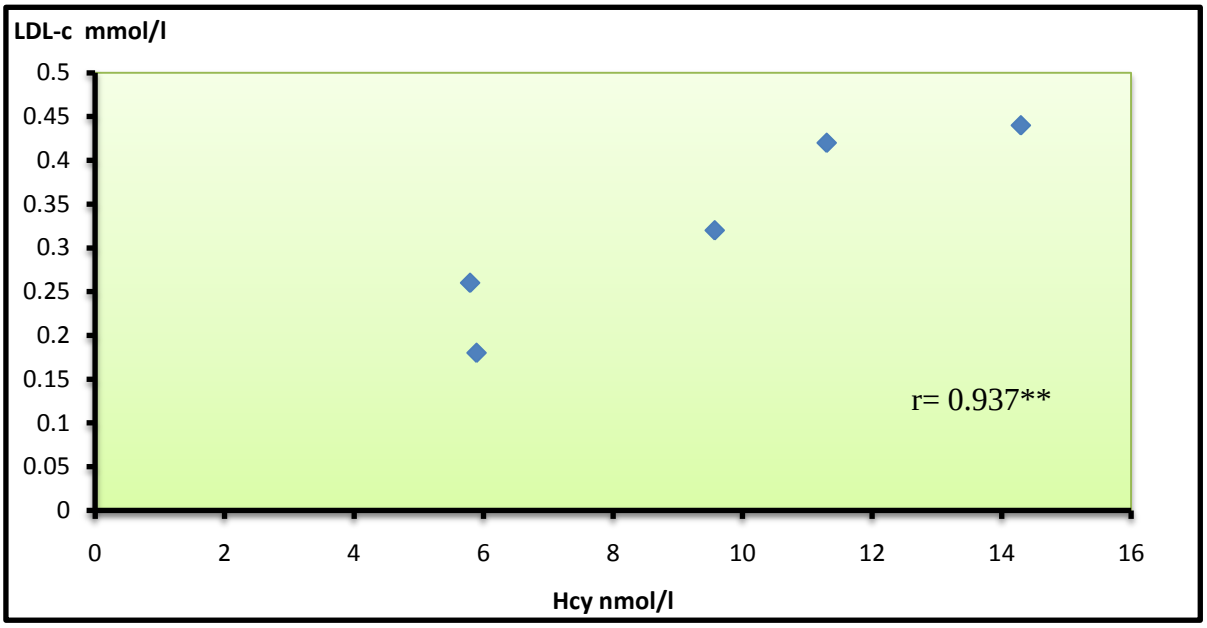

Figure 24: Correlation between lipoprotein LDL-c and homocysteine in MO group

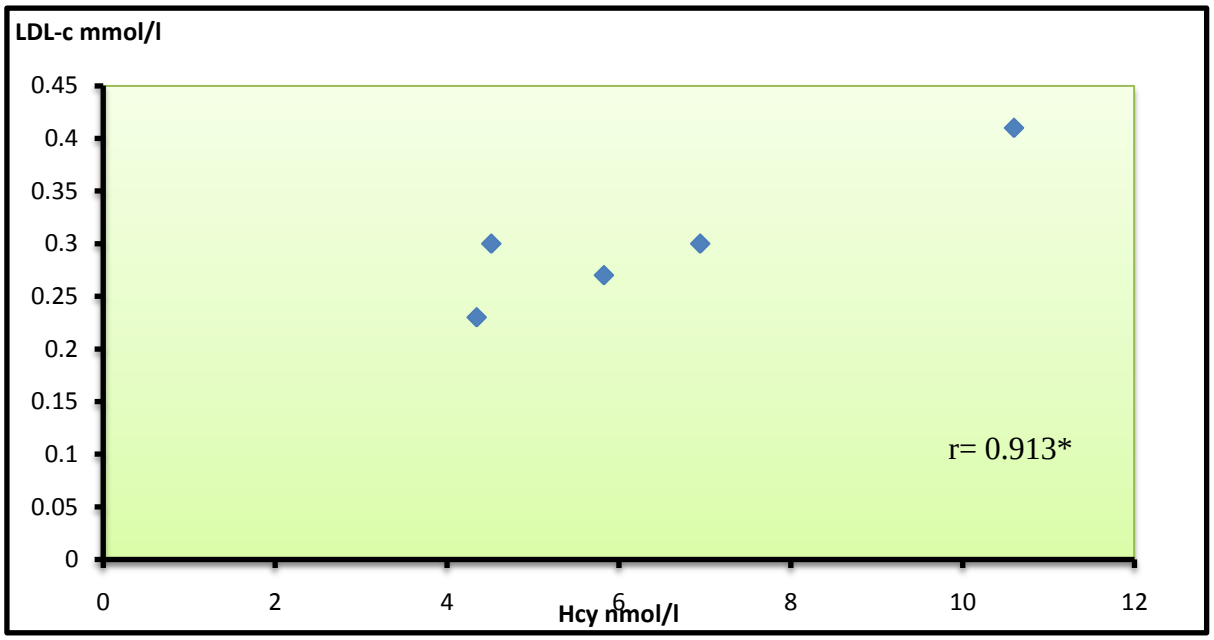

Figure 25: Correlation between lipoprotein LDL-c and homocysteine in OO group.

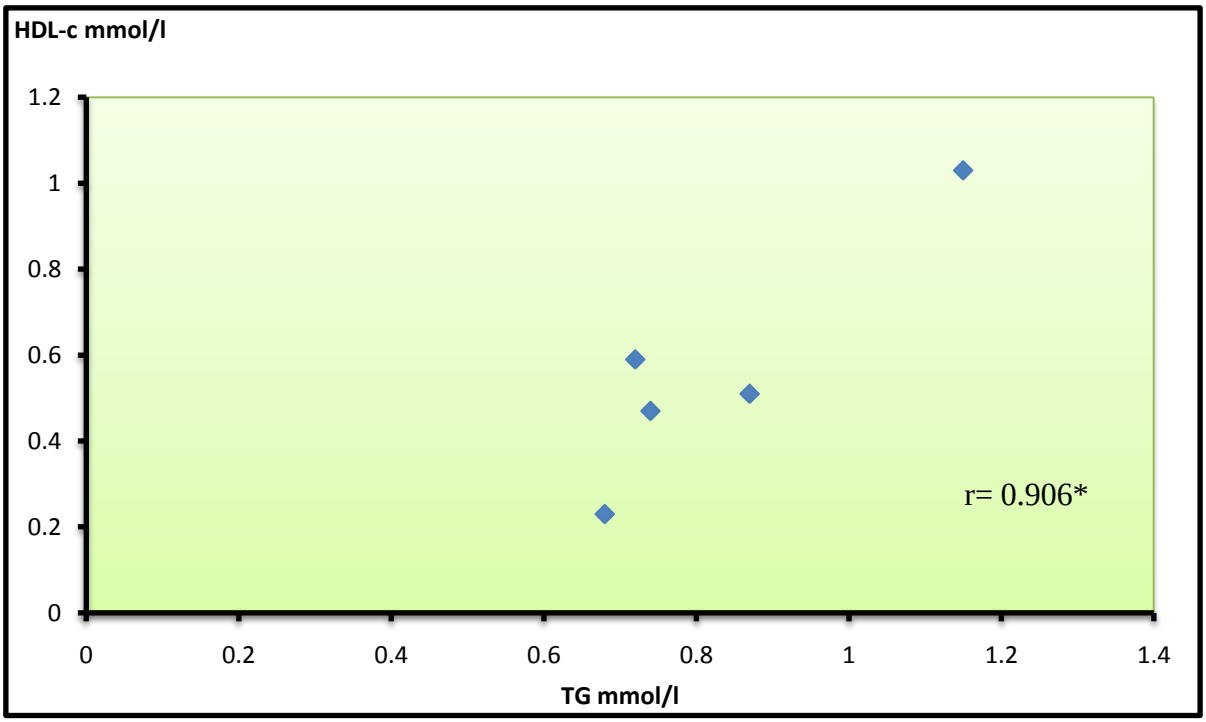

Figure 26: Correlation between lipoprotein HDL-c and TG in OO group.

3- Histological investigation

3-1 Aorta

3-1-a *Abdominal aorta*

The abdominal aorta of animal in (M) group showed degeneration and desquamation of endothelial cells, we also observed in the media lysis, some region of necrosis, fenestration, formation of foam cells and oval nuclei **(figure 28 a, b, c)**.

However, in the control (F) group the abdominal aorta had intact endothelium and spindle shaped mediocytes nuclei **(Figure 27 a, b)**, these results were observed in the positive control group (OO) **(Figure 30 a, b)**.

Observation of abdominal aorta in (MO) group showed intact endothelium, and spindle shaped mediocytes with the absence of foam cells formation. Nevertheless some oval nuclei and a few lysis regions were observed **(Figure 29 a, b).**

3-1-b *Iliac aorta*

Microscopic observation of the control (F) group presents an intact intima-lined epithelium. The media appears thicker composed of smooth muscle cells with fusiform nuclei. The adventitia is formed by a relatively thin layer of connective tissue containing fibroblasts. The elastic fibers are concentrated in the inner and outer elastic limiting **(Figure 31 a, b).**

The iliac arteries of the OO group showed the same observation as in the control group **(Figure 34 a, b).**

However there was clear differences at histology between controls and hyperhomocysteinemic animals, resulting in desquamation of endothelial cells, smooth muscle cell proliferation formation of foam cells and change of the appearance of the core fusiform muscle cells to a rounded appearance **(Figure 32 a, b and c).**

The iliac arteries of the MO group showed intact endothelium, spindle nuclei and absence of foam cells formation. Nevertheless some oval nuclei were observed **(Figure 33 a, b).**

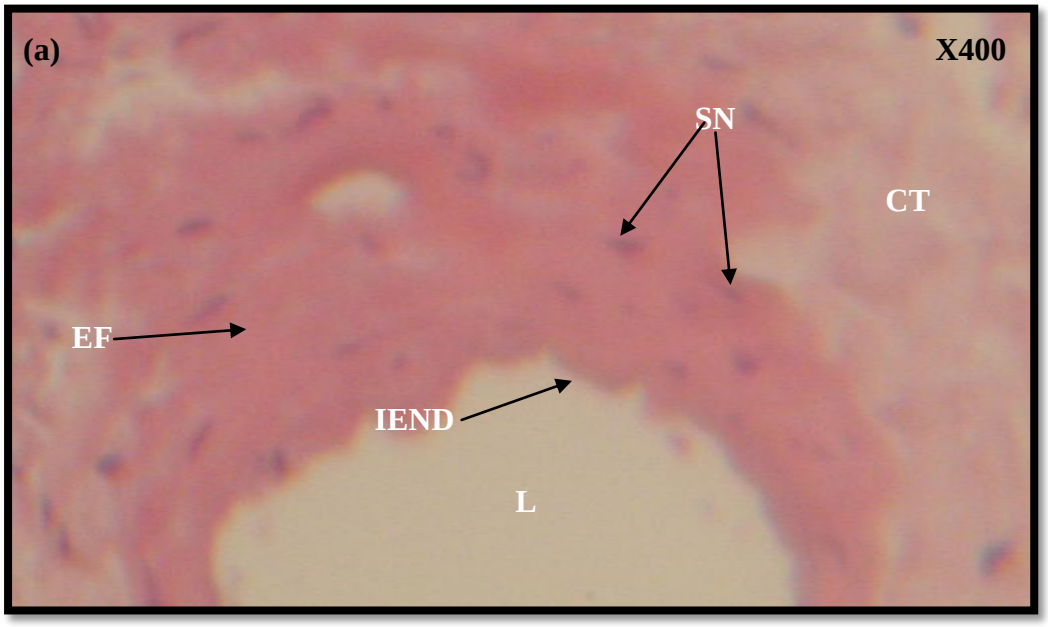

Figure 27 (a): Histological sections of the abdominal aorta, F group Hematoxylin eosin staining.

