Molecular study of Mitochondrial MT-TL1 Leucine gene and some Biochemical parameters in association with Coronary Heart Disease in patients attending Erbil cardiac center

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ABSTRACT

The present study was carried out between December 2013 and April 2014, in Hawler cardiac center. Sixty patients with coronary atherosclerotic patients were enrolled in our study (40 males and 20 females), their age ranged between 41-78 years, in addition, 30 healthy individuals (15 males and 15 females) were enrolled as control, their ages were ranged from 36-75 year. The statistical analysis showed significant difference at the level of 0.05 between coronary atherosclerotic patients and normal healthy control in terms of the mean of diabetic (for females 169.200 ± 16.510 and males 179.150 ± 13.252 mg/dl in coronary atherosclerotic patients, for females 97.733 ± 2.289 and males 94.200 ± 3.806 mg/dl in normal healthy groups respectively), triglycerides (for females 190.650 ± 12.117 and males 202.750 ± 12.105 mg/dl in coronary atherosclerotic patients, for females 108.866 ± 6.677 and males 144.733 ± 9.087 mg/dl in normal groups respectively), total cholesterol (for females 207.750 ± 10.685 and males 217.400 ± 9.571 mg/dl in coronary atherosclerotic patients, for females 107.600 ± 4.182 and males 170.466 ± 8.487 mg/dl in normal groups respectively), low density lipoprotein (for females 132.100 ± 6.307 and males 141.475 ± 5.809 mg/dl in coronary atherosclerotic patients group, for females 98.666 ± 4.744 and males 123.866 ± 1.166 mg/dl in health groups respectively) and high density lipoprotein (for females 37.750 ± 1.798 and males 34.575 ± 1.349 mg/dl in coronary atherosclerotic patients group, for females 44.260 ± 1.462 and males 47.066 ± 1.318 mg/dl in health groups respectively) (biochemical tests) (P<0.01). Percentage between coronary atherosclerotic patients and healthy control in smoking (life style) shown, most of coronary atherosclerotic patients (52%) were nonsmoker, while (48%) were smoker. Also, there was a percentage in patients with hypertension (62%) were had hypertension, while (38%) were had normality blood pressure. The results of MT-TL1 Leucine UUR gene sequencing were clearly showed no polymorphic mutation to be linked with regards to the disease status or development with those published in the NCBI database, indicating that this gene is not responsible and it is not counted as marker for this disease in our study.

1. INTRODUCTION

Cardiovascular diseases are leading causes of morbidity and mortality in many industrialized societies. Atherosclerosis is the major risk factor for the development of cardiovascular disease in general based on arterial endothelial dysfunction caused by the
impairment of endothelial-dependent dilation. Atherosclerosis is a disease associated with remodeling of the arterial intima, in part as a result of an initial protective inflammatory response following lipid uptake into the vessel wall and endothelial injury. Mutation of the mitochondrial genome remained out of focus for a long time, although they may play a pathogenic role in formation of atherosclerosis lesions of human arteries causing various defects in the protein chains of some respiratory protein complex and transfer RNA (tRNA), synthesized directly in the mitochondria. This lead to a decrease or blockage a number of proteins since their (tRNA) absent or total dysfunction, which contributes to the development of oxidative stress and increased the probability of occurrence and development of atherosclerosis (Chistiakove et al., 2012). Human mitochondrial tRNAs are highly susceptible to point mutations, which are the primary cause of the mitochondrial dysfunctions associated with a wide range of pathologies. During the last two decades, an increasing number of single nucleotide substitutions within the human mitochondrial transfer RNA (hmtRNA) genes have been linked to a variety of diseases showing pleiotropic effects. More than 220 mutations have been associated with diseases in the 22 genes of hmtRNAs and 40 mutations types in hmtRNAsLeu (Juhleng et al., 2009; Brandon et al., 2005). Mitochondrial transfer leucine (MT-TL1) gene coding for mitochondrial tRNAleu (UUR) [mttRNAleu (UUR)] was first identified as a genetic cause of mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes (MELAS) (Goto et al., 1990). It is one of the most common mitochondrial pathogenic mutations with a carrier frequency, like many mutations affecting mitochondrial respiratory chain (Ruiz-Pesini et al., 2007). Therefore this study aimed to study association between some risk factors like age, smoking, hypertension, gender and diabetes mellitus with coronary atherosclerotic disease, also to investigate contribution of a specific genetic causes of atherosclerosis at level of mitochondrial genome [MT-TL1 gene coding for mitochondrial tRNAleu (UUR)].

