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From the Editor

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This is the second issue this year with a number of topics from the region.

The biological markers study of acute and chronic myloid leukemia were studied using twenty two acute myelgeneous leukemia (AML) and 20 chronic myelgeneous leukemia (CML) patients , who were admitted to Medical City Hospital, in addition to 35 healthy individual as control group. Vitamin C and vitamin D ,trace and essential elements cobalt, and Nickel magnesium were evaluated in the sera of all above individuals, using high performance liquid chromatography and atomic absorption spectrophotometry for evaluating vitamins and elements. The results revealed a significant decrease in vitamin C, vitamin D and Mg levels in both patients groups compared to control and a significant difference in CO and Ni levels in sera of AML and CML were found. The authors concluded that vitamins, trace and essential elements abnormalities occur in both patients groups compared to Control.

Another study compared levels of specific divalent trace elements in hyperlipidemia and hepatitis sera patients. Sera of both hepatitis (40) and hyperlipidemia (40) were collected from the central lab of Sulaimaniyah-Kurdistan region/ Iraq. Age, sex, duration of infection, medication used and lipid profile using computerized database program, as characteristics information of patients were done. The levels of Cr, Cu, Zn, Mn, V and Se were determined in sera of both patients and compared with healthy controlled (n=30), using Inductively Coupled Plasma-Optical Emission Spectrometry (ICP-OES). Hepatitis samples yielded concentration in ppb of (7.34±0.01) Cr, (171.7±2.51) Cu, (138±3.41) Zn, (1.309±0.99) Mn, (184.7±6.12) V and (218.9±3.14) Se respectively, while hyperlipidemia samples yielded concentration (ppb) of (5.22±0.45) Cr, (197.01±2.34) Cu, (160.3±4.01) Zn, (0.068±1.91) Mn, (247.01±0.65) V and (185.5±3.11) Se respectively. Controlled samples reflects the following concentrations (ppm), (5.324±1.34) Cr, (131.2±4.34) Cu, (216.6±0.83) Zn, (0.024±1.75) Mn, (235.3±0.18) V and (223.2±3.15) Se respectively.

A prospective longitudinal randomized controlled study was designed in rat tibia to test the effects of various types of bisphosphonates on fracture healing. The Bisphosphonates are unique class of drugs that inhibit bone resorption, however recent studies also suggest their stimulatory effect on osteoblast formation.. 48 skeletally mature female Wistar rats with a mean weight of 340 (316-351) g were used. Rats were allocated into four syudy groups, 12 animals in each group. Right tibial diaphysis was then fractured and fracture was stabilized with long leg cast. No other treatment was given to the control group, other groups received; risedronate 0.2 mg/kg/day, raloxifene 1.0 mg/kg/day, alendronate 0.2 mg/kg/day separately. Animals were sacrified week four of experiment. Fractured tibia was evaluated radiologically and mechanically and histologically. Radiographic study showed that bony callus was present in all the fractures and bisphosphonates led not to a larger callus as in the other studies. Mechanical testing revealed that ultimate load of alendronete group was significantly higher than other groups. The result suggest that systemic alendronat treatment induces stronger callus formation in rats.

A paper from KSA investigated the role of beneficial effect of L-carnitine in healthy and streptozotocin (STZ) N?-nitro-L-arginine methyl ester (L-NAME) induced diabetes mellitus and hypertension in rats. The authors stressed that Diabetes mellitus and hypertension are a global health problem due to its serious complications, along with oxidative stress were shown to contribute to endothelial dysfunction. Results showed that diabetic hypertensive (DH) rats had significant increase in the level of plasma glucose, malondialdhyde (MAD), cholesterol (CH), triglycerides (TG), urea, creatinine, and the activity of serum liver enzymes (AST, ALT) as to normal control rats. While, blood glutathione (GSH) content and erythrocyte superoxide dismutase (SOD) activity and nitric oxide level (NO), were significantly lowered. Supplementation of L- carnitine for 6 weeks improve plasma glucose, lipids, liver and kidney functions, In addition, both normal healthy rats and DH rats treated with L- carnitine showed increase in blood GSH and SOD activity and serum nitrate level (stable product of nitric oxide NO) as compared with healthy control and DH respectively. Histopathological and immunochemical study of heart confirm the biochemical results. The authors concluded that it was concluded that administration of L-carnitine reduces or delays oxidative stress in diabetic hypertensive rats.

Biological markers study of acute and chronic myeloid leukemia

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ABSTRACT

Twenty two acute myelogenous leukemia (AML) patients and 20 chronic myelogenous leukemia (CML) patients, were admitted to Medical City Hospital, in addition to 35 healthy individuals as the control group. Vitamin C and vitamin D, trace and essential elements cobalt, and Nickel magnesium were evaluated in the sera of all above individuals, using high performance liquid chromatography and atomic absorption spectrophotometry for evaluating vitamins and elements. The results revealed a a significant decrease in vitamin C and vitamin D and Mg levels in both patients groups compared to controls and a significant difference in CO and Ni levels in sera of AML and CML was found.

Conclusion: Vitamins, trace and essential elements abnormalities occur in both patient groups compared to Controls.

Key words : Acute, Chronic leukemia, vitamins C, D, cobalt, Nickel, Magnesium

Introduction

Leukemia is a type of cancer of the blood or bone marrow characterized by an abnormal increase of immature white blood cells called “blasts. It is classified into several types. Acute and chronic could be considered as the essential type and includes many types under each term such as lymphocytic, granulocytic and monocytic leukemia((1). Acute leukemia, is one of the two major categories of leukemia, with symptoms including anemia, fatigue, weight loss, easy bruising, thrombocytopenia, and granulocytopenia ,while chronic leukemia is characterized by the excessive build up of relatively mature, but still abnormal, white blood cells (2). The maintenance of optimal health proteins, lipids, macronutrients, micronutrients, vitamins and trace elements.(3) Vitamins are an organic compound required by an organism as a vital nutrient. In limited amounts ,vitamins serve multiple roles and they work together to perform many functions, from reducing infection to aiding metabolism. (4) Vitamin C (Ascorbic Acid) is an important antioxidant found in the biological system. It is the first line antioxidant defense in plasma.(5) Proposed mechanisms of vitamin C activity in the prevention and treatment of cancer include : enhancement of the immune system by increased lymphocyte production, stimulation of collagen formation necessary for “walling off” tumors , inhibition of hyaluronidase, keeping the ground substance around the tumor intact and preventing metastasis, inhibition of oncogenic viruses, correction of an ascorbic deficiency, and is often seen in cancer patients.(6,7,8)