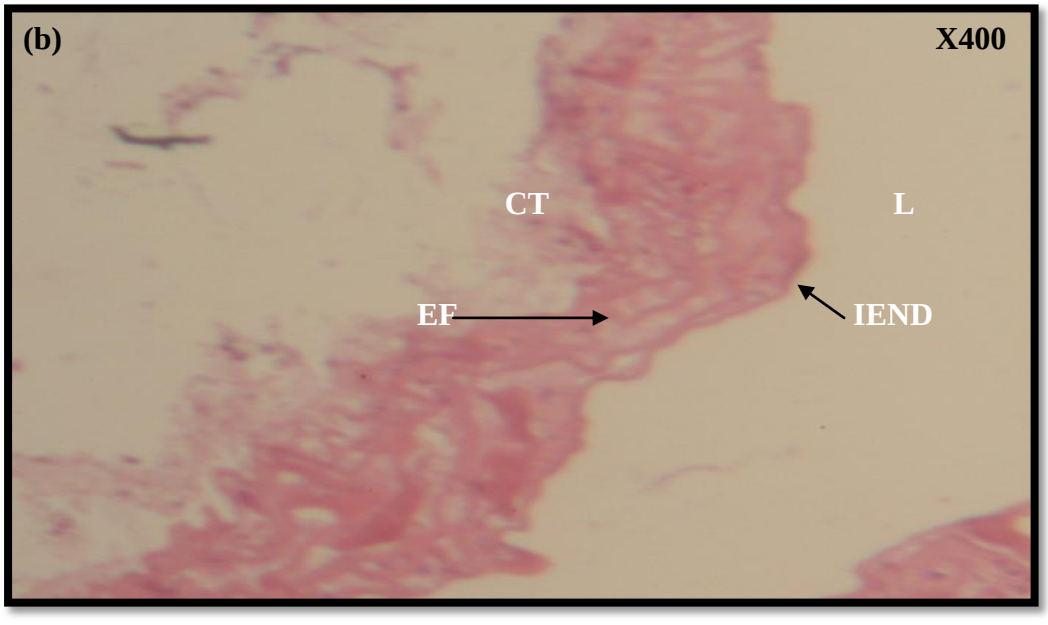

Figure27 (b): Histological sections of the abdominal aorta, F group Hematoxylin eosin staining.

L: Lumen, CT: Connective tissue, IEND: Intact Enothelium, SN: Spindle nuclei, EF: Elastic Fibers.

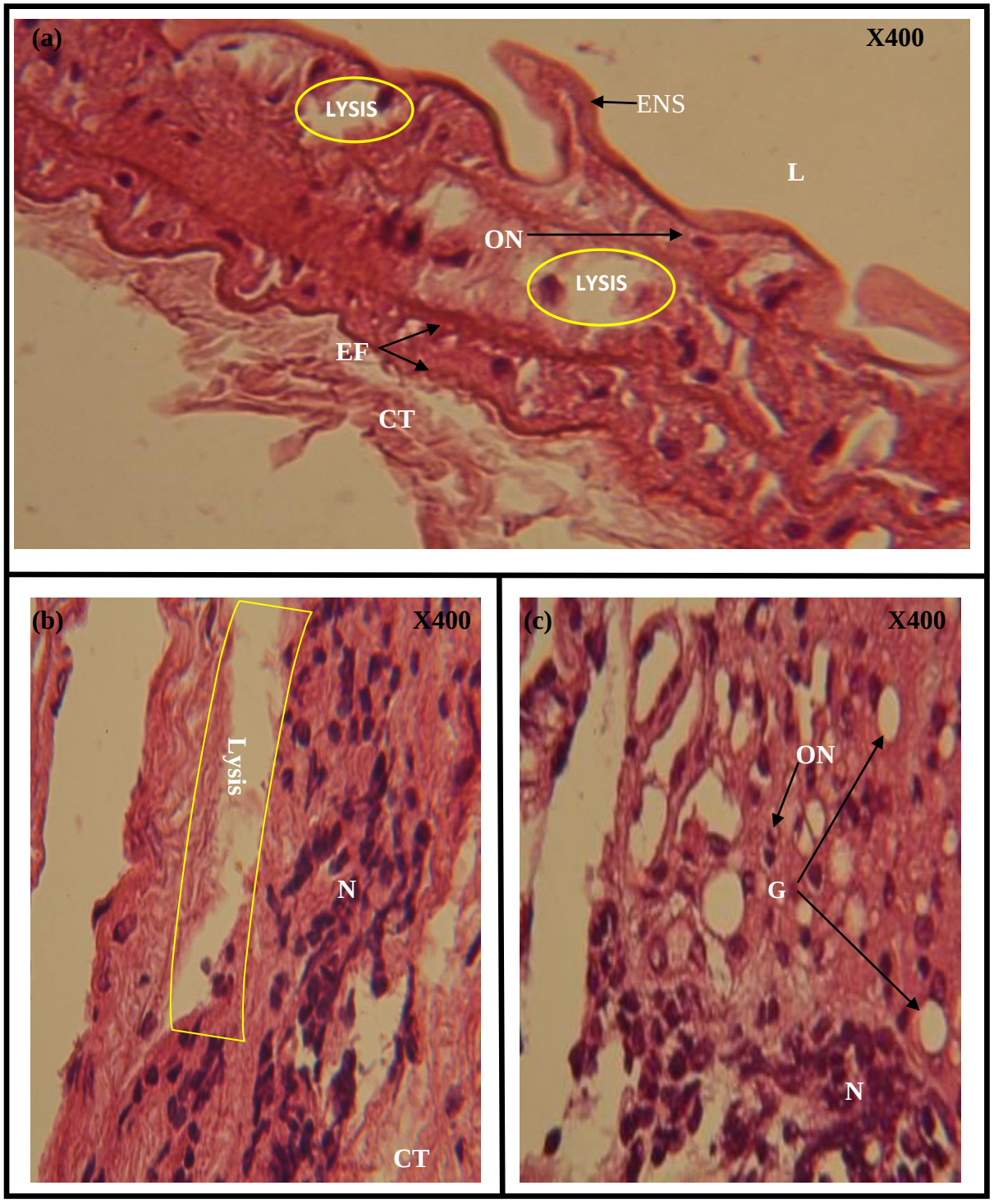

Figure 28 (a, b, c): Histological sections of the abdominal aorta, M group. Hematoxylin eosin staining.

L: Lumen, CT: Connective tissue, ON: Oval nuclei,

ENS: Endolysis, N: Necrosis, G: Gaps,

EF: Elastic Fibers.

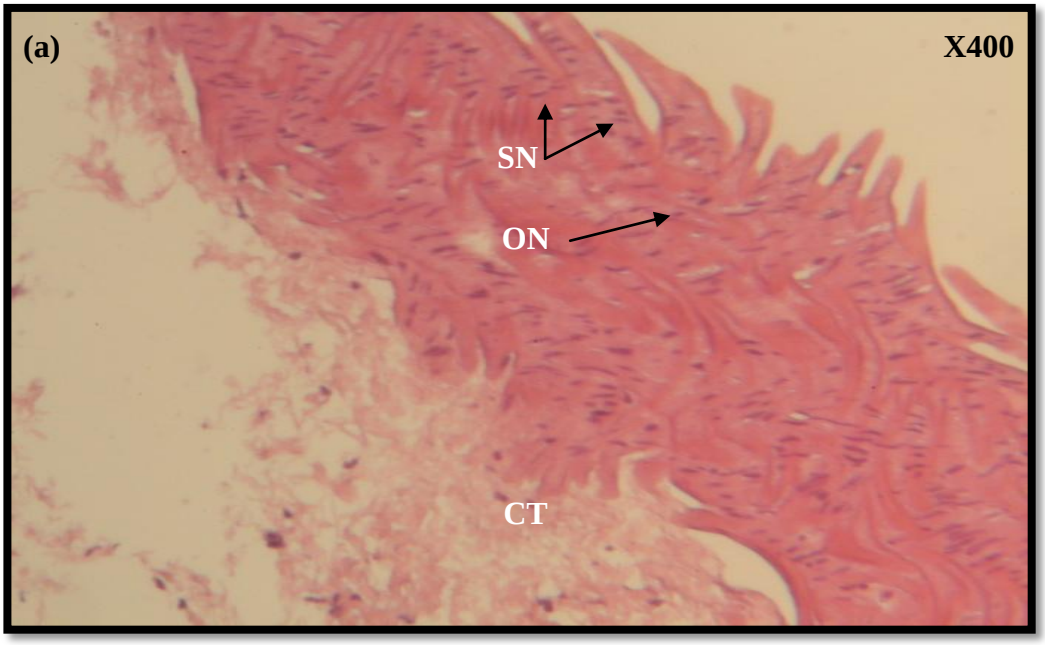

Figure 29 (a): Histological section of the abdominal aorta, MO group Hematoxylin eosin staining.

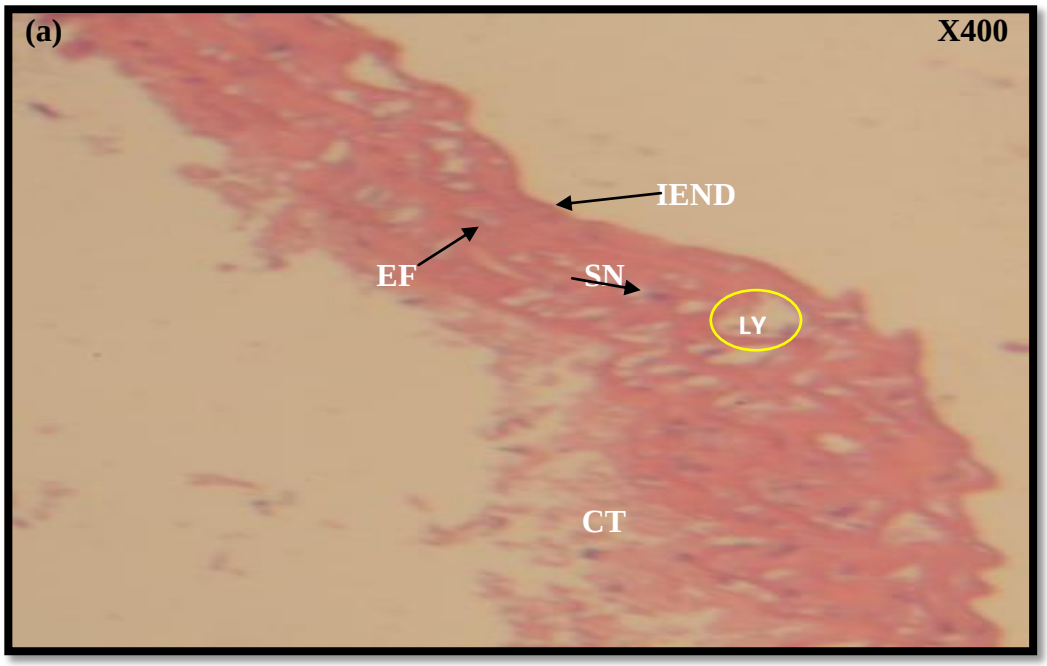

Figure 29 (b): Histological section of the abdominal aorta, MO group Hematoxylin eosin staining.

L: Lumen, CT: Connective tissue, ON: Oval nuclei, LY: Lysis

IEND: Intact Enothelium, SN: Spindle nuclei, EF: Elastic Fibers.

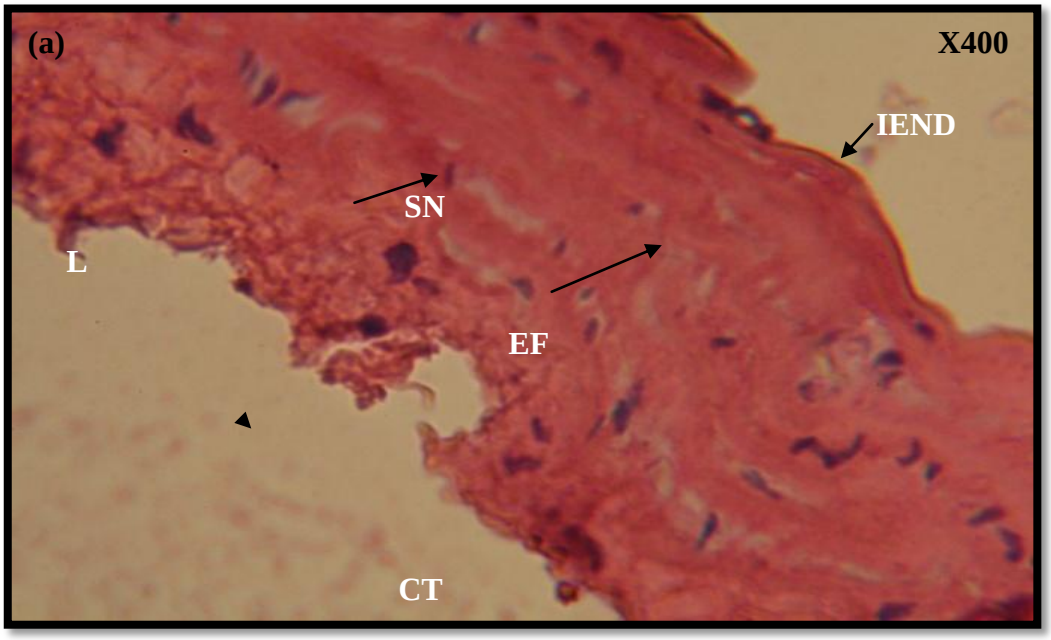

Figure 30 (a): Histological sections of the abdominal aorta, OO group Hematoxylin eosin staining.