2. MATERIALS AND METHODS
2.1. Coronary atherosclerotic patient group
This group consisted of 60 patients (40 males and 20 females); their age ranged between (41-78) years, attending Hawler Cardiac Center Hospital. All patients were subjected to personal interview through especially designed questionnaires format. Coronary angiograms were performed for all patients at first day, blood samples were collected prior to coronary angiography. Patients with acute coronary syndrome were excluded in this study.

2.2. Healthy control (HC)
Thirty (15 males and 15 females) ; their age ranged between (36-75) years, apparently healthy individuals who have no history of clinical evidence of any acute or chronic disease, which angiography pictures are normal.

2.3. Sample collection
A venous blood sample was obtained from each individual of the study group through vein puncture. Three to Five ml of blood was collected using a disposable syringe and sterile non-coagulated test tube (containing EDTA). The blood samples were transferred on ice and kept freezing at Microbiology Laboratory of the Education college in Salahaddin University/ Hawler, for later use.

2.4. Determination of Serum Glucose, Total Cholesterol, Triglyceride (TG) and High-Density Lipoproteins (HDL)
The enzymatic colorimetric method was used for fasting serum glucose, total cholesterol, triglyceride (TG) and high-density lipoproteins (HDL) concentration determination using the Cobas c 311 Diagnostic kit by Cobas c 311 fully automated biochemistry analyzer, Germany.

2.4.1. Procedure

One ml of the serum was added to flexor tube and determination of fasting serum glucose, total cholesterol, triglyceride (TG) and high-density lipoproteins (HDL) concentration is analyzed by using automatic chemical analyzer (BIOLABO biochemical analyzer) use only (2) µL of serum for each parameter.

2.5. Determination of Low Density Lipoproteins (LDL)

The lipid profile does not measure LDL level directly but instead estimates it via the Fried Ewald equation:

\[ \text{LDL Cholesterol (mg/dL)} = (\text{Total Cholesterol} - \text{HDL Cholesterol} - \text{VLDL}) \]

2.6. Statistical analysis

The collected data were analyzed by using computer program software SPSS (Statistical Package for Social Sciences); version 19.0. Descriptive statistics which include: 1. Mean and 2. t-test used for statistically significant difference between two groups means was tested.

2.7. Genomic DNA extraction protocol (Geneaid)

Three hundred µl of blood transferred to a 1.5 ml microcentrifuge tube. Nine hundred µl of RBC lysis buffer were added to micro centrifuge contained blood then mixed by multiple inversions. Do not vortex. Incubated the tube for 10 minutes at room temperature. Centrifuged for 5 minutes at 3000 rpm then removed the supernatant completely. One hundred µl of RBC lysis buffer were added to re-suspend the leukocyte pellet. Two hundred µl of GB buffer were added to the 1.5 ml micro centrifuge tube then shake vigorously. Incubated at 60°C for last 10 minutes to ensure the sample lysate was cleared. During incubation, inverted the tube every 3 minutes. At this time, pre-heat the required elution buffer (200 µl per sample) to 60°C (for step DNA elution). Two hundred µl of W1 buffer were added to GD column then Centrifuged for at 14-16000 rpm for 30-60 seconds. Discarded the flow-through then placed the GD column backed in the 2 ml collection tube. Six hundred µl of wash buffer (ethanol added) were added to GD column. Centrifuged for at 14-16000 rpm for 30-60 seconds then discarded the flow-through. Placed the DG column backed in 2 ml collection tube. Centrifuged again for 3 minutes at 14-16000 rpm to dry the column matrix. Transferred the dried GD column to a clean 1.5 ml micro centrifuge tube. One hundred µl of pre-heated elution buffer were added or ET to the center of the column. Stand for 3 minutes ensuring elution buffer/TE was absorbed. Centrifuged for at 14-16000 rpm for 30-60 seconds to eluted the purified DNA.