Cholecalciferol is a prohormone that is synthesized in the skin by photochemical conversion of 7-dehydrocholesterol. It is subsequently hydroxylated to 25-hydroxycholecalciferol [25(OH)D3] in the liver and in the kidney finally to the active metabolite, 1,25 didydroxcholecalciferol [1,25(OH)2D3. (9 ,10)

Trace elements (TE) are a group of elements that are present in the human body in very small amounts (milligrams per day), but are nonetheless important to good health .(11)Their action is that (TE) are constituents of, or interact with enzymes, cofactors , phosthetic groups or hormones that regulate the metabolism of much larger amounts of biochemical substrates(12).

Cobalt is an essential element for humana, as a part of vitamin B12 (coblamin) - micro flora of the lumen intestine.1(13,14,15,)

Nickel is one of the ultra trace elements; Nickel ions used in the iron transport system across small intestine related with ferritin, thereby cause anemia;the deficiency of nickel causes alteration of folate and vitamin C .(16)

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Magnesium (Mg) is essential to the basic nucleic acid chemistry of life and thus is essential to all known living organisms. Mg is important for maintaining of DNA. Magnesium is extremely necessary for proper ATP synthesis(17,18,19).

Aim of Study

The aim of the study is to evaluate vitamin C, vitamin D and trace and essential elements of Co, Ni, Mg, levels in the sera of patients with acute and chronic leukemia compared to controls.

- Experimental Part
- Subjects:

Blood samples were collected from the patients with acute myelogenous leukemia (AML) and patients with chronic myeloblastic leukemia (CML) from men, who were admitted to Medical City Hospital. Their age range was 32-52 years. The patients were diagnosed by specialist doctors. Thirty five healthy individuals were matched aged 30-50.

Instrumentation : Vitamin C was measured using high performance liquid chromatography (Shimadzu Japan C18 column) . Vitamin D was separated on FLC (Fast liquid chromatograph column) 3µm particle size stored column, obtaining optimum conditions, temperature, flow rate, eluent composition were used for detection of vitamins . The concentration of each vitamin was estimated through measurement of area under the curve of chromatograms of standard vitamins and for patients and control serum samples.

$C_{standard}$ = Standard concentration of the measured vitamin in ug/ml.

$$\text{Vitamin concentration in } \upsilon g/ml = \frac{A_{sample}}{A_{standard}} \times C_{standard}$$

A_{sample} = Area under the curve of sample.

$A_{standard}$ = Area under the curve of standard

Atomic absorption spectrophotometry (model AA46-schma-dzo-japan{AAS) : AAS was used for determination of CO, Ni and Mg. A standard calibration curve is used for measurement of CO, Ni and Mg in the sera of groups under this study AML, CML, and controls.

Statistical analysis

The means and standard deviations, and student t test was used to compare the significance of the difference in the mean values of any two groups.

($P \leq 0.05$) was considered statistically significant (20).

Result and Discussion

Table 1 (opposite page) shows low serum levels of vitamin C (3.06, 3.46 µg|ml) for AML, ACML compared with controls (4.2µg|ml).

It was reported that toxic reactive oxygen species increase during leukemia(21). Ascorbate is a powerful reducing agent capable of rapidly scavenging a number of reactive oxygen species, so our results are in agreement with results which illustrated that the cancer incidence and mortality is associated with low levels of ascorbic acid.(22) We can speculate that this protective effect may occur through antioxidant pathways or through enhancement of immune function. (21) There is also some recent evidence suggesting that ascorbate exerts a selective cytotoxic effect against malignant cells; all these observations indicate that supplemental ascorbate should be of some therapeutic value in the treatment of cancer.(22)

Table 1 shows levels of vitamin D (3.46, 3.63 µg|ml) for AML, ACML compared with controls (4.73µg|ml). The reduction of vitamin D level in AML and CML is in agreement with Drake, MT, Maurer MJ(23). Numerous epidemiological and preclinical studies support a role of vitamin D compounds in cancer prevention and treatment in colorectal, breast, prostate, ovarian, bladder, lung and skin cancers and leukemia(24).The newly recognized use of 1,25(OH)2D3 analogues as immunomodulatory agents is based on the ability of these analogues to influence gene expression in cells of the immune system and cytokine expression by other cells(25). In addition some non traditional roles ascribed to vitamin D include anti-flammatory and immune-modulating effects. (26)

Table 2 (opposite page) shows the results of cobalt, Nickel and magnesium, levels in sera of controls, AML and CML groups. The result of cobalt level in serum of patients with AML, CML, were (0.012 58 , 0.0154 ug/l), respectively, which are lower than serum level of controls (0.027930 ug/l). This result is in agreement with published results (0.03 ug/l) (27). It appears that the depletion of Cobalt in serum of patients is related to vitamin B12. (15) In addition, biological research that has been done suggests a relationship between cobalt and cancer(28).

The Nickel level in serum of patients with Acute CML were 0.01163 and 0.01390 respectively, which is lower than level in serum of controls, (0.01445 ug/l). This result is compared with Nickel level of patients and controls with published values (0.3 ug/l) (29, 15). In another study, high serum concentration of Nickel causes cancer because itdamages the chromosomes , and on a molecular basis Nickel induces DNA damage (DNA strand breaks and crosslink's infidelity of DNA replication, inhibition of DNA repair, and the helical of B-DNA to Z-DNA) by binding Nickel to the nuclear protein (30). These events reflected the effects of Nickel in two states of concentrations, in the statement of exposure to and the statement of lower levels, tended to cause cancer (31).

The Mg level in patient groups is significantly lower than controls. The decrease in Mg concentration in the many

Table 1: Serum concentration (µg|ml) of vitamin C and D in AML, CML and healthy control group.