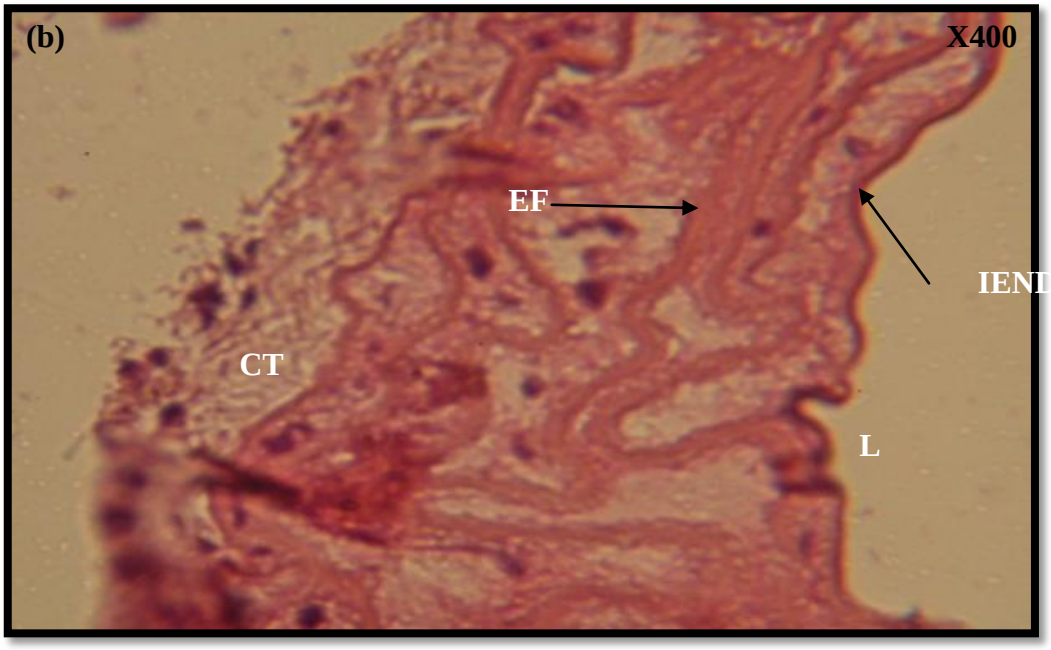

Figure 30 (b): Histological sections of the abdominal aorta, OO group Hematoxylin eosin staining.

L: Lumen, CT: Connective tissue, IEND: Intact Enothelium, SN: Spindle nuclei, EF: Elastic Fibers.

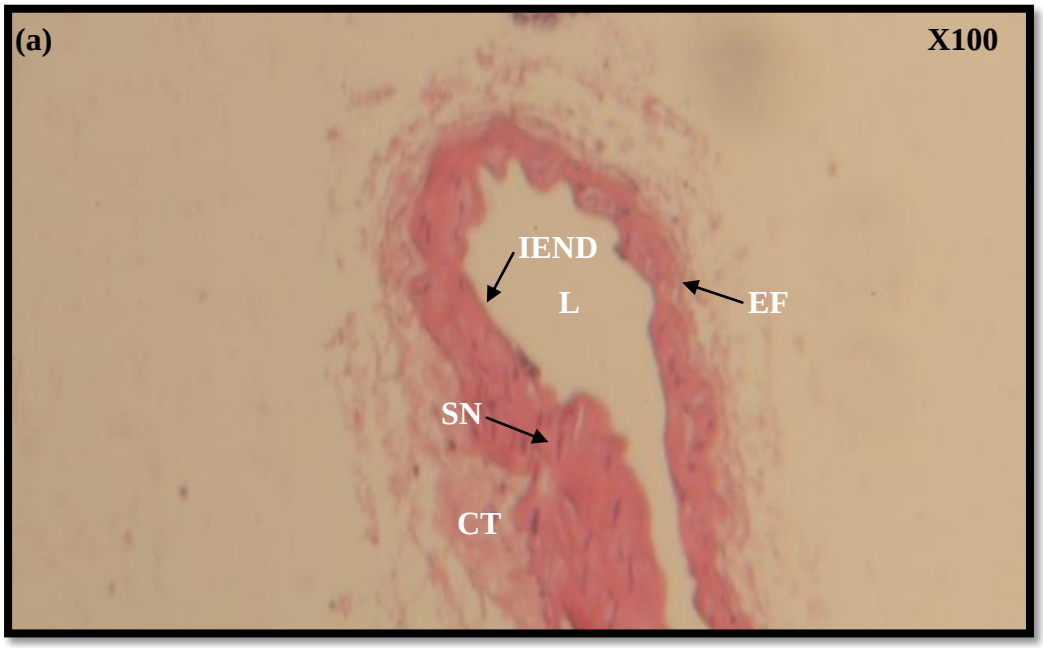

Figure 31(a): Histological section of the iliac aorta, F group

Hematoxylin eosin staining

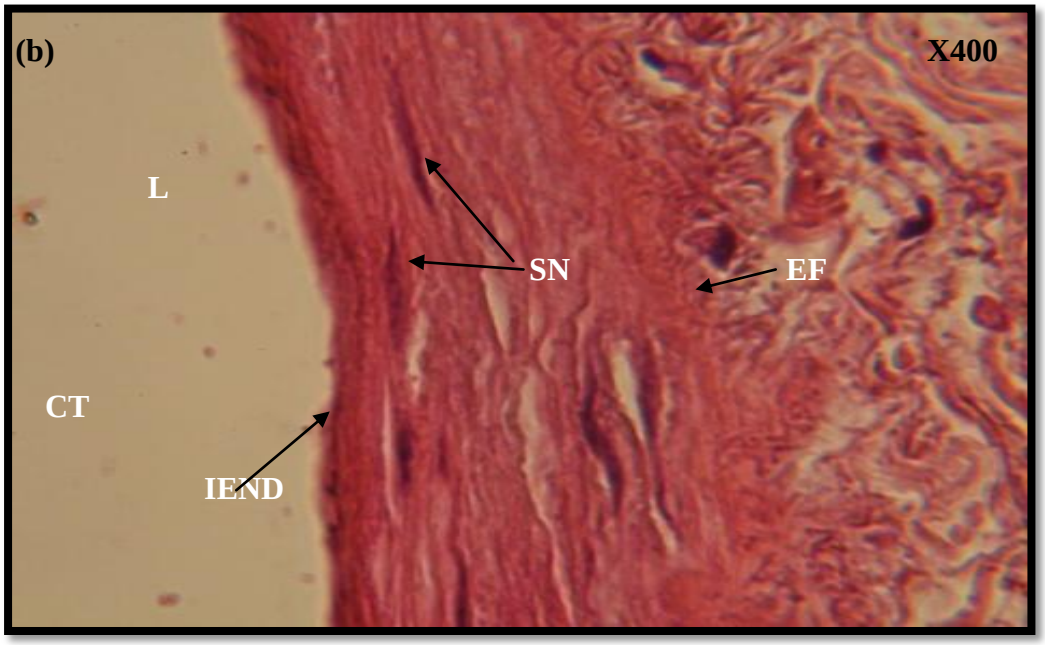

Figure 31(b): Histological section of the iliac aorta, F group

Hematoxylin eosin staining.

L: Lumen, CT: Connective tissue IEND: Intact Enothelium, SN: Spindle nuclei, EF: Elastic Fibers

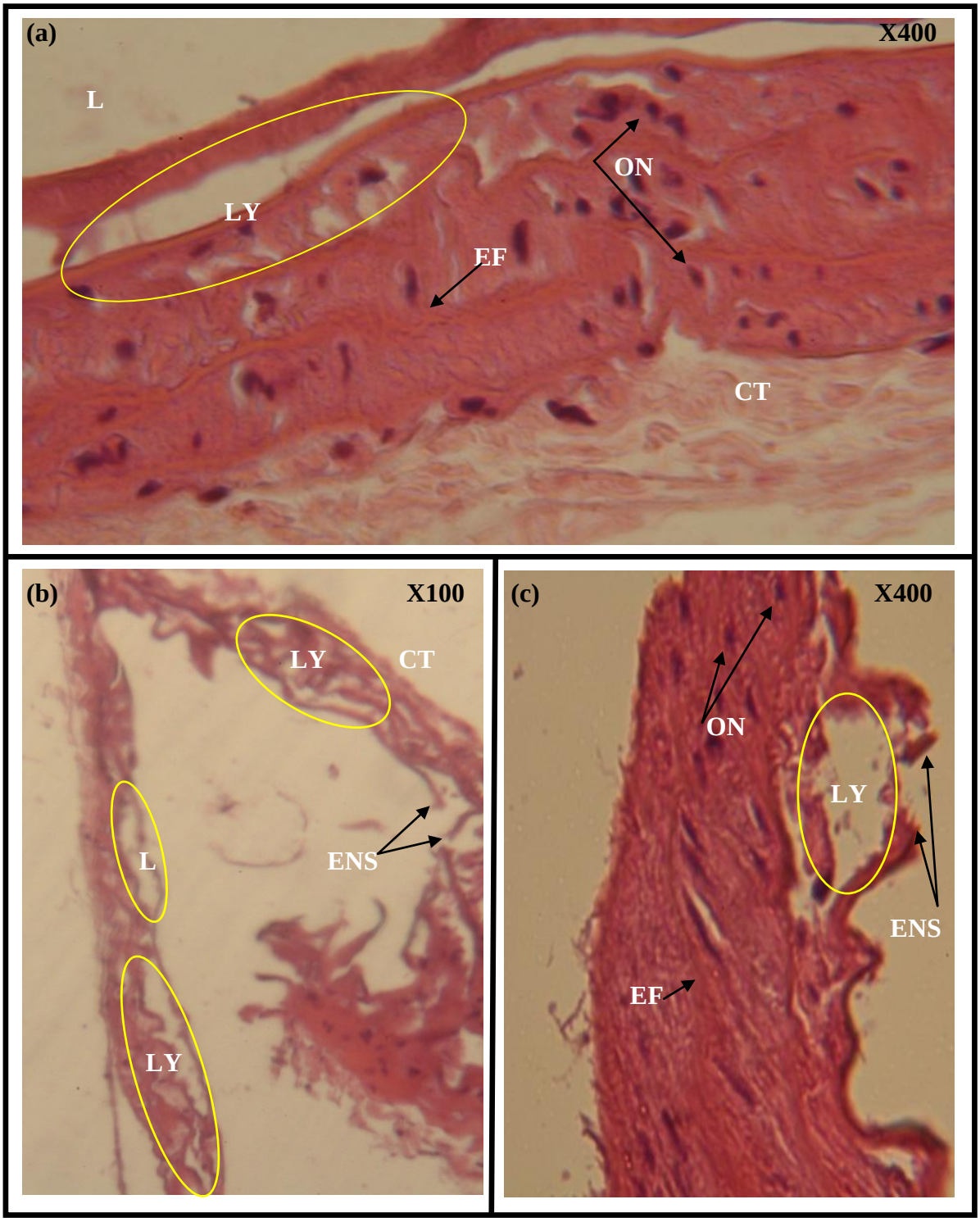

Figure 32 (a, b, c): Histological section of the iliac aorta, M group Hematoxylin eosin staining

L: Lumen, CT: Connective tissue, ON: Oval nuclei, ENS: Endolysis, SN: Spindle nuclei, EF: Elastic Fibers

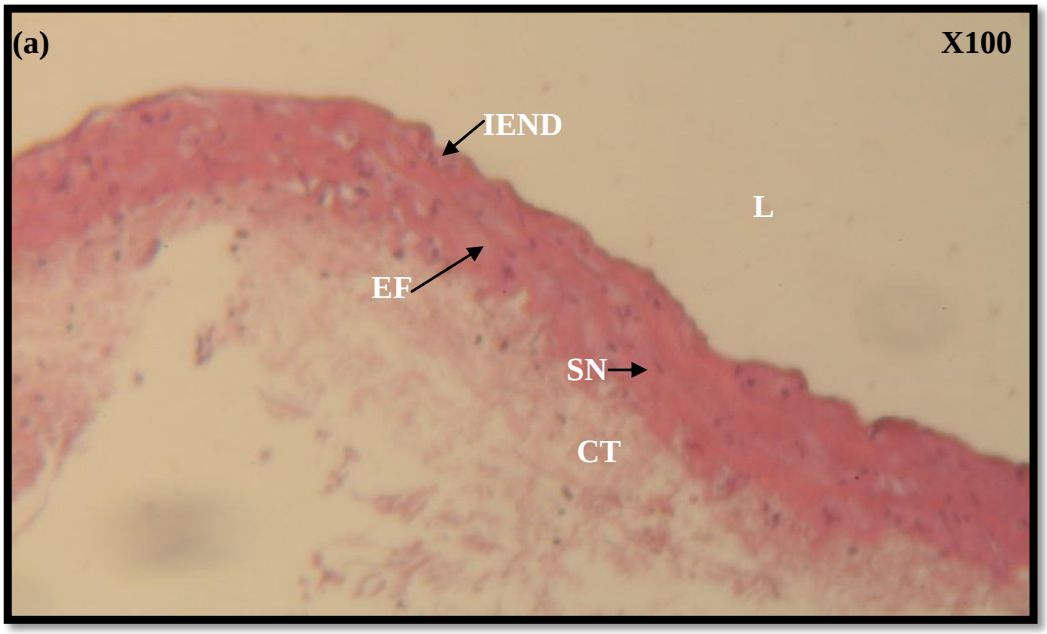

Figure 33 (a): Histological section of the iliac aorta, MO group Hematoxylin eosin staining.