2.8. Polymerase Chain Reaction (PCR)

In this study the mtDNA MT-TL1 gene of mitochondrial human genome (75) bps was amplified by specific primer MT-TL1Fa

5’ AGGACAAGAGAAAATAAGGCC 3’ ,

MT-TL1Rb

5’ ACGTTGGGGCCTTTGCGTAG3’ (Sobenin 2012a). The PCR master mix contained all the components needed for the PCR, i.e. 2X GoTaq Green. Taq Green is a premixed containing Taq DNA polymerase; 400 mM Deoxynucleosid Triphosphates (dNTPs)), 3 mM MgCl2, reaction buffers (pH8.5) and two dyes (blue and yellow) which functions as loading dyes, at optimal concentrations for efficient amplification of
DNA templates (human genome). PCR was performed in a 50µl of reaction volume. Master Mix 45 µl, forward Primer 1.5 µl, reverse Primer 1.5 µl, and template DNA 2 µl.

2.8.1. The amplification programs

The PCR cycle parameters were as follows; one cycle of initial denaturation at 95°C for 2 min; then 35 cycles of denaturation at 95°C for 30 sec, annealing at 72°C for 1 minute and extension at 72°C for 1 min; and followed by one cycle of final extension at 72°C for 2 min (Lim et al., 2008). Then amplified DNA was running by 1.5% agarose gel electrophoresis to be sure that the PCR amplicon were correct (Reineke et al., 1998).

PCR products were analyzed by agarose gel electrophoresis in 1X TBE buffer at 100 V for 120 min in gel composed of 1.5% agarose. 100bp ladder DNA Marker was run with PCR products for sizing of the bands. Gels were stained with ethidium bromide solution (concentration of 0.5 µg/ml) for 30 min, then visualized with a UV transilluminator and photographed (Sambrook and Russell, 2001).

2.8.2. PCR product purification (PCR clean up method)

PCR- amplified samples were purified with the Geneaid Gel/PCR DNA Fragments Extraction Kit protocol (Geneaid/ Taiwan) as follow: Twenty-five 1µl of reaction product (PCR product) transferred up to a 1.5 microcentrifuge tube. One hundred twenty-five µl of DF buffer were added to 1.5 microcentrifuge tube and mixed by vortex. Placed a DF column in a 2-ml collection tube. The sample mixture (PCR and DF buffer) were transferred to the DF column and centrifuged at 14-16000 rpm for 30 seconds. The flow-through discarded and placed the DF column back in 2 ml collection tube. Centrifuged again for 3 minutes at 14-16000 rpm to dry the column matrix. The dried DF column transferred to a new 1.5 ml microcentrifuge tube. Thirty µl of elution buffer or TE was added into the center of matrix. Stand for 2 minutes or until the elution buffer or TE was completely absorbed by the matrix. Centrifuged again for 2 minutes at 14-16000 rpm to elute the purified DNA.

2.8.3. Quality checking of PCR production after PCR purification

Five µl of DNA samples gently loaded to the respective lanes in the gel with (1.5%) agarose gel. A first lane was left for loading five µl of 100 bps molecular weight DNA Ladder in to the first lane of the gel, and then running and staining (Sambrook and Russell, 2001). The PCR product band of the expected size 294bp) were considered as positive sample.

2.8.4. Determination of DNA Concentration and Purity

Nano Drop 1000 UV-Vis spectrophotometer (Nano Drop, USA) used for determinate genomic DNA concentration and purity.