Vitamin Groups	*Vitamin C concentration µg|ml Mean ±SD*	*Vitamin D concentration µg|ml Mean ±SD*	*P*
Healthy group N =35	4.2±2.0	4.73±2.11	
AML N = 22	3. 06±1.01	3.46±1.21	P<0.05
CML N = 20	3.46±1.21	3. 63±1.40	P<0.05

Table 2: The serum concentration(µg|l) of CO, Ni and Mg in AML, CML and healthy control group

Trace element Groups	CO concentration µg|l Mean ± SD		Ni concentration µg|l Mean ± SD		Mg concentration µg|l Mean ± SD	P
Healthy control N=35	0.027930 ±0.011		0.01445±0.0090		40.8±15.4	
AML N= 22	0.01258±0.0054	P<0.05	0.0116302 ±0.0028	P<0.05	33.7±10.3	P<0.05
CML N=20	00154±0.0035	P<0.05	0.01389±0.00 65	P<0.05	34.6±11.7	P<0.05
P*		P*>0.05		P*>0.05		P*>0.05

P* value between AML and CML

studies suggests the diminished Mg due to the high ATP requirement of the leukemia since Mg is known to be an important part as a cofactor in most of the energy producing reactions(32).

Figure 1, Figure 2 and Table 3 (next 2 pages) show the correlation relation between vitamin D and CO, Ni in sera of AML, CML and healthy control group; positive correlation between vitamin D and CO, Ni in healthy control group with r values (0.987,0.799), positive correlation between vitamin D and CO in AML and CML with r values (0.980,0.970); positive correlation between vitamin D and Ni in AML and CML with r values (0.948, 0.870)

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Table 3: Correlation relation between Vitamin D and Co, Ni in AML, CML and healthy control group.

Trace element groups	*Co-Concentration µg/L Mean ±SD (P-value and r)*	*Ni-Concentration µg/L Mean ±SD (P-value and r)*	*Vit.D-Concentration µg/ml Mean ±SD*
Healthy n = 35	0.027930 ±0.011 (3.55×10^{-13}, 0.987355)	0.01445± 0.00090 (3.42×10^{-13}, 0.799952)	4.73± 2.11
AML N = 22	0.01258 ± 0.0054 (6.27×10^{-17}, 0.980622)	0.0116302 ± 0.0028 (6.22×10^{-17}, 0.948111)	3.46 ± 1.21
CML N = 20	0.0154 ± 0.0035 (2.71×10^{-14}, 0.973719)	0.01389± 0.0065 (2.7×10^{-14}, 0.870364)	3.63 ± 1.40