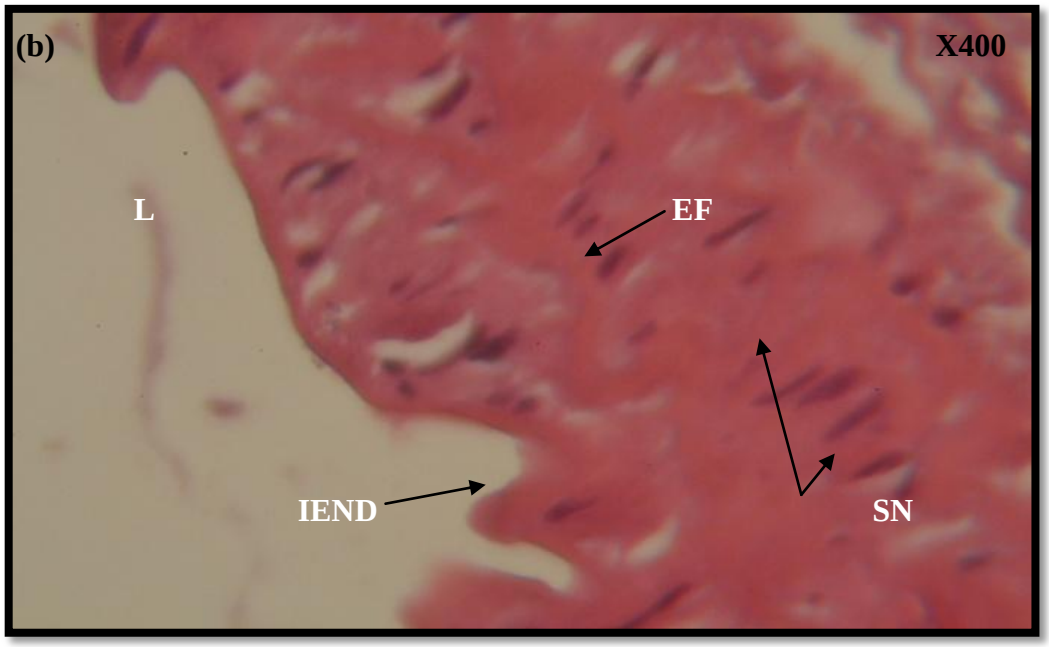

Figure 33 (b): Histological section of the iliac aorta, MO group Hematoxylin eosin staining.

L: Lumen, CT: Connective tissue, IEND: Intact Enothelium, SN: Spindle nuclei, EF: Elastic Fibers

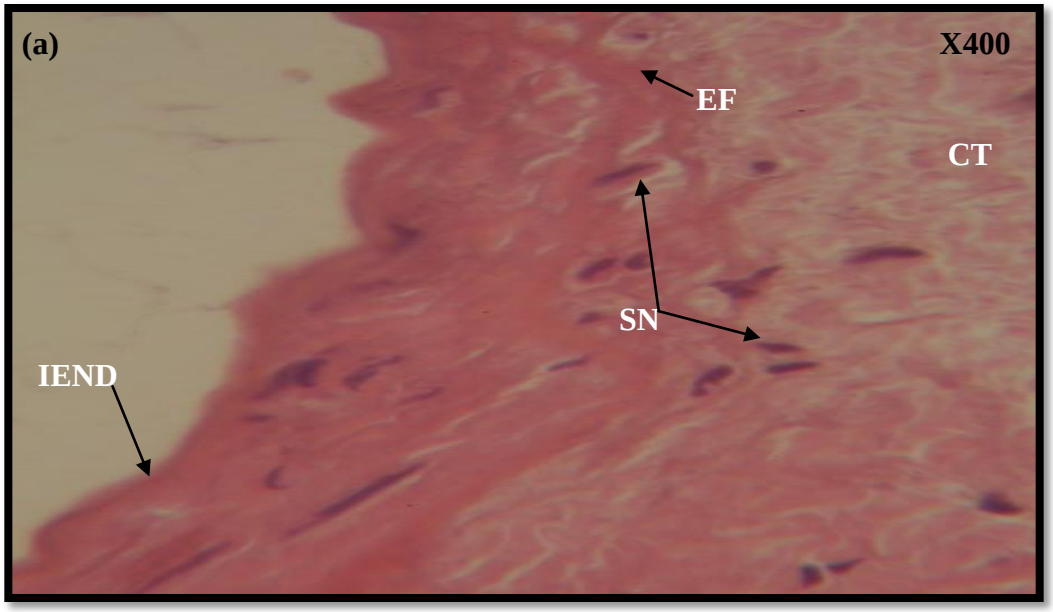

Figure 34 (a): Histological section of the iliac aorta, OO group
Hematoxylin eosin staining.

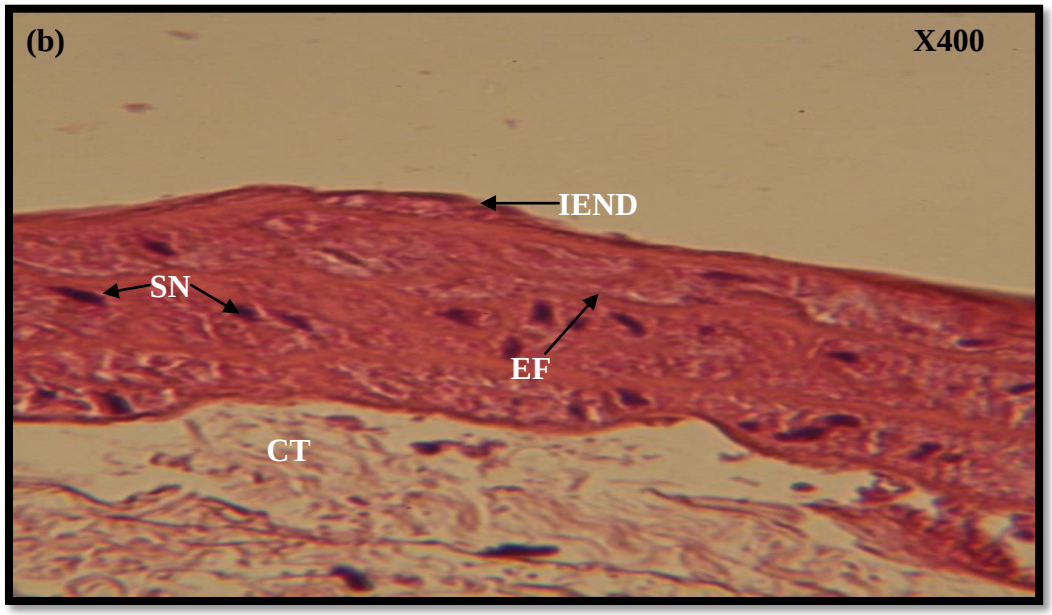

Figure 34 (b): Histological section of the iliac aorta, MO group
Hematoxylin eosin staining.

L: Lumen, CT: Connective tissue, ON: Oval nuclei, ENS: Endolysis
IEND: Intact Enothelium, SN: Spindle nuclei, EF: Elastic Fibers

3-2 Heart

Microscopic observation of the methionine group presented lysis in structure of muscular fibers in heart **(Figure 36a, b, c, d)** in contrast to control group whose tissues structure were intact **(Figure 35a, b)**.

In the other group, we have not observed any alteration **(Figure 37a, b and 38a, b)** in contrast to methionine group.

3-3 liver

The liver of control group showed a normal structure **(Figure 39 a, b)**. The histologic section of liver revealed intact hepatic lobules separated by interlobular septa and traversed by portal veins. The hepatocytes were polyhedral. The nuclei were round and the size was roughly uniform.

However, in the methionine group resulted in severe pathologic liver injury marked by necrosis, dilatation of sinusoids, inflammation, and strong vacuolization of hepatocytes **(Figure 40 a, b, c, d).**

A similar morphology of the hepatocyte nuclei was observed in the other group compared with controls **(Figure 41 a, b and 42 a, b).**

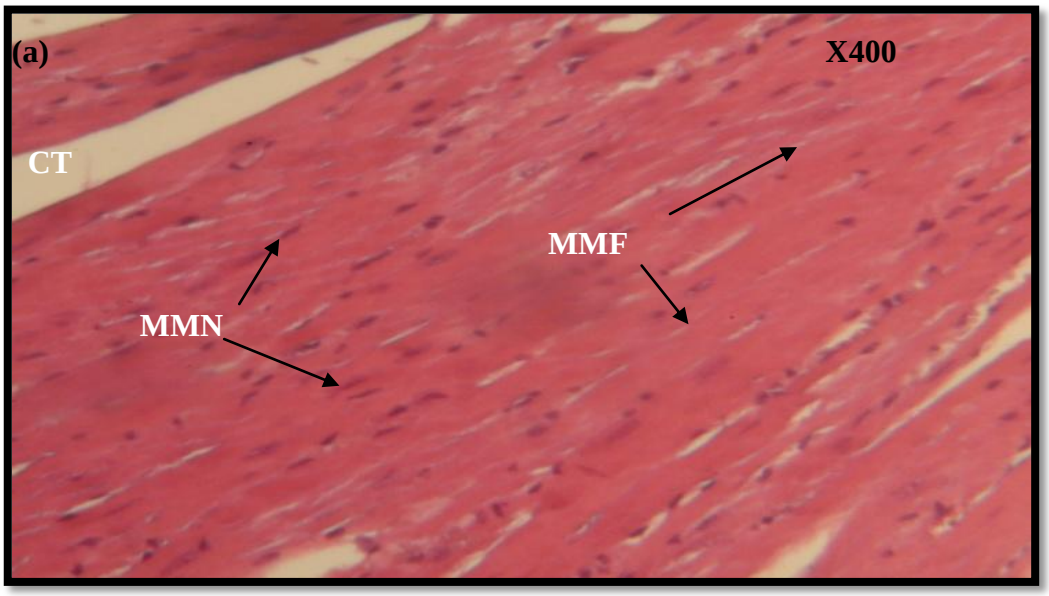

Figure 35 (a): Histological section of the Heart, F group Hematoxylin eosin staining.