2.9. DNA Sequencing and Nucleotide Alignment

All Sequencings of the MT-TL1 gene coding for tRNA leucine (UUR) of mitochondrial human genome for the studied mutations were performed by the Macrogen company (Korea). Sequence alignments were performed with Clustal W (using default settings) in MEGA 4.0.2 software (Tamura et al., 2007, Kumar et al., 2008).

3. RESULTS AND DISCUSSION
3.1.1. The age distribution in patients group and normal healthy group

Sixty patients with coronary atherosclerotic patients were selected randomly which included 40 males and 20 females. Their ages were ranged from intervals 41-78 years. Most coronary atherosclerotic patients 37% were in the age range 57-64 years, while lowest percentage 8% of them were in age range 41-48 years. For normal group thirty clinically normal healthy individuals were selected randomly 15 males and 15 females. Their age was ranged from 36-75 year. Highest percentage of normal healthy group 37% their age were in age interval 65-74 years, while lowest percentage 10% of them were in age range 41-48 years. Regarding the age, it has been found that the average age of patients in current study seems to be similar with those participated in other studies such as Jousilhati et al., (1996) due to most frequently complications of this age group. Our study shown that the age between 57-64 years has highest percentage 37% with coronary atherosclerotic patients between 54-65 years. The results of our study are consistent with previous study done by Brunk (2002) who state that Age-specific mortality rates from coronary atherosclerotic patients increase exponentially with age in people older than 65 years. Ageing is associated with a plethora of pathological changes leading to a progressive decline in cellular function and manifestation of a number of degenerative diseases. The mitochondrial–lysosomal axis theory of ageing implicates imperfect autophagocytosis leading to accumulation of damaged mitochondria and generation of ROS. Recent studies suggest that increased generation of ROS contributes to accelerated senescence of endothelial cells. Endothelial cell senescence may impair the regenerative capacity of the endothelium, its reactivity and promote the progression of atherosclerosis overexpression of several proteins that typify the proinflammatory/pro-thrombotic phenotype of the endothelium (Erusalimsky, 2009).

3.1.2. Biochemical test (Serum glucose concentration)

Table (1) shows the mean of serum glucose concentration in coronary atherosclerotic patients and health groups. The results obtained revealed that the mean serum glucose levels were for females 169.200 ± 16.510 and males 179.150 ± 13.252 mg/dl in coronary atherosclerotic patients, for females 97.733 ± 2.289 and males 94.200 ± 3.806mg/dl in normal healthy groups respectively. The values obtained in coronary atherosclerotic patients group significantly elevated level compared to the normal healthy group.

| Sex     | Group                        | Glucose mg/dl Mean ± SE | Normal range |
|---------|------------------------------|-------------------------|--------------|
| Female  | Healthy control              | 97.733 ± 2.289          | 80-110 mg/dl |
| Female  | Coronary atherosclerotic      | 169.200 ± 16.510        |              |
| Male    | Coronary atherosclerotic      | 180.200 ± 13.252        |              |
|         | patient                      |                         |              |
|         | Healthy control              | 94.200 ± 3.806          |              |

These data cleanly implies that glucose level is elevated in coronary atherosclerotic patients. This is an indication that diabetes mellitus (both types) in the major risk factor for the atherosclerosis coronary heart disease, the same results were observed in earlier studies(Shishehbor and Bhatt, 2004). Diabetes acts through inflammatory and non-inflammatory pathways through non enzymatic glycation of macromolecules, advance
glycation end products (AGE) bind with their receptor. These AGE associated modifications can lead to increased expression and production of inflammatory mediators and proinflammatory cytokines (Shishehbor and Bhatt, 2004). Diabetes is a significant risk factor for atherosclerosis; the diabetic state promotes oxidative stress mediated by ROS, these consume NO and lead to endothelial dysfunction (Shishehbor and Bhatt, 2004).