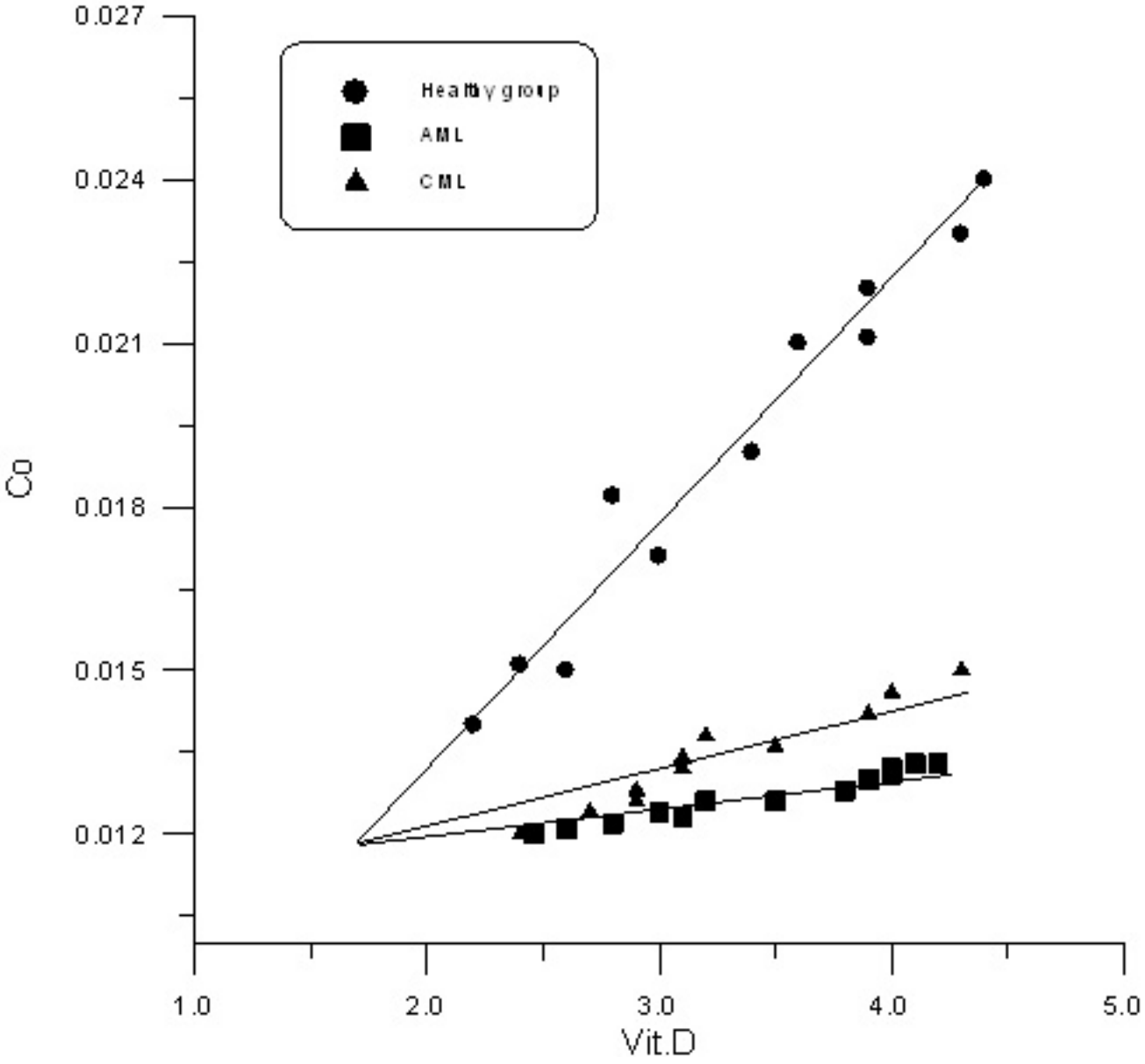

Figure 1

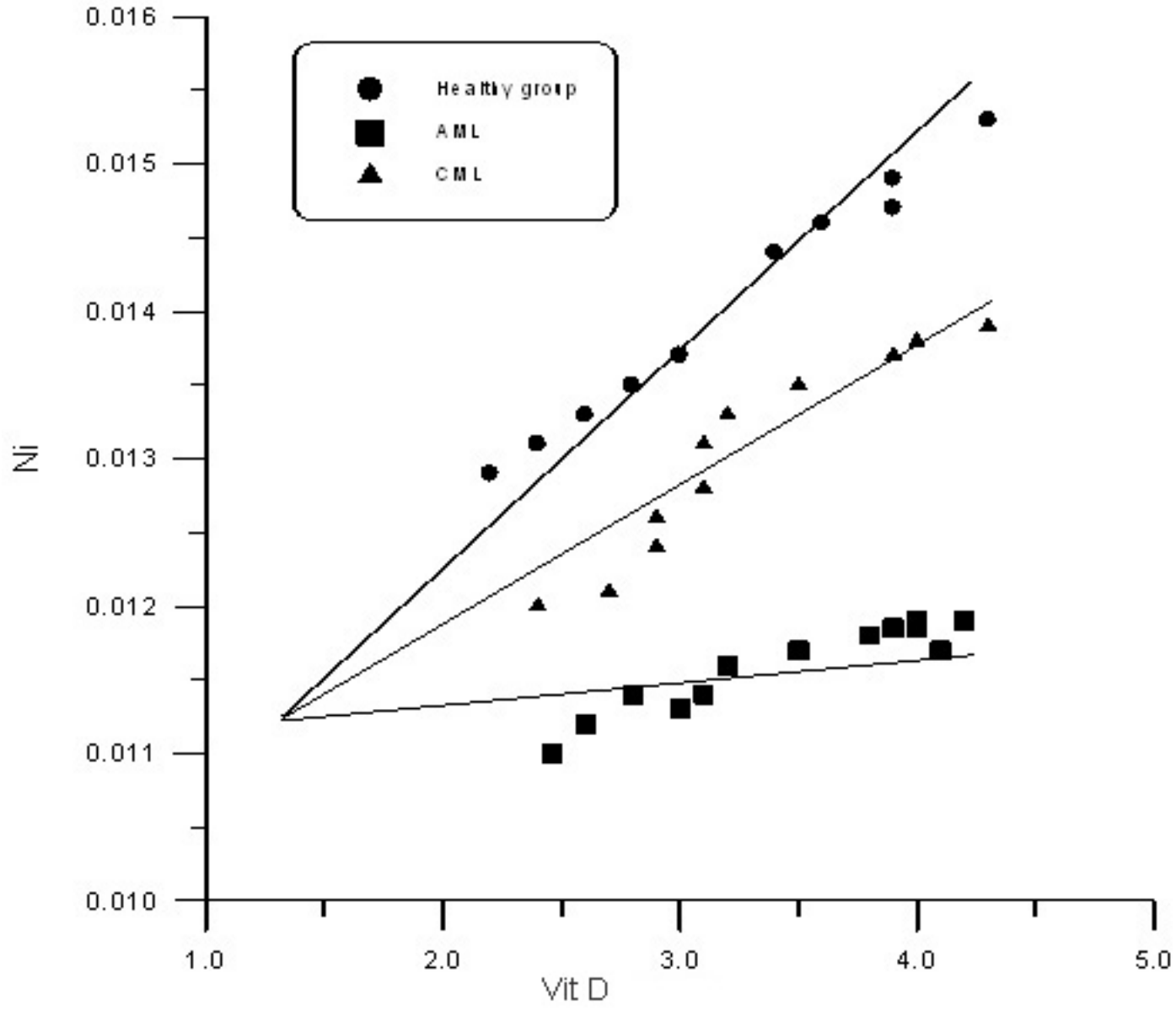

Figure 2

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Compared levels of specific divalent trace elements in hyperlipidemia and hepatitis sera patients

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ABSTRACT

Patients with hepatitis infection can also have hyperlipidemia. Sera of both hepatitis (40) and hyperlipidemia (40) were collected from the central lab of Sulaimaniyah, Kurdistan region, Iraq. Age, sex, duration of infection, medication used and lipid profile using computerized database program, as characteristics information of patients were done. The levels of Cr, Cu, Zn, Mn, V and Se were determined in sera of both patients and compared with healthy controls (n=30), using Inductively Coupled Plasma-Optical Emission Spectrometry (ICP-OES). Hepatitis samples yielded concentration in ppb of (7.34±0.01) Cr, (171.7±2.51) Cu, (138±3.41) Zn, (1.309±0.99) Mn, (184.7±6.12) V and (218.9±3.14) Se respectively, while hyperlipidemia samples yielded concentration (ppb) of (5.22±0.45) Cr, (197.01±2.34) Cu, (160.3±4.01) Zn, (0.068±1.91) Mn, (247.01±0.65) V and (185.5±3.11) Se respectively. Control samples reflect the following concentrations (ppm), (5.324±1.34) Cr, (131.2±4.34) Cu, (216.6±0.83) Zn, (0.024±1.75) Mn, (235.3±0.18) V and (223.2±3.15) Se respectively. The aim of the study focused on evaluating metal ions physiological role in the body, and as liver was responsible for synthesizing lipid, thus lipid profiles were suggested to be investigated along the study.