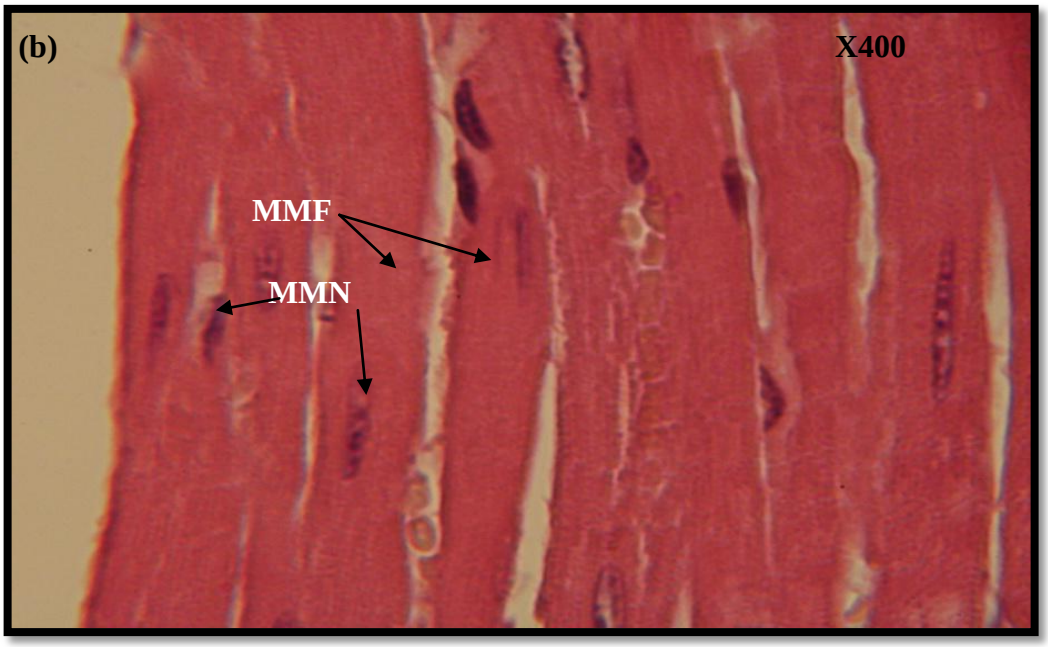

Figure 35 (b): Histological section of the Heart, F group Hematoxylin eosin staining

MMF: muscular myocardnuclei,

MMN: muscular myocardfibers, CT: connective tissue

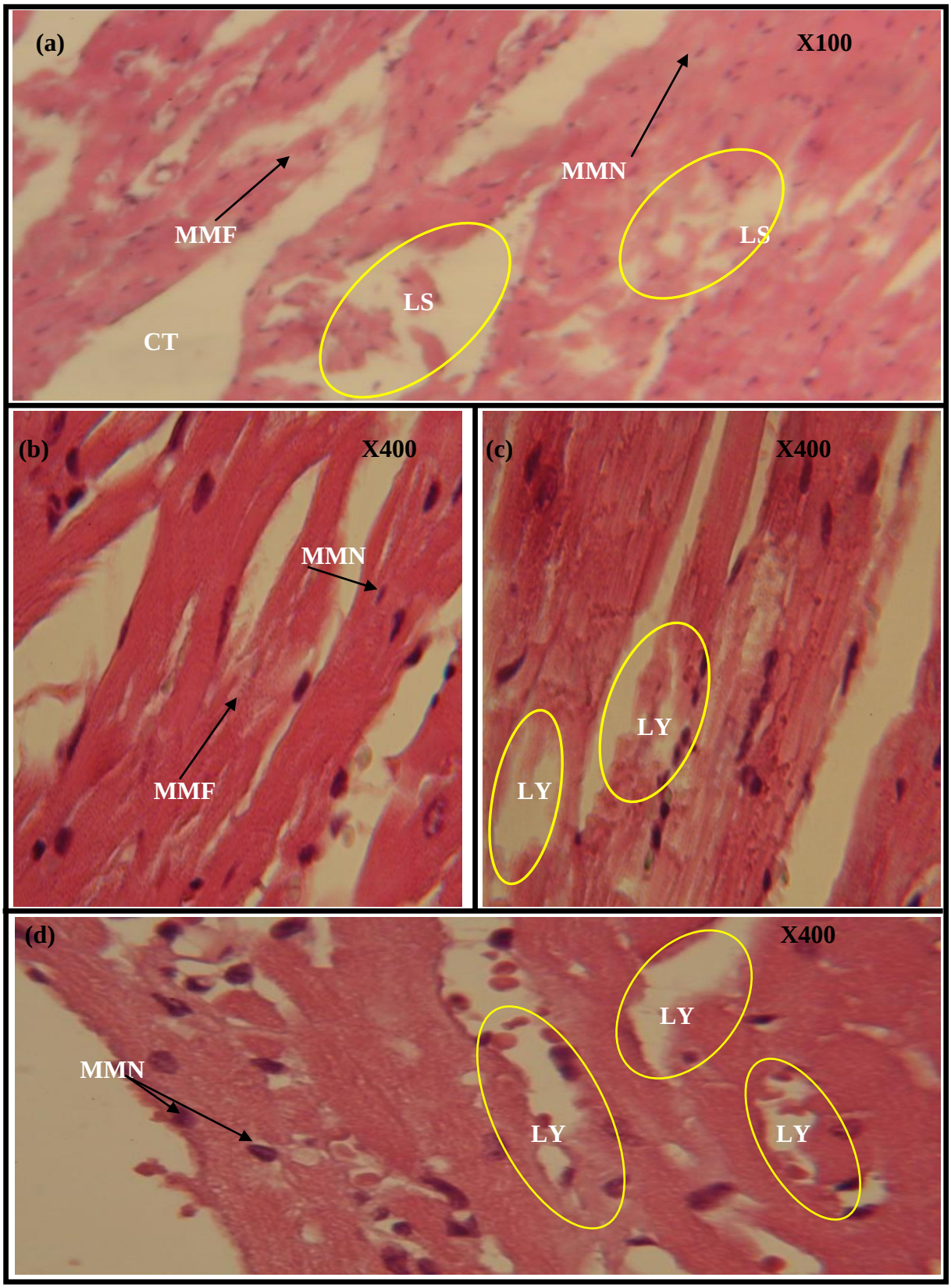

Figure 36 (a, b, c, d): Histological section of the Heart, M group Hematoxylin eosin staining.

MMN: muscular myocard nuclei, MMF: Muscular myocard fibers,

CT: connective tissue, LS: lysis.

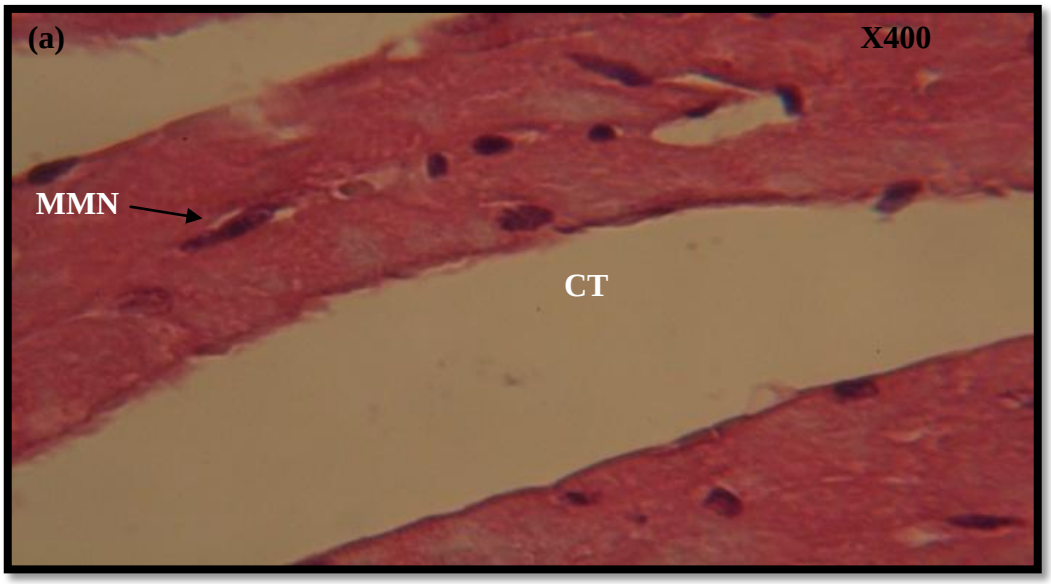

Figure 37 (a): Histological section of the Heart, MO group
Hematoxylin eosin staining.

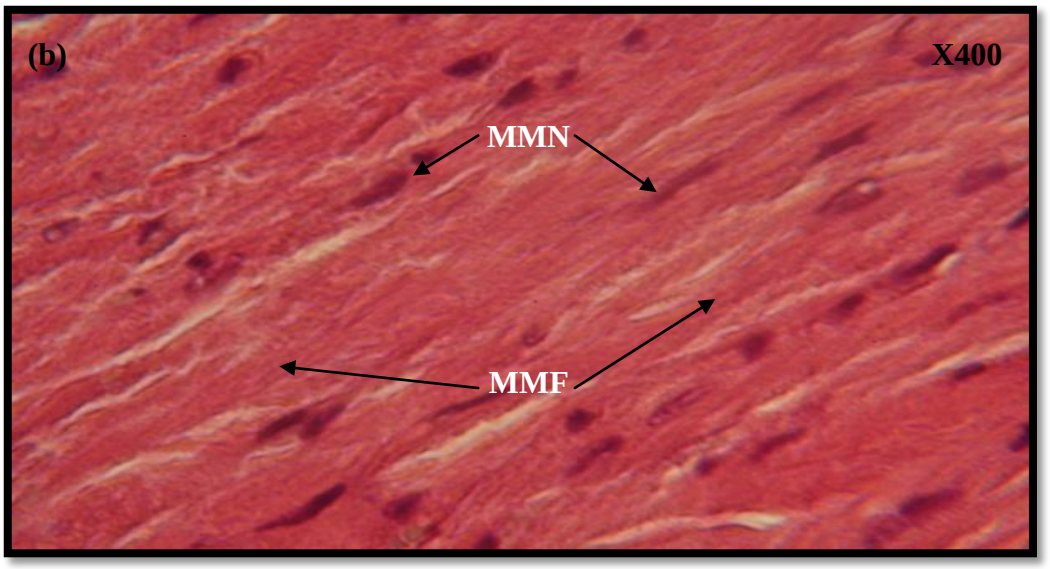

Figure 37 (b): Histological section of the Heart, MO group
Hematoxylin eosin staining.

MMN: muscular myocard nuclei,
MMF: muscular myocard fibers, CT: connective tissue.

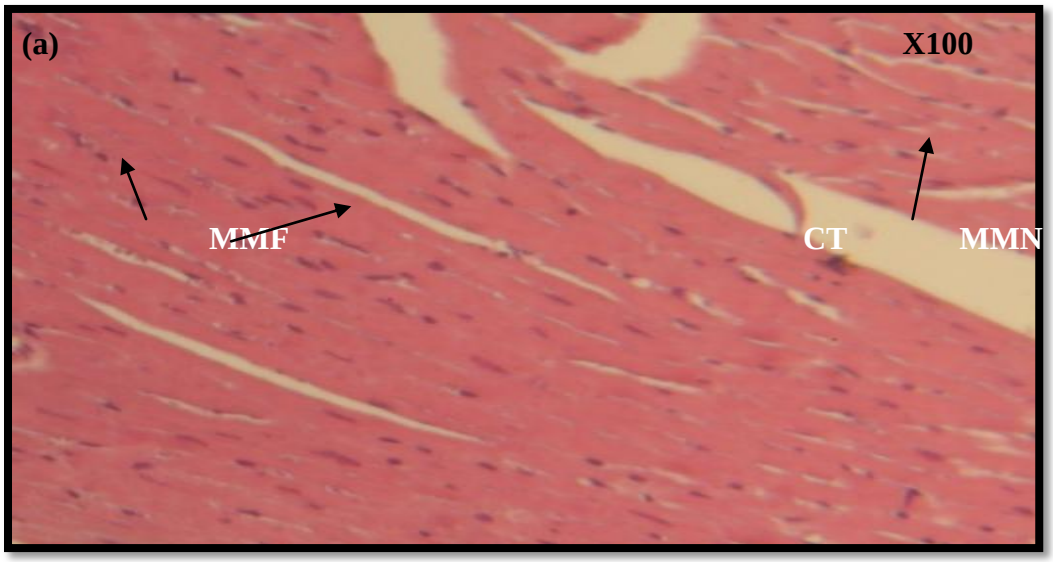

Figure 38 (a): Histological section of the Heart, OO group Hematoxylin eosin staining.