3.1.3. Biochemical test (Serum triglyceride concentration)

Table (2) shows the mean of fasting serum triglyceride concentration in coronary atherosclerotic patients and normal healthy groups. The results obtained revealed that the mean serum Triglyceride levels were for females 190.650 ± 12.117 and males 202.750 ± 12.105 mg/dl in coronary atherosclerotic patients, for females 108.866 ± 6.677 and males 144.733 ± 9.087 mg/dl in normal groups respectively. The values obtained in coronary atherosclerotic patients significantly exceeded values of normal healthy group. This result data indicate that TG level is elevated in coronary atherosclerotic patients. Therefore, it is counts as a risk factor for the disease development. This finding is in a concordance to the record data presented in preview publication (Botham et al., 2007). Several species of triglyceride-rich lipoproteins (TRLs) including VLDL and VLDL remnants, as well as CM (metabolized chylomicron) remnants appear to promote atherogenesis independently of LDL.

Remnant species result from partial hydrolysis by lipoprotein lipase (LPL) of TRLs of hepatic and intestinal origin that have picked up cholesterol esters from HDL through the action of cholesterol ester transfer protein (Brewer, 1999). Similar to oxidized LDL, these cholesterol enriched, TG-poor species are subject to endothelial accumulation and uptake by macrophages to form foam cells (Botham et al., 2007). Foam cells promote fatty streak formation, the precursor of atherosclerotic plaque.

3.1.4. Biochemical test (Serum cholesterol concentration)

Table (3) shows the mean of serum cholesterol concentration in coronary atherosclerotic patients and normal healthy group. The results obtained revealed that the mean serum Cholesterol levels were for females 207.750 ± 10.685 and males 217.400 ± 9.571 mg/dl in coronary atherosclerotic patients, for females 107.600 ± 4.182 and males 170.466 ± 8.487 mg/dl in normal groups respectively. The values obtained in coronary atherosclerotic patients significantly increased as compared to normal healthy group.

Our result shows that cholesterol is another risk factor for coronary atherosclerotic patients, elevation almost doubled in females and elevated by 35% in males (Table 3). Previous studies have shown cholesterol contributing a risk factor for coronary atherosclerotic patients. Our data confirm data finding in our society.
Table 3: Mean± S.E. of serum cholesterol in coronary atherosclerotic patients disease and health group which done by T-test at level P< 0.01= (highly significant)

| Sex      | Group                          | Cholesterol mg/dl Mean ± SE | Normal range |
|----------|--------------------------------|-----------------------------|--------------|
| Healthy control |                                |                             |              |
| Female   | Coronary atherosclerotic patient| 207.750 ± 10.685            | Up to 200    |
|          | Healthy control                 | 170.466 ± 8.487             | mg/dl        |
| Male     | Coronary atherosclerotic patient| 217.400 ± 9.571             |              |

3.1.5. Biochemical test Low Density lipoprotein (Serum LDL concentration)

Table (4) shows the mean of fasting serum LDL concentration in coronary atherosclerotic patients group and normal health groups. The results obtained revealed that the mean serum LDL levels were for females 132.100 ± 6.307 and males 141.475 ± 5.809 mg/dl in coronary atherosclerotic patients group, for females 98.666 ± 4.744 and males 123.866 ± 1.166 mg/dl in health groups respectively. The values obtained in coronary atherosclerotic patients group significantly exceeded values of normal health group.

Table 4- Mean± S.E. of serum low density lipoprotein in coronary atherosclerotic patients disease and health group which done by T-test at level P< 0.01= (highly significant)

| Sex      | Group                          | LDL mg/dl Mean ± SE | Normal range |
|----------|--------------------------------|---------------------|--------------|
| Healthy control |                                | 98.666 ± 4.744      |              |
| Female   | Coronary atherosclerotic patient| 132.100 ± 6.307      | Up to 130    |
|          | Healthy control                 | 123.866 ± 1.166      | mg/dl        |
| Male     | Coronary atherosclerotic patient| 141.475 ± 5.809      |              |