Key words: hepatitis, hyperlipidemia, trace elements, ICP-OES technique

Introduction

Hepatitis and hyperlipidemia have become a major human health problem worldwide [1]. It is not clear what the prevalence of hyperlipidemia is and how often hyperlipidemia is treated in patients with hepatitis. In addition, in those patients receiving cholesterol-lowering medication, it is not clear whether it is associated with worsening of liver synthetic function or not. In 1978, a comprehensive compilation of literature values was published by Iyengar et al. [2]. It served as a guideline for approximate concentrations of many elements in numerous tissues. In 1983, Woittiez [3] exclusively investigated the problem of establishing reference values for 28 elements in human serum. Trace elements such as Cr, Cu, Zn, Mn, V and Se are essential nutrients for humans and are required in very small amounts for many physiological functions, including immune, antioxidant function, growth and reproduction [4]. It has been determined that humans need nearly 72 trace elements, including very low concentrations of heavy metals, such as Cu, Se, V, Cr, Mo, Mn and Co. Most metals are toxics at high concentrations, while others provoke deleterious effects at low concentrations [5]. For example, Vanadium (VI) revealed a biological interest due to its biotoxicity [9]. The significance of the biochemical and nutritional roles of trace elements is widely recognized, since metals are found as constituent components of many metalloproteins and metalloenzymes. Some trace elements such as Copper act as cofactors against hepatic fibrosis in chronic liver diseases. Trace elements also affect many aspects of lipids metabolism through enzymes action and have modulator effects on the synthesis and metabolism of lipids [10]. Zinc for example, functions as an antioxidant and stabilizes membranes; Selenium is an essential micronutrient for human health [11-13]. The human body contains approximately ten milligrams of Mg most of which is found in the liver, bone and kidneys; it is a cofactor for a number of important enzymes, including arginase, pyruvate carboxilase and several phosphatase, peptidases and glycosyl transferase. Low levels of Mg have been associated with Atherosclerosis [14]. We conducted a study to assess the relationship and changing of some trace elements between hyperlipidemia and hepatitis.

Materials and Methods

The randomly selected study group comprised 40 patients with hepatitis that included 25 males and 15 females (aged 30±15.2), ranging between 25 and 60 years. Forty patients with hyperlipidemia were also included, 23 males and 17 females aged 35±12.3 years. The control group comprised 30 healthy individuals , which included 19 males and 11 females aged between 20 and 63 years. Sera of patients and controls were isolated from blood at the central lab (Sulaimaniyah- Kurdistan region, Iraq). All sera were collected in the morning after fasting 8 hours. Patients with hepatitis were diagnosed based on clinical, biochemical and histological data. Serum with hyperlipidemia was also diagnosed based on increased concentration of cholesterol, triglyceride, HDL

and LDL. Standard solution of the metals, 1000 µg/ml of Cr, Cu, Mn, Zn, V and Se, were prepared. Other chemicals were purchased from Fluka. Standard solutions were prepared freshly from the stocks, with diluted nitric acid (3 %v/v). In order to achieve ICP-OES responses, the experiments were performed using different concentration levels.

Sample Digesion:

1ml of serum was transferred to a Teflon beaker and 10ml of concentrated nitric acid and 2.5ml concentration perchloric acid were added. The sample was then brought very slowly to boiling on a hot plate and heated to dryness. If sample blackening occurred during the fuming stage, nitric acid was added drop wise, then the sample was cooled, dissolved again in distilled water and concentrated HCl (10:1) and brought to a volume of 25ml in a volumetric flask. The solution was analyzed against calibration curve [16].

ICP-OES:

An inductively coupled plasma-Optical Emission Spectrometer has been extensively used in the analysis of major, minor and trace elements in biological material because of its high sensitivity, accuracy, low matrix effect and simpler operation. The presence of various elements in the sample was identified by determining the wavelength of the emitted radiation (Cu: 327.393nm, Se: 196.026nm, Zn: 213.857nm, Cr: 267.716, Mn: 257.610, V: 290.880) and the concentration was calculated by intensity of the radiation, which might be sufficiently low for certain applications with a simple matrix. Sample and standard were analyzed in triplicate [17]. Statistical analysis, using STATISTICAL program (statsoft) was applied for data analysis. A p-value of < 0.05 was considered statistically significant [18].

Results

Serum concentrations of total cholesterol, HDL, LDL and triglyceride are represented in Table 1 (below). Patients with hyperlipidemia show a significant decrease ($p<0.05$) in Zn serum (160.3±4.01), (0.068±1.91) Mn and Se (185.5±3.11) ppb respectively compared with controls, while levels of Cu and V increased significantly ($p<0.05$) compared with those of controls. No significant changes were found in case of Cr.

Serum Cr, Cu, and Mn of hepatitis patients were significantly higher (17.53±0.01), (171.7±2.51) and (1.309±0.99) ppb respectively compared to normal (Cr=5.324±1.34), (Cu=131.2±5.93) and (Mn=0.204±1.75) ppb. The serum Zn, V and Se level was (Zn=138.3±3.41), (V=184.7±6, 12) and (Se=218.9±3.41) ppb in patients with hepatitis which was higher than normal, (216.6±0.83), (235.3±0.18) and (223.2±3.15) respectively, as shown in Table 2. Results of this study have been summarized and show the overall comparison between trace elements in both cases of hyperlipidemia and hepatitis in Figure 1.

Discussion

Damage of hepato cells can result in fluctuation of body cell constituents. Lipid profiles are of importance and can be affected by liver damage and the process results in variation in lipid levels. Trace elements are used as a diagnosing tool during disease; it is important to know whether the balance is changed in free or bound elements. The results of the present study have shown a significant increase ($p< 0.05$) in Cr, Cu and Mn in hepatitis and Cu and V in hyperlipidemia, while there were no significant changes in serum Cr in hyperlipidemia patients. These metals are members of one of the major subgroups of the micronutrients that have attained prominence in human nutrition and health. The biological role of trace metals, especially serum Zn, Cu, Cr and Mn, in different physiologic conditions has been extensively investigated in recent years (19). Similar observations were made by Lin CC et al. (20), and Pramoolsinsap C (21). They reported statistically significant decreased levels of serum Mn, Cu and Se in patients with hepatitis. But different observations have been reported by Saghir M. et al. (22), and show that Cu level decreased in hepatitis. It is clear that deficiencies of some trace elements, such as Cu, Cr, Zn and