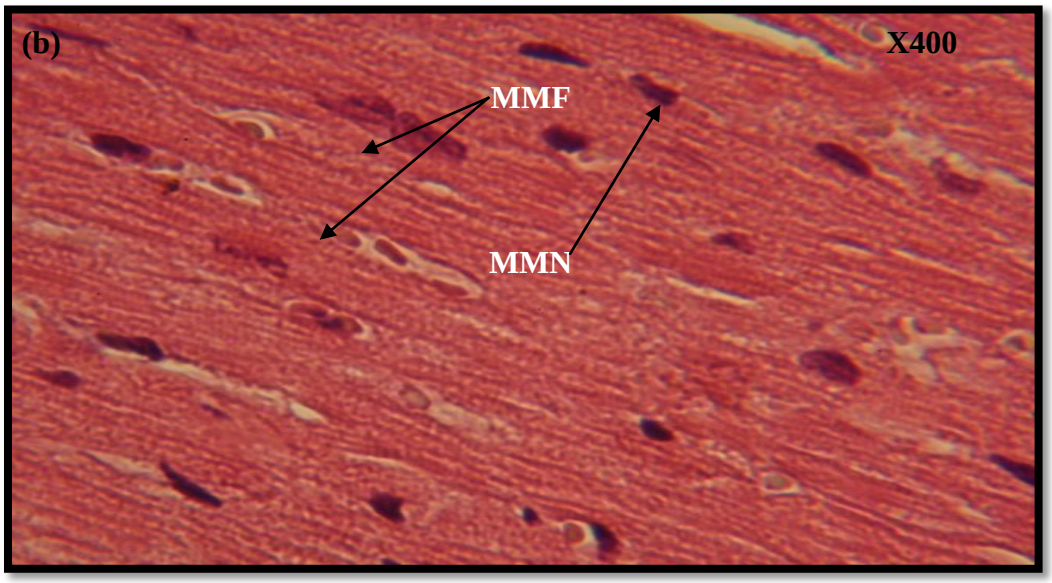

Figure 38 (b): Histological section of the Heart, OO group Hematoxylin eosin staining.

MMF: muscular myocardnuclei,

MMN: muscular myocardfibers, CT: connective tissue.

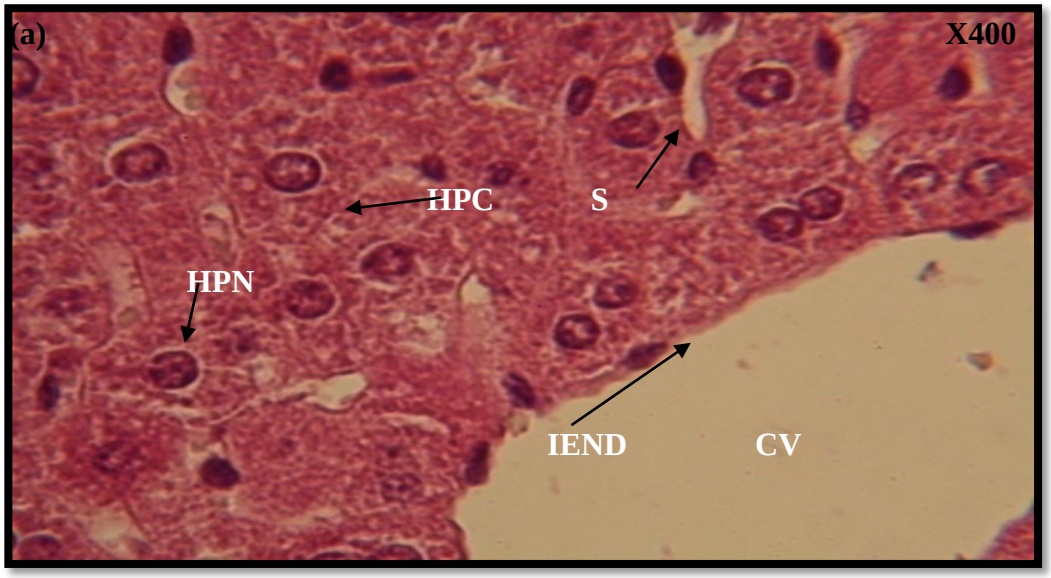

Figure 39 (a): Histological section of the liver, control group Hematoxylin eosin Staining.

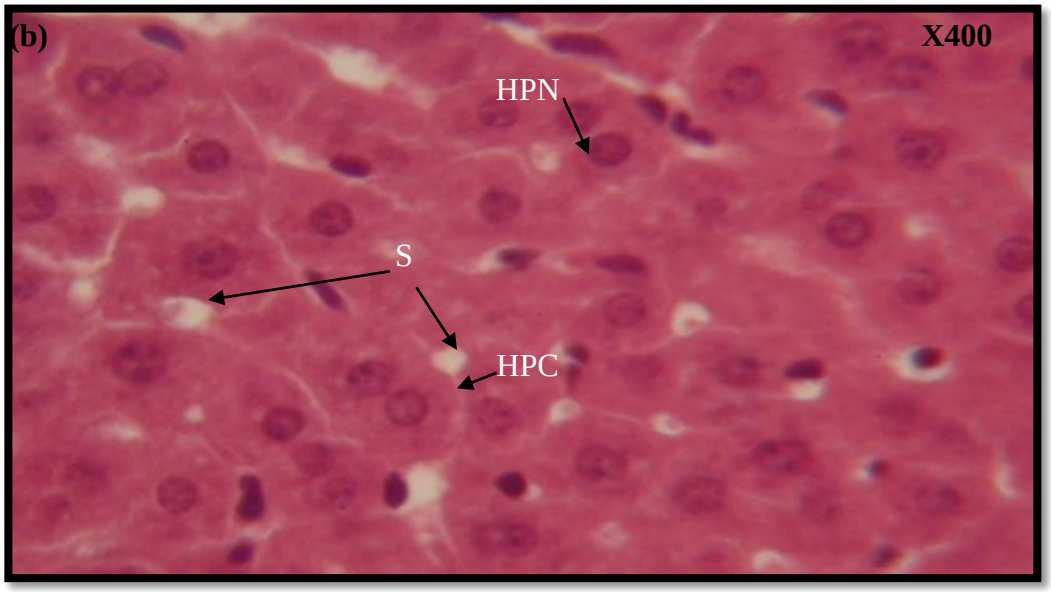

Figure 39 (b): Histological section of the liver, F group Hematoxylin eosin Staining.

HPN: Hepatocyte nuclei, HPC: Hepatocyte cell, S: sinusoid, IEND: Intact endothelium, CV: Central vein

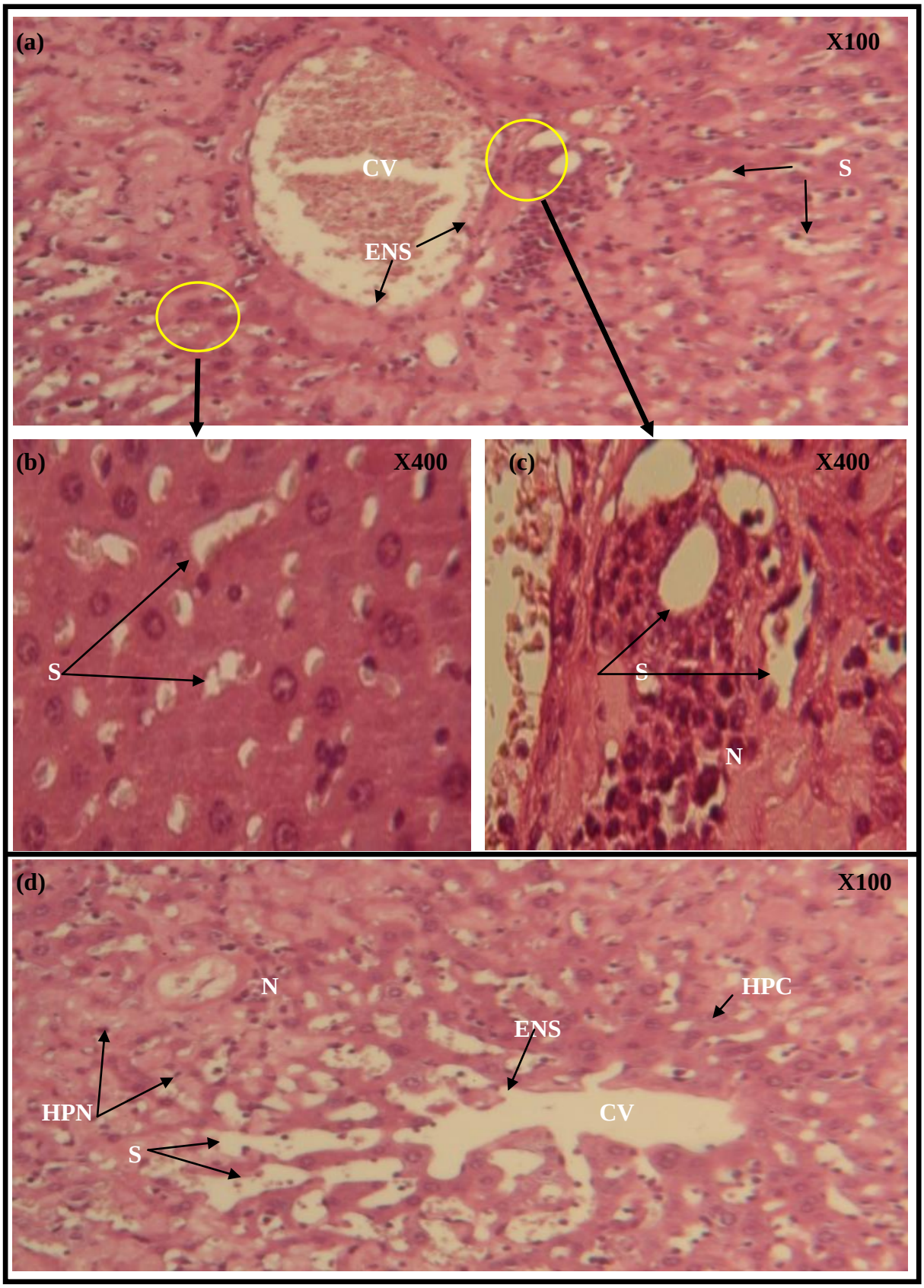

Figure 40 (a, b, c, d): Histological section of the liver, M group Hematoxylin eosin staining.

HPN: Hepatocyte cell, HPC: Hepatocyte cell, S: sinusoid, EDS: Endolysis, CV: central vein, N: Necrosis.

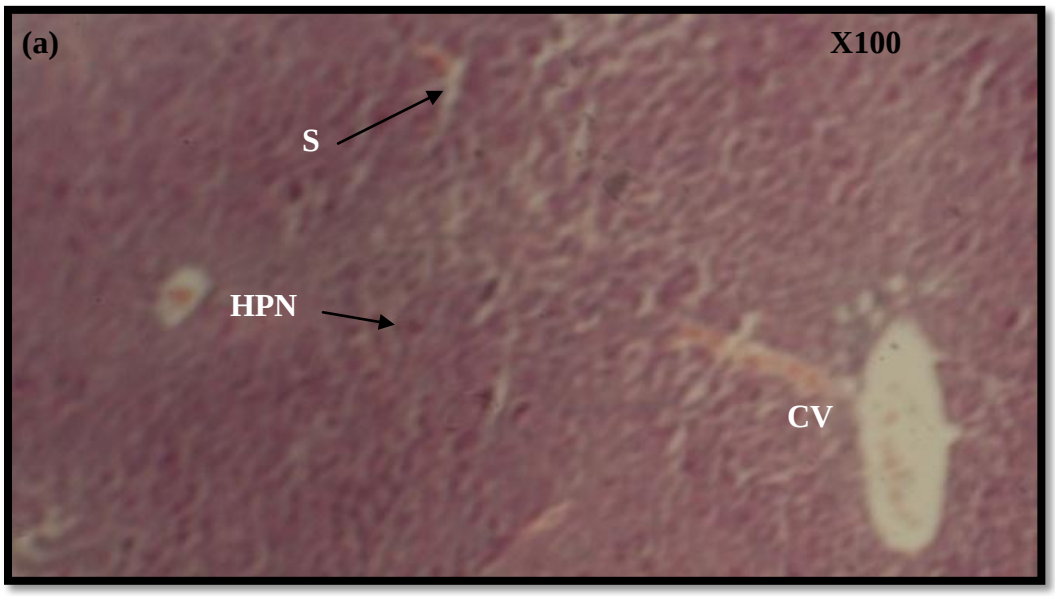

Figure 41 (a): Histological section of the liver, MO group Hematoxylin eosin staining.