3.1.6. Biochemical test High Density lipoprotein (Serum HDL concentration)

Table (5) shows the mean of fasting serum HDL concentration in coronary atherosclerotic patients group and normal health group. The results obtained revealed that the mean serum HDL levels were for females 37.750 ± 1.798 and males 34.575 ± 1.349 mg/dl in coronary atherosclerotic patients group, for females 44.260 ± 1.462 and males 47.066 ± 1.318mg/dl in health groups respectively. The values obtained in coronary atherosclerotic patients group significantly exceeded values of health group. The data obtained in our study elevated of cholesterol and LDL and decreased of HDL in disease group shows that in general that cholesterol essential factor for development and progression of the atherosclerosis coronary heart, our result similar to the previous study which Shown that cholesterols contributed and motivated to development of coronary atherosclerotic patients.

Table (5) Mean± S.E. of serum high density lipoprotein in coronary atherosclerotic patient’s disease and health group which done by T-test at level P< 0.01= (highly significant)

| Sex      | Group                          | HDL mg/dl Mean ± SE | Normal range |
|----------|--------------------------------|---------------------|--------------|
| Healthy control |                                | 44.260 ± 1.462      |              |
| Female   | Coronary atherosclerotic patient| 37.750 ± 1.798      | 40-59        |
|          | Healthy control                 | 47.066 ± 1.318      | mg/dl        |
| Male     | Coronary atherosclerotic patient| 34.575 ± 1.349      |              |

High LDL levels can decrease NO available in the vessel by reducing the amount and activation of the enzyme that is involved in the production of NO, or enhancing NO breakdown. This decrease in NO is the primary cause of endothelial dysfunction, which is considered one of the initiating events in
atherosclerosis, and precedes the entry of LDL within the intima (Badimon and Vilahur 2012). More recent studies (Goldbourt et al., 1997) confirmed this relationship, and found that patients with HDL <35 mg/dL had an incidence of cardiovascular events eight times higher than individuals with HDL >65 mg/dL. Similar to our study that the mean of HDL concentration was decreased in patients with coronary atherosclerosis. Elevated levels of circulating OxLDL are strongly associated with documented CAD in the general population (Tsimikas et al., 2005). Normal HDL clears OxLDL from the endothelium: abnormal pro-inflammatory HDL associate with accelerated atherosclerosis. There are many mechanisms designed to clear OxLDL from the sub endothelial space, including macrophage engulfment using scavenger receptors, and enhanced reverse cholesterol transport mediated by lipoprotein transporters in HDL (Remaley et al., 2003). In addition to reverse cholesterol transport, HDL removes ROS from LDL (via anti-oxidant enzymes in the HDL, such as paroxonase), thus preventing the formation of OxLDL and the subsequent recruitment of inflammatory mediators (Navab et al., 2000). Elevated and modified LDL, TG, cholesterol and decreased HDL level caused by cigarette smoking, hypertension and diabetes, in addition a chronic and systemic pro-inflammatory stimulus can be due to the increased production of cytokines in adipose tissue (Mertens and Holvoet, 2001). In our study the mean concentration of LDL, TG and cholesterol, were elevated and HDL was decreased in patients with coronary atherosclerosis, these results consistent with previous study (Mertens and Holvoet, 2001).

3.1.7. Life style (patient smoking)

Sixty patients with coronary atherosclerotic patients were selected randomly. The smoker distribution in coronary atherosclerotic patients group. Most of coronary atherosclerotic patients 52% were no smoker, while 48% were smoker. Data obtained from our results indicate that most patients were non-smoker, which disagrees with similar study that has reported increase in cardiovascular events among smoker males (De et al., 2003). Explanation of our obtained results can be based upon the fact that not only smoking but also several other risk factors may be involved in cardiovascular diseases including hypertension, diabetes and age. The smokers as risk factor are not only the dominant factor, most probably other parameters are contributing.