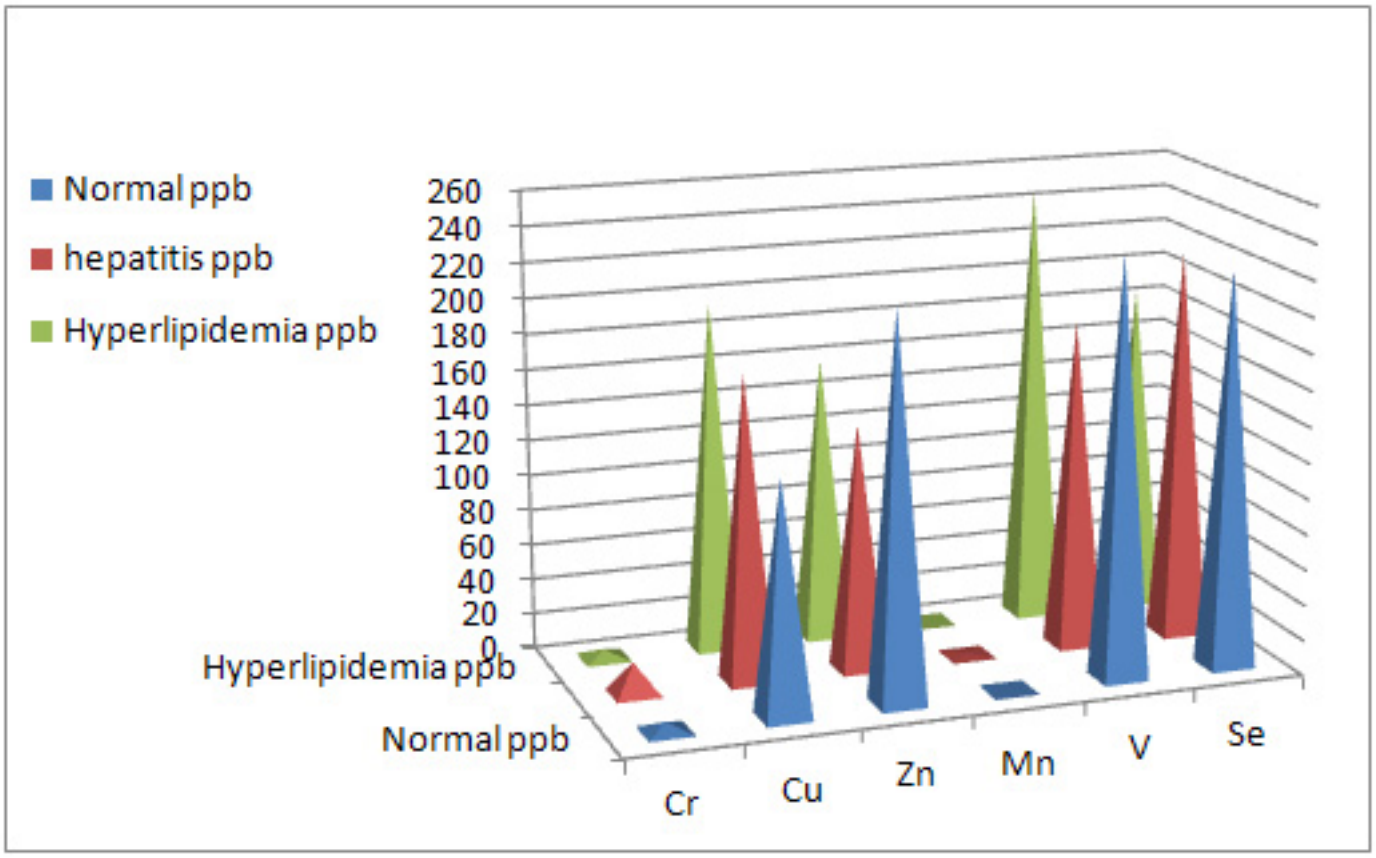
Table 1 : Characteristics of both patients which show the same features

No.	parameter	Mean ± SD
1	Body mass (kg)	55-85
2	Duration of disease (month)	5-15
3	S. cholesterol (mg/dl)	265-400
4	S.LDL	160-170
5	S.HDL	60-65
6	S. triglyceride (mg/dl)	200-499

Table 2: Concentrations of Cr, Cu, Zn, Mn, V and Se in healthy control hepatitis and hyperlipidemic patients

Metals	Normal ppb	Hepatitis ppb	Hyperlipidemia ppb
Cr	5.324±1.34	17.53±0.01	5.22±0.45
Cu	131.2±4.34	171.7±2.51	197.01±2.34
Zn	216.6±0.83	138.3±3.41	160.3±4.01
Mn	0.204±1.75	1.309±0.99	0.068±1.91
V	235.3±0.18	184.7±6.12	247±0.65
Se	223.2±3.15	218.9±3.41	185.5±3.11

Figure 1



Mn can result in marked alterations in lipid and lipoprotein metabolism (23). To the best of our knowledge, there has been no previous research regarding the correlations of serum trace elements with lipids and lipoprotein in hyperlipidemia patients. Several studies reported an inverse relation between serum Cu and cholesterol in rats during Cu deficiency (4), while Koo and Williams found no significant correlation between the serum Cu and cholesterol levels in non Cu deficient rats (11). Decrease in serum Se might indicate the development and progression of hepatitis, it also links to the disease progress of some viral agents in relation to the biosynthesis of selenoproteins (24), and decrease in serum Se significantly increases the risk of cancer mortality. Four-year animal studies showed that dietary supplement of Se reduced the hepatitis infection by 77.2% (25). El-Hendy et al, showed that Zn deficiency increases serum cholesterol in a dose-dependent rat (26). Manganese is critical for lipid and lipoprotein metabolism; it has been demonstrated that Mn enhances cholesterol synthesis in the liver. The above results show that serum Cu concentrations of hepatitis patients are higher than normal individual serum concentrations. These elevated serum Cu levels indicate an alteration of Cu metabolism during the acute phase of uncomplicated hepatitis (27). It may be explained by the release of copper from damaged necrotic hepatocytes (28).