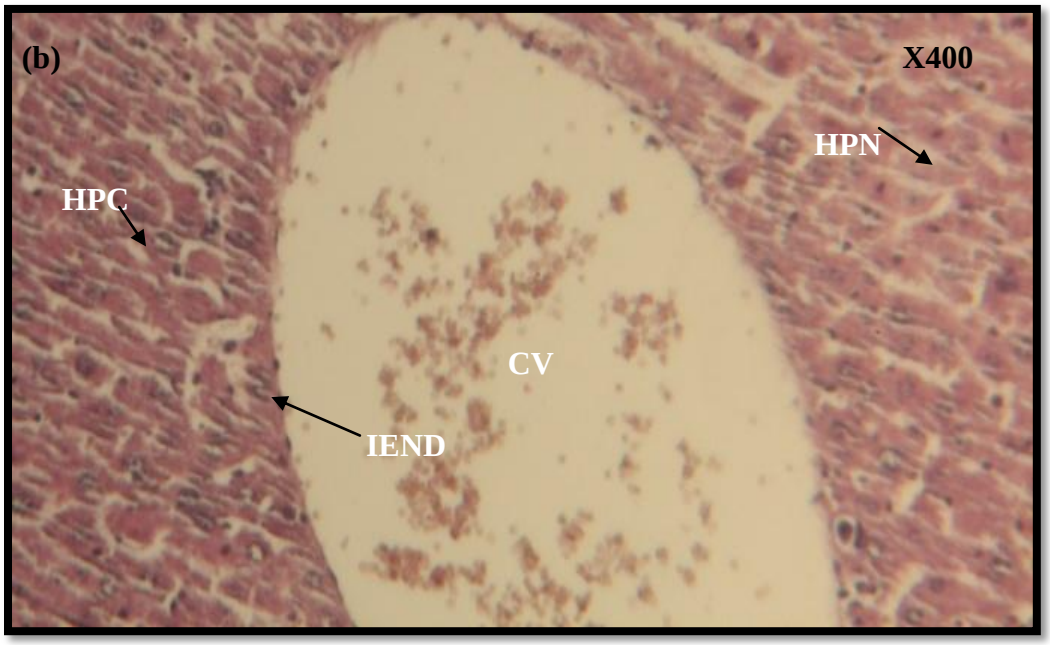

Figure 41 (b): Histological section of the liver, MO group Hematoxylin eosin staining.

HPN: Hepatocyte cell, HPC: Hepatocyte cell, S: sinusoid,

IEND: Intact endothelium, CV: central veine.

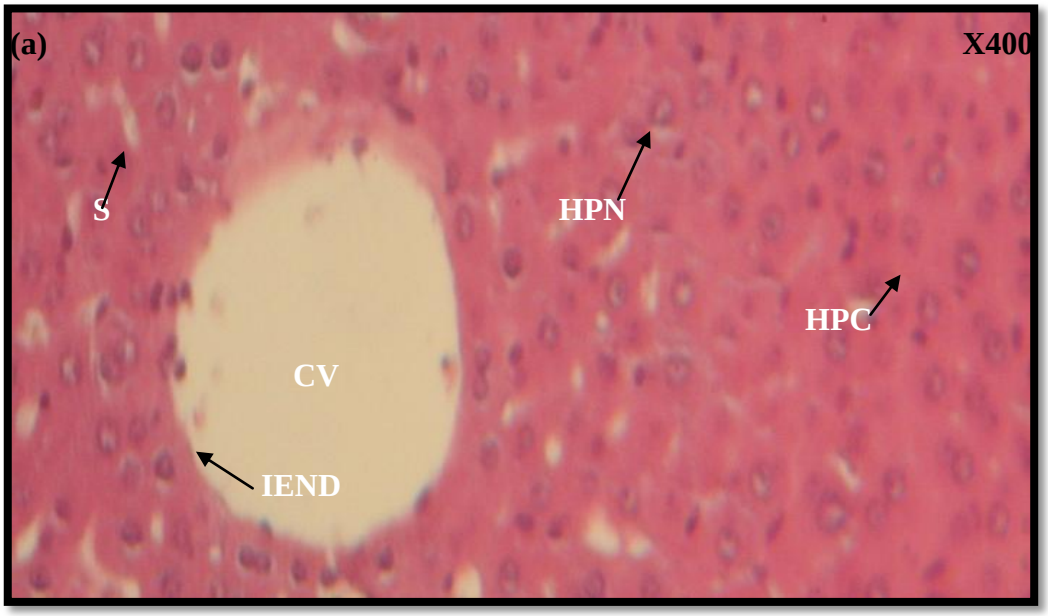

Figure 42 (a): Histological section of the liver, OO group
Hematoxylin eosin staining

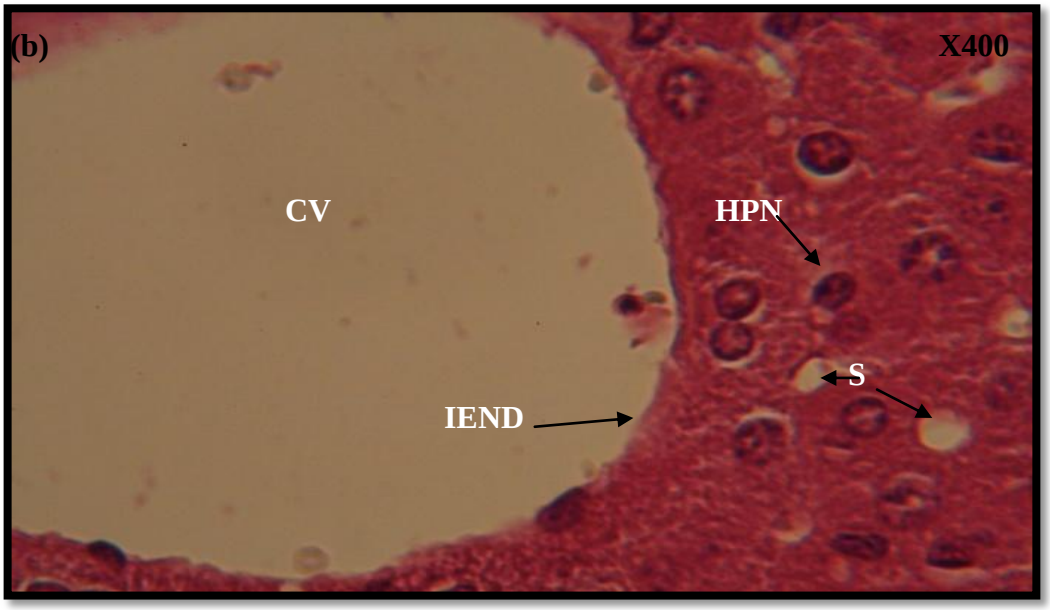

Figure 42 (b): Histological section of the liver, OO group
Hematoxylin eosin staining

HPN: Hepatocyte cell, HPC: Hepatocyte cell, S: sinusoid,
IEND: Intact endothelium, CV: central veine.

Discussion

Discussion

In addition to classical risk factors of cardiovascular diseases, hyperhomocysteinemia received much attention as independent risk factors (Zerizer and Naimi, 2004).

As mentioned in the introduction, the L-methionine produced the homocysteine in human and mammals. We have respected this metabolism pathway, and for this reason, we have administered the animals with L-methionine rather than the homocysteine; so the objective of the present work was undertaken to clarify the effects of high a dose of L-methionine on weight and diet consumption , some biochemical parameters (Hcy, T-ch, TG, HDL-c, LDL-c and glutathione reduced) and on histological structure of some organs (abdominal and iliac aorta, heart and liver) in mice and explore the therapeutic effect of the olive oil (olea europaea) on the anomalies caused by the high dose of L-methionine (400mg/kg) during 21 days of treatment.

The first and second experiments evaluate the effect of L-methionine on the weight and diet consumption of mice. The results showed that there is an increased significantly in weight of mice in all groups, however in group of (OO) the weight increased but not significantly. The study of (Zerizer, 2006) reported an increased in the weight of rats treated with 200mg/kg of L-methionine for 21 days. Also, the study of (Boudebouz, 2013) showed an increase in the weight of mice treated with 400mg/kg of L-methionine for 21 days. However, the work of (Benmebarek, 2013) indicated a significant decrease in the weight of mice treated with 200mg/kg of L-methionine for 21 days. Given the conflicting results, we cannot conclude that there is a relationship between hyperhomocysteinemia and the weight of mice.

Concerning the diet consumption of mice, the results showed that the consumption diet in group methionine increased significantly and decreased significantly in mice treated with olive oil.

These results indicated a probable relationship between olive oil and mice appetite.

Following the oral administration of L-methionine, it’s results of degradation, the high levels of homocysteine when it is compared to the control group. In addition to the increased levels of Hcy, the L-methionine treatment caused an important increase in Total cholesterol, triglyceride and LDL-c and a decrease in HDL-c and glutathione reduced, which indicated a change in the permeability of hepatic cells.

Discussion

Homocysteine's toxic effects have been documented on the heart, liver, adrenals joints, nerves and blood vessels (Taravati *et al.*,2013), Our results showed that L-methionine exerted an angiotoxic activity on the abdominal and iliac aorta **(Figure 28a, b, c and 32a, b, c)** and a toxic effect on the heart **(Figure 36a, b, c, d).** This was observed through the loss and degeneration of endothelium, formation of foam cells in the different sections of the aorta, alteration of cardiac muscle and histopathologic liver changes **(Figure 40a, b, c, d)** such as vacuolization, necrosis, marked leukocytic infiltration, and widened sinusoidal spaces.

Our results are in agreement with the previous experimental study of (Zerizer and Naimi, 2004), (Boudebouz, 2013) and (Benmebarek, 2013) who reported structure alterations in the aorta, heart and liver caused by administration of high doses of L-methionine. Also the study of (Taravati *et al.*, 2013) demonstrate that the rabbits receiving experimental diet containing 1.2% methionine by weight in the diet for 3 months have a toxic effect on heart muscle cells, liver and kidney.

It seems that hypomethylation associated with hyperhomocysteinemia is responsible for lipid accumulation in tissues. Because Hcy metabolism is not restricted to the liver, one might assume that other tissues such as the endothelial system that are prone to accumulate lipids might be sensitive to hyperhomocysteinemia. In contrast, in the endothelial system where the role of betaine homocysteine methyltransferase in Hcy metabolism is probably negligible, hyperhomocysteinemia might enhance the accumulation of lipids (Obeid and Herrmenn, 2009).

In the group of mice administered with L-methionine and treated with olive oil showed a decrease at the level of homocysteine in the plasma. At our knowledge this result is an original work never done before.

The treatment with olive oil corrected the structural abnormalities observed in the studied organs and reduced the levels of total cholesterol, triglyceride, LDL-c and increased the level of HDL-c.

Our results confirmed the finding of EUROLIVE study (the effect of olive oil consumption on oxidative damage in European populations) (De La Cruz *et al.*, 2000) who provided that olive oil with different phenolic content were given to 200 individuals (25ml/day) from five Europeans countries in intervention periods of 3 weeks preceded by 2-week washout periods

increased high-density lipoprotein (HDL)-cholesterol and the ratio between reduced and oxidized forms of glutathione, and decreased triglycerides and DNA oxidative damage.

The study of (Roche *et al.*, 2000) demonstrated that the lower incidence of coronary heart disease in Mediterranean countries was associated with high consumption of olive oil.

In experimental studies, minor components of the unsaponifiable fraction of olive oil, such as α-tocopherol, β-sitosterol, and triterpenes, and phenolic compounds have been shown to have anti-inflammatory and anti-endothelial activation properties (Perona *et al.*, 2006).

Increased levels of LDL is a major risk factor for cardiovascular disease (Obeid and Herrmenn, 2009), as they facilitate transport of cholesterol to arteries (Huang and Sumpio, 2008). As mentioned above, oxidation of LDL to ox LDL, contributed to foam cell formation and thereby inhibited the egression of macrophages from plaques.

Conversely HDL-c are antiatherogenic. Unlike their larger counterpart, HDL primarily delivers cholesterol to the liver to be metabolized and excreted or reduced. It is also hypothesized that HDL are able to dislodge cholesterol molecules from atheromas in arterial walls (Huang and Sumpio, 2008).

We found in this study that the lipoprotein LDL-c is correlated positively and significantly with homocysteine in control group (r =0.937), M group (r =0.987), MO group (r =0.973) and OO group (r =0.913) **(Figure 22, 23, 24, 25)** also we found that lipoprotein HDL-c correlated positively and significantly with TG (r =0.906) in positive control group **(Figure 26)**.

These results suggested a probable relationship between hyperhomocysteinemia and hyperlipidemia. Our results are in agreement with the previous experimental study of (Lee *et al.*, 2002; Obeid and Herrmenn, 2009; Tselmin *et al*., 2013; Boudebouz, 2013). However, it's differed from studies of (Zerizer, 2006 and Zerizer *et al.*, 2008) who observed no relationship between hyperhomocysteinemia and lipids status.