3.1.8. Blood pressure

Sixty patients with atherosclerosis coronary heart disease were selected randomly. The blood pressure distribution in coronary atherosclerotic patients group shown in Figure (1). Most of coronary atherosclerotic patients 62% were had hypertension (high blood pressure), while 38% were had health blood pressure.

![blood pressure distribution](image)

**Figure 1: coronary atherosclerotic patients distribution according to blood pressure**

Our study has shown that 62% were having hypertension (high blood pressure) with which agrees with other studies consistence with previous studies our study has shown that the majority of patients were hypertensive with the mean of their SBP ≥ 140 and DBP ≥ 90. It seems likely that hypertension will remain the most common risk factor for atherosclerosis and heart attack (Wang and Vasan, 2005).
People with high blood pressure are more likely to develop coronary artery disease, because high blood pressure puts added force against the artery walls. Over time, this extra pressure can damage the arteries, making them more vulnerable to the narrowing and plaque buildup associated with atherosclerosis. The narrowed artery limits or blocks the flow of blood to the heart muscle, depriving the heart of oxygen (Zureik et al., 1999)

3.2 Molecular studies

In our study, we selected \textit{MT-TLI} gene coding for mitochondrial \textit{tRNALeucine} (UUR) [\textit{mt-tRNALeu} (UUR)] that the function of \textit{MT-TLI} is a 75 nucleotide RNA (position 3230-3304 in the mitochondrial map) that transfers the amino acid leucine to a growing polypeptide chain at the ribosome site of protein synthesis during translation. According to previous study done by Sobenin et al., (2012b), who reported that the level of C3256T mutation (heteroplasmy) at the \textit{MT-TLI} gene in mitochondrial genome, in human white blood cells, is a biomarker of mitochondrial dysfunction and strong association for progression of coronary atherosclerosis. Genomic DNA was isolated for the 90 human blood samples with Genomic DNA Mini Kit for Blood/Cultured Cell (Geneaid - Taiwan). The purity was also found to good ranging between (1.7-1.8) determined by spectrophotometer ratio A260/A280. The amplified product of the \textit{MT-TLI} gene segment migrates with third band of DNA marker (~300 bps). The exact size of the PCR product is 294 bps and it shows less than 300 bps in the agarose gel as shown Figure (2).

Figure 2: Agarose gel electrophoresis (ethidium bromide stained) for some purified PCR \textit{MT-TLI}Leucine product using DNA purification kit. L: 100 bps DNA Marker.

3.2.1. Sequence Analysis of the \textit{MT-TLI} gene PCRs

All samples of the PCR products, the \textit{MT-TLI} gene segment, were sent to the Macrogen company/Korea, for DNA sequencing. The raw data files were obtained and utilized for bioinformatics sequence alignment to identify mutation positions. The complete sequences of the nucleotide of the gene are validated after visual inspection of the strength nucleotide-peak signals of each sample DNA sequence files and it is presented in Figure (3). The sequences obtained were further analyzed using the BLAST tool of bioinformatics and alignment appear at http://www.ncbi.nlm.nih.gov homepage. The nucleotide sequences of each gene showed high 99%-100% identity to the reports Homo sapiens isolate M25 mitochondrion (Figure 2). The nucleotide sequence resulting from all the coronary atherosclerosis patient samples shared 100% identity with the previously corresponding published sequences of the human (accession numbers KM102156.1).
The BLAST search tool for nucleotide confirmed that these sequences are corresponding to exact position of human mitochondrial genome. Additional analyses of multiple DNA sequence alignments of all our clinical isolates were done by Clustal W (using default settings) in MEGA 4.0.2 software and Genius software version 6.1.6. Again, the alignment data revealed high nucleotide sequence identity as BLAST search tool results. Indeed, the results confirmed the identity of the MT-TL1 gene isolated from the patients’ samples with that formerly deposited in the NCBI nucleotide database based on the nucleotide BLAST search. Furthermore, the data showed conservation of the gene at least between our patient and healthy control isolates obtained locally.