Vanadium has a role in the regulation of the metabolism of lipids and other constituents of importance. The major concern is that excessive levels of vanadium have been suggested to be a factor in manic depression, as increased levels of vanadium is found in hair samples from manic patients, and these values fall towards normal levels with recovery (29).

Conclusion

The real mechanism is not known but abnormal results of trace elements may damage the liver by oxidative stress. Our study results suggest trace element supplementation may be complementary therapy to hyperlipidemia and hepatitis patients, so some of these trace elements might be considered as a marker of normal liver function. Dietary intake of these elements or vegetable and food which are considered as rich sources of these elements are necessary to reduce these two syndromes.

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Effects of Risedronate, Alendronate, and Raloksifene on Fracture Healing in Rats

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ABSTRACT

Bisphosphonates are a unique class of drugs that inhibit bone resorption, however recent studies also suggest their stimulatory effect on osteoblast formation. There are still controversies about the effects of bone resorption inhibitors during fracture healing. A prospective longitudinal randomized controlled study was designed in rat tibia to test the effects of various types of bisphosphonates on fracture healing. 48 skeletally mature female Wistar rats with a mean weight of 340 (316-351) g were used. Rats were allocated into four study groups, 12 animals in each group. Right tibial diaphysis was then fractured and fracture was stabilized with long leg cast. No other treatment was given to the control group, other groups received; risedronate 0.2 mg/kg/day, raloxifene 1.0 mg/kg/day, alendronate 0.2 mg/kg/day separately. Treatment began immediately after an experimental tibial fracture. Animals were sacrificed week four of the experiment. Fractured tibia was evaluated radiologically and mechanically and histologically. Radiographic study showed that bony callus was present in all the fractures and bisphosphonates led not to a larger callus as in the other studies. Mechanical testing revealed that ultimate load of alendronete group was significantly higher than other groups. Histologically, process of fracture healing time was shorter with significantly higher osteoblast in alendronte treated group (P <0.05). The result suggest that systemic alendronat treatment induces stronger callus formation in rats.

Key words: **Bisphosphonate; Fracture healing; Mechanical testing; Histopathology**

Introduction

Antiresorptive and anabolic drugs are two currently available therapeutic options for the treatment of osteoporosis. Antiresorptive agents inhibit bone resorption and bone formation to stabilize bone structure and increase bone mass [1,2]. Osteoporosis most frequently affects postmenopausal women, placing them at a significant risk for fractures. Osteoporotic fractures in women consist of vertebral fractures, wrist and forearm fractures, hip fractures, rib fractures, humeral fractures, pelvic fractures, clavicular, and tibial and fibular fractures, scapular, and sternum fractures [3]. Bisphosphonates, and selective estrogen receptor modulators currently, are used widely in the treatment of osteoporosis in postmenopausal women [2,4]. Animal studies using bisphosphonates such as alendronate, zoledronic acid, incandronate and pamidronate generally indicate an increase in callus size and structural biomechanical changes [5-12]. Risedronate improves bone biomechanical properties through alterations of trabecular structure, especially its shape and connectivity and it is effective in the treatment and prevention of postmenopausal osteoporosis in women and corticosteroid-induced osteoporosis in men and women [13-15].

Bisphosphonates have also been used in the treatment of diseases involving extreme osteoclast mediated bone resorption such as Paget's disease, tumor-induced hypercalcemic and metastatic bone diseases [16,17]. Alendronate in mature dogs did not show any adverse effects on fracture healing, mineralization and mechanical properties [18]. Recent studies with another bisphosphonate, incadronate, showed an enlarged callus that was strong, but incadronate delayed callus remodelling in the fractured femora of rats [19,20]. The size of the callus was either not influenced or was increased but never decreased [21-23]. The influence of bisphosphonates on fracture repair may depend on the mode and dosage of the administration [19]. Possible effects of selective estrogen receptor modulators in fracture healing are not well understood. They suppress bone resorption activity in ovariectomized rats secondarily suppress bone formation activity resulting in lower bone remodeling [23]. The purpose of the study was to investigate the effects of risedronate, alendronate, and raloksifene on early stages of fracture healing and mechanical properties of callus.

Material and Methods

Animals

The experimental protocols were approved by the local animal ethical committee. Forty-eight female, 12-week-old Wistar rats with a mean weight of 340 (316-351) g were used. The animals were housed in a cage (floor area 900 cm2 and height 20 cm) with free access to tap water and standard laboratory rodent diet (with 1.1 % calcium, 0.8 % phosphorus and 1500IU/kg vitamin D3) in a 12 h/12 h light-dark cycle.

Experimental protocol

The animals were anesthetized with combination intramuscular injections of ketamine HCL (50 mg/kg, Ketalar; Parke-Davis, Morris Plains, NJ) and xylazine (10mg/kg, Rompun; Bayer, Istanbul, Turkey). All animals were subjected to a standardized right tibial fracture, using a specially designed fracture forceps [24]. The fractures were stabilized by long leg cast. The fractures were left without further immobilization. All rats resumed full weightbearing of the fractured limb within 1 week as confirmed by the absence of a visible limp.

Experimental groups

The animals were randomly allocated into four groups: one control group and three treatment groups with the same body weight, 12 rats per group. Treatment began immediately after an experimental tibial fracture.

Group 1 (control);

Group 2 (Risedronat); 0.2 mg/kg/day,

Group 3 (Raloxifene); 1.0mg/kg/day,

Group 4 (Alendronate); 0.2 mg/kg/day,

The drugs were administered orally on a daily basis with 16 gauge stainless-steel gavage needle. The compounds were prepared, in sterile 0.9% saline. All dosing solutions were stored refrigerated at approximately 50C. Solutions were warmed to room temparature before administration.

Four weeks after fracture, the rats were sacrificed, and tibias cleaned of all soft tissues, while leaving the callus of the right tibia intact.

Radiography

The anteroposterior soft radiographs of all fractured tibias were taken (30 Kvp, 2 mA) with a Siemens X-ray machine (Model number: 4803404, Germany). Callus maturity was evaluated, described by Goldberg [25].