The effect of Hcy on HDL-cholesterol is probably related to inhibiting enzymes or molecules participating in HDL-particle assembly (Obeid and Herrmenn, 2009), Also The study of (Tselmen *et al.*, 2013) suggests that the decrease in HDL is due to an inhibition synthesis of Apo AI, the major apolipoprotein of HDL by homocysteine.

Discussion

In healthy humans, the short-term consumption of olive oil decreased serum oxidative stress and their isolated lipoprotein fractions; LDL and HDL were shown to be enriched with oleic acid and resistant to oxidation (Nakbi *et al.*, 2010).

During our study we have shown that treatment with olive oil has beneficial effect in lipids status and glutathione reduced. This protection related to the decrease level of Hcy and therefore the suppression of their cytotoxic effects on different organs, these corrections related to the antioxidants and anti-inflammatory components of Algerian olive oil.

Ramirez-Tortosa *et al.* (1999) showed that oleic acid reduces oxidised LDL uptake by macrophages and decreases susceptibility of lipoproteins to undergo lipid peroxidation because LDL enriched in oleic acid is protected against oxidative damage

The protective mechanism of oleic acid-rich diets on inflammation has been attributed to a decrease in the LDL linoleic acid content. The low oxidability of oleic acid, and the scavenging capacity of olive oil minor compounds, could decrease the activation of pro-inflammatory transcription factors, such as NFkB, by reducing reactive oxygen spices and peroxyl radicals (Covas, 2007).

Visioli *et al.* (2001) demonstrate that hydroxytyrosol, administered to animals is able to increase their plasma antioxidant capacity, and exert other biological activities including inhibition of platelet aggregation and potentiation of the nitric oxide-mediated macrophagic immune response.

Ruano *et al*. (2005) reported that a meal containing high-phenolic virgin olive oil improved the endothelial-dependent vasodilatation during postprandial state more than when the meal was taken with a similar olive oil, but with low-phenolic content.

Mascitelli *et al*. (2010) suggest that the beneficial antioxidant action of olive oil is mediated by interactions of olive oil derived polyphenols and iron. A plausible mechanism for this interaction is related to a reduction of iron absorption induced by polyphenols.

Histological sections of mice treated with L-methionine and olive oil revealed a partial correction in the aorta, thus probably explained that the dose administered by this treatment is not sufficient to completely remove the side effects of HHcy.

Conclusion and Perspectives

Conclusion and perspectives

In this study we have explored the possibility of influencing hyperhomocysteinemia by increased level of homocysteine. This was done by administration of high dose of L-methionine (400mg/kg) during 21 days.

Following oral administration of L-methionine in high dose, its degradation product homocysteine caused angiotoxic activity on the abdominal and iliac aorta, and a toxic effect on the heart and liver in mice. This was observed through the loss and degeneration of endothelium, formation of foam cells, the alteration of the cardiac muscle, and hepatic tissue necrosis. These alterations were not seen in the organs of animal treated with Algerian olive oil (M and MO groups).

For a number of years, some researchers demonstrated that vascular disease of various forms is associated with abnormal methionine metabolism, leading to elevated plasma levels of Hcy (Huang *et al.*, 2008) and our results confirmed the assertion that elevated plasma Hcy could induce atherosclerosis.

A diet enriched with (7ml/60kg/days) of Algerian olive oil prevented the endothelial alteration, and the heart and liver damages in L-methionine treated mice.

Our results suggested that there is a probable relationship between HHcy and Hyperlipidemia (positive correlation between LDL-c and Hcy, increased level of LDL-c, TG, TC and decreased level of HDL-c in M group in contrast with the control goup)

This study demonstrated that olive oil has very novel beneficial effects on postprandial lipid metabolism and oxidative stress. Habitual dietary olive oil consumption was associated with endothelial activation, decreased serum TG and LDL-c, increased HDL-c and lowered the oxidative stress by increased level of glutathione reduced.

The beneficial effects of olive oil have been attributed to its content of many antioxidants and anti inflammatory namely vitamins such as α- and γ-tocopherols, β-carotene, phytosterols, squalene, pigments, terpenic acids, flavonoids such as luteolin and quercetin, and phenolics usually referred to as polyphenols.

Conclusion and perspectives

We have to evaluate many topics in the future work:

- We will determine the biochemical and molecular basis of Algerian olive oil
- We will measure other biochemical markers to understanding the relationship between HHcy and hyperlipidemia
- We will study the gene expression of antioxidant enzymes.
- We will develop endothelial cell culture in the aim to explore the relationship between homocysteine and endothelial dysfunction of molecular level.

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Appendix

Appendix

Preparation of PBS:

- NaCl ……...……. 8g
- KCl ……...….. 0,2g
- NaH_2PO_4 ……. 1,15g
- KH_2PO_4 ……..... 0,2g
- $MgCl_2$ ……..... 0,132g
- Dissolve these continents in sufficient quantity of distilled water……1L

Preparation of alcoholic Bouin

- Picric acid (1%) diluted in ethanol 95%.....……...45ml
- Formol ……..…… 26ml
- Acetic Acid…… ……. 7ml
- Distilled water ……. 22ml

Hematoxylin eosin coloration

Staining Procedure:

1- Dip the lame in the alcohol solution for 5 minutes.

2- Rinse with tap water.

3- Immerse sections in Hematoxylin for 4 minute.

2. Rinse with tap water.

3. Exchange tap water until the water is clear.

4. Immerse sections in EOSIN stain for 10 minutes.

5. Rinse with tap water.

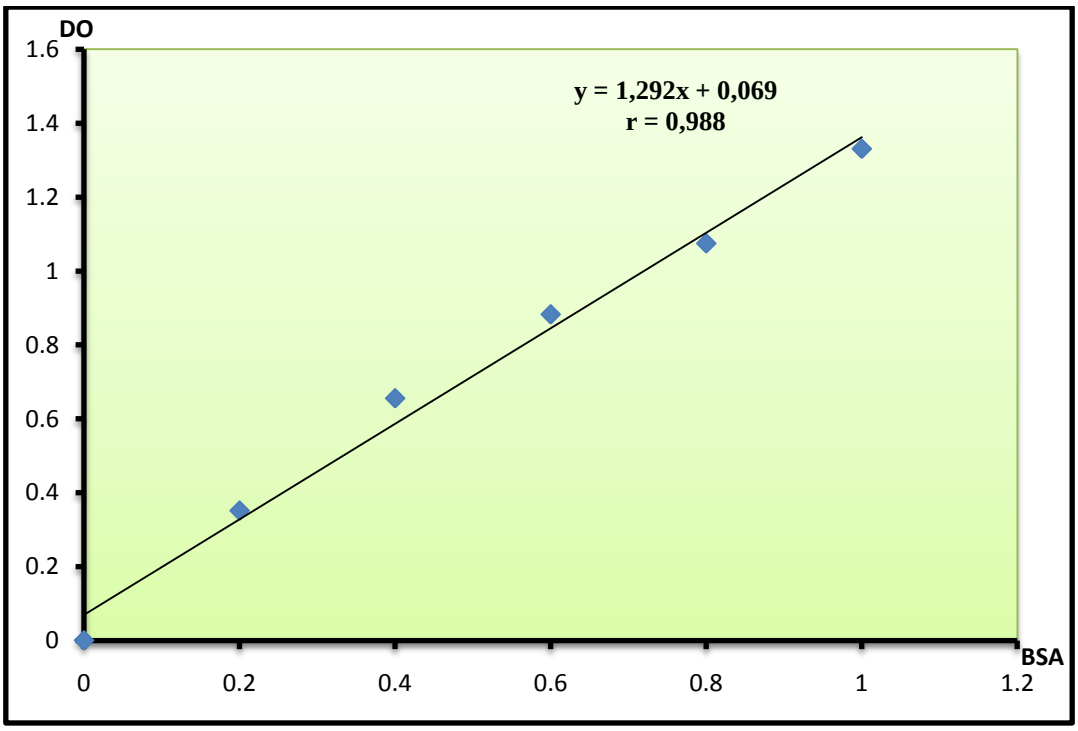
6. Exchange tap water until the water is clear.

7. Dehydrate in alcohol solutions for 1 min.

8. Clear with xylene.

Nuclei and other basophilic structures are blue. Cytoplasm and acidophilic structures are light to dark red.

Appendix



The curve of BSA

Appendix

Table N°1: shows the weight and food taken by mice in F group during treatment

days	Average weight (g)	Average per week (g)	diet consumed (g)	Average per week(g)
D_1	32,41		112,30	
D_2	31,55		92,30	
D_3	32,26		101,10	
D_4	31,94	**31.61±0.66**	98,60	**106.30±12.23**
D_5	31,27		121,40	
D_6	30,44		121,40	
D_7	31,44		95,15	
D_8	31,11		95,15	
D_9	31,52		113,10	
D_{10}	32,03		108,40	
D_{11}	33,26	**32.23±0.90**	106,00	**109.09±7.78**
D_{12}	33,4		110,00	
D_{13}	31,6		110,00	
D_{14}	32,75		121,00	
D_{15}	33,8		108,20	
D_{16}	33,98		108,50	
D_{17}	34,2		115,10	
D_{18}	33,9	**34.06±0.36**	113,00	**109.15±6.36**
D_{19}	33,68		95,90	
D_{20}	34,8		112,90	
D_{21}	34,06		110,50	

Appendix

Table N°2: shows the weight and food taken by mice in M group during treatment

Days	Average weight (g)	Average per week (g)	Diet consumed (g)	Average per week(g)
D_1	33,22		62,00	
D_2	33,12		53,30	
D_3	31,68		130,20	
D_4	32,59	**32.63±0.63**	108,30	**101.51±31.18**
D_5	33,28		108,30	
D_6	32,63		124,25	
D_7	31,92		124,25	
D_8	32,73		137,40	
D_9	32,5		141,40	
D_{10}	32,38		136,90	
D_{11}	32,4	**32.36±0.22**	127,20	**137.78±8.71**
D_{12}	32,47		127,20	
D_{13}	31,98		142,70	
D_{14}	32,45		151,70	
D_{15}	32,58		152,10	
D_{16}	33,45		113,50	
D_{17}	32,48		141,00	
D_{18}	33,13	**33.28±0.62**	145,55	**146.57±28.40**
D_{19}	34,11		145,55	
D_{20}	33,28		203,30	
D_{21}	33,98		125,00	

Appendix

Table N°3: shows the weight and food taken by mice in MO group during treatment

Days	Average weight (g)	Average per week (g)	Diet consumed (g)	Average per week(g)
D_1	30,31		87,2	
D_2	29,8		92,7	
D_3	29,61		112,4	
D_4	31,75	**30.87±0.96**	86,2	**113.24±28.81**
D_5	31,1		152,8	
D_6	31,62		152,8	
D_7	31,94		108,6	
D_8	31,1		72	
D_9	30,6		121,95	
D_{10}	30,1		121,95	
D_{11}	31,94	**31 .32±0.74**	107,4	**108.00±17.24**
D_{12}	31,74		114,4	
D_{13}	31,81		114,4	
D_{14}	32		103,9	
D_{15}	32,17		96,4	
D_{16}	31,4		83,9	
D_{17}	32,35		83,9	
D_{18}	32,21	**32 .00±0.34**	67,8	**80.62±11.65**
D_{19}	32		62,6	
D_{20}	32,21		81,8	
D_{21}	31,68			

Appendix

Table N°4: shows the weight and food taken by mice in OO group during treatment

Days	Average weight (g)	Average per week (g)	Diet consume (g)	Average per web(g)
D_1	32,43		49,5	
D_2	31,96		50,6	
D_3	31,54		50,6	
D_4	29,7	**31.32±1.05**	103,1	**82.95±31.34**
D_5	31,92		122,6	
D_6	29,98		102,15	
D_7	31,74		102,15	
D_8	31,68		95,2	
D_9	31,75		112,05	
D_{10}	30,88		112,05	
D_{11}	31,3	**31.39±0.46**	138,3	**111.64±13.21**
D_{12}	30,88		112,1	
D_{13}	31,15		105,9	
D_{14}	32,09		105,9	
D_{15}	31,7		121,3	
D_{16}	30,98		95	
D_{17}	31,24		92,4	
D_{18}	32,51	**32.08±0.76**	86,2	**97.25±12.06**
D_{19}	32,9		86,2	
D_{20}	32,65		97,8	
D_{21}	32,6		101,9	