3.2.2. mtDNA mutations in coronary atherosclerosis

Our result of DNA sequencing (Figure 3) shown that have no mutations were located in MT-TL1 gene coding for mitochondrial tRNALeucine (UUR) [mttRNALeu (UUR)] isolated from 60 coronary atherosclerotic patients. Our findings seem to be similar with two other studies (Botto et al., 2005), who reported absence of heteroplasmic mutations of mtDNA in the MT-TL1 gene. They demonstrated that the common mtDNA mutation del4977 resulted in a deletion of a nearly 5-kb mtDNA region between nucleotides 8470 and 13459. Although the 5-kb segment contains five coded regions for tRNA and seven genes coded for respiratory-chain subunits, but no significant difference in heteroplasmia levels was found between healthy controls and patients with coronary atherosclerosis. Despite the importance of the mutation C3256T of MT-TL1 gene found in Russia (Sozanova et al., 2009), Our study shows entirely wild-type sequence on the MT-TL1 gene of coronary atherosclerosis patients and healthy controls, as compared to NCBI-sequences. Furthermore, our study is significantly with high-confidence (100%) found that the mutation does not exist in our locality, while it is a polymorphic association in Moscow-Russia (Sobenin et al., 2013a). They assessed association between the level of heteroplasmia of the mtDNA mutation C3256T in human white blood cells, extent of carotid atherosclerosis, and presence of coronary heart disease (CHD), the major clinical manifestation of atherosclerosis. The highly significant relationship between the C3256T heteroplasmy level and susceptibility to atherosclerosis was observed (Sobenin et al., 2013b). The level of C3256T heteroplasmia of mitochondrial genome in human white blood cells was considered to be a polymorphic marker of mitochondrial dysfunction and risk factor for atherosclerosis (Sobenin et al., 2013b). Therefore, it was suggested as an informative marker of genetic susceptibility to atherosclerosis, coronary atherosclerotic, and myocardial infarction (Sobenin et al., 2013c).

In a study performed by Mueller et al., (2011) in 482 in patients with coronary heart disease (CHD), 505 diabetic patients, and 1481 healthy individuals it has been demonstrated that only of the prevalence of T16189C mutation in mitochondrial genome was significantly higher in patients with CHD, as well as in diabetics. Similar with this study our
study showed no found heteroplasy levels of mutations in MT-TL1 gene of coronary artery disease and diabetic patients. On the other hand Qin et al., (2014) performed a clinical and genetic evaluation and mutational screening of 22 mitochondrial tRNA genes in a cohort of 80 genetically unrelated Han Chinese subjects and 125 members of 4 families with coronary heart disease and 512 Chinese control subjects. This analysis identified 16 nucleotide changes among 9 tRNA genes. Of these, the T5592C mutation creates a highly conservative base pairing (5G-68C) on the acceptor stem of tRNA\text{Gln}, whereas the G15927A mutation destabilizes a highly conserved base pairing (28C-42G) in the anticodon stem of tRNA\text{Thr}. However, the other tRNA variants were polymorphisms. The pedigrees of BJH24 carrying the T5592C mutation, BJH15, and BJH45 harboring the G15927A mutation exhibited maternal transmission of coronary heart disease. Sequence analysis of their mitochondrial genomes revealed the presence of T5592C or G15927A mutation but the absence of other functionally significant mutations in all matrilineal relatives of these families. Theses result coincide with our study in the genome of mitochondria in which several mutations in another locations than mutation in MT-TL1 gene may responsible for atherosclerosis. In conclusion the Mitochondrial MT-TL1-leucine gene is not a genetic marker for coronary heart disease in our locality (Kurdistan region), since we do not carry polymorphic signature in this location at all indicating that the location polymorphisms are possibly spread over various regions in the mitochondrial DNA.

CONCLUSIONS

The present work was designed to determine the histological effect of fluoxetine on liver, kidney and cerebrum of male rats. The light microscopic examination showed that orally administration of fluoxetine for a period of one month had a bantam histological effect of liver, kidney and cerebrum of male rats.

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