Statistical Analysis

Data were analyzed using the Statistical Package for Social Sciences version 15.0 (SPSS for Windows 15.0, Inc., Chicago, IL, USA).

Biomechanical Testing

Both the fractured right tibias were tested by the three-point bending method using a mechanical testing machine (Zwick/Roell, 1446, Germany). The tibia was placed, facing its anterior surface down, on the two lower support bars (12 mm apart) with loading bar positioned at the fracture site, or middle tibia. Load was applied until breakage ultimate load was determined by a connected computer. All specimens were consequently loaded to failure point at a static rate of 20 mm/min and force versus displacement data was recorded. Biomechanical data were studied using the One-Way ANOVA and post hoc Bonferroni test. A P value of less than 0.05 was considered statistically significant for mechanical results.

Histopathology

After mechanical testing, fractured tibias were repositioned, the specimens were fixed in 10% formaldehyde, decalcificated with 10% formic acid, and embedded in paraffin, stained

with hematoxylin-eosin. 5 micron thick cross-sections were cut. All histological specimens were examined under light microscope by the blinded pathologist. Histological evaluation was performed according to the grading system of the fracture healing. A point value was assigned to each phase of healing in a continiuum, such that 10 points would represent the most mature repair and 1 point the most immature [26]. One-Way ANOVA and post hoc Bonferroni test was used to evaluate the histologic results. A P value of less than 0.05 was considered statistically significant for histologic results.

Results

Of the total 48 rats, one was excluded because of infection in raloxifen group. After fracture, the rats resumed normal activity within a week, and drugs did not cause any side effects. Soft X-ray observation showed external callus formation. Fracture line disappeared in all groups. Callus width was same in all groups. In the alendronate group, at 4 weeks histologic observations of callus showed that there was more woven bone than other groups ($P<0.05$). [The histologic score for risedronate, alendronate, raloksifene, and control groups were 5 (3-6), 7 (6-8), 4 (3-6), 4.5 (3-6) respectively.] In the three point bending test, all the fractured tibias failed along the original fracture line. Ultimate load of fractured tibias in alendronate (14,16 ±1,02) group was higher than raloxifene (12,1±0.93), risedronate (12,13±0,91) and control (11,84±0,88) groups. The differences in ultimate load were statiscally significant ($P<0.05$). There was no statistically significant difference between the other groups ($P > 0.05$).

Discussion

Fracture healing was always been a main medical problem and it has been the aim of physicians to shorten the healing time and to prevent nonunion. The effort to develop drugs to promote bone formation have not been succesfull yet. Invention of bone-forming growth factors, such as the transforming growth factors, fibroblast growth factors, bone morphogenetic protein and others gives hope that soon we shall have use of their anabolic properties [27]. Madsen et al. have confirmed that tibial diaphyseal fracture model is sufficient to be used to investigate the effects of bisphosphonates on the processes of fracture repair [28]. The fracture healing process includes various stages such as endochondral ossification, woven bone production, and callus remodelling to lamellar bone, and fracture callus is heterogeneous with respect to the tissue composition especially in the early stage. These situations make histological evaluation of fracture callus very hard [29]. During fracture healing, osteoclasts play an important role in endochondral ossification and remodelling of woven bone to lameller bone [30-32]. Bisphosphonates inhibit osteoclast activity and their continuous long-term use inhibits osteoclast differentiation [33-35]. The inhibitory effect may be directly on the osteoclast, partly mediated by other cells, especially osteoblast [36]. Currently, Alendronate, estrogen, Risedronte, and Raloxifene are avaliable therapies used to treat postmenopausal osteoporosis [4,37,38]. The present study showed that process of fracture healing progressed not only in the control group, but also in other groups as evidenced by histological observations. Callus formation evaluated by radiographs showed that all fractures healed with external osseous callus. It confirms that these drugs do not inhibit mineralization of fibrocartilage. Risedronate, alendronate, and raloksifene treatment led not to a larger callus as evidenced by radiographs. In a previous study alendronate treatment increased the size of the callus compared with other groups [39]. In our study size of the callus was not affected by alendronate. In the early process of fracture healing, the bone mineral turnover is high, and the alendronate effect on osteoclast function at this stage could explain the increased callus size observed by other authors [39]. The reason why our study could not confirm these findings might be the relatively short fracture healing period of 4 weeks. The animals used were not osteopenic. Bending tests are often used to determine mechanical properties because they are faster and convenient [40]. It is possible to locate the loading bar at the fracture site to test the part of the bone by using the three-point bending test [24,41]. The three-point bending test was also used in the present study. There was difference in ultimate load between the alendronate group and other groups. Mechanical strength of fractured bone might be unaffected under treatment with alendronate, tiludronate and clodronate but it affected use with etidronate [18,21,28,42]. Taken together, the previously mentioned studies further confirm that whether bisphosphonates interfere with fracture repair and mechanical strength of fractured bone varies based on their chemical structure, dosage, potency and duration. Our mechanical study indicated that the ultimate load of alendronate group was higher than the other treatment groups and control. Continuous treatment with risedronate, alendronate, and raloksifene appears not to delay the fracture healing at any time point after fracture. In contrast, short-term continuous treatment with alendronate shortened the healing time in the early stages of fracture repair processes as evidenced by histopathology. There was less fibrocartilage and more woven bone than the other groups. The recruitment of periosteal cells to the fracture site, differentiation of these cells to chondrocytes and osteoblasts, and the process of mineralization of fibrocartilage were normal under new bisphosphonates treatment (14,35,36).[22,42,43]. The production of the mineralization matrix in callus by endochondral bone formation and in growth plate was increased by clodronate and incadronate treatment [42,44]. A study by Giuliani et al. have showed that bisphosphonates could directly stimulate formation of osteoblast precursors and mineralized nodules in both murine and human marrow cultures in vitro [45]. Raloksifene and risedronate had similar effects on fracture healing and were also similar to control.

In conclusion, alendronate induced the formation of strong fracture calluses in rats and short term continuous treatment shortened the healing time in the early stages of fracture repair processes. Risedronate and raloksifene had no effect on progression of fracture repair. Risedronate, alendronate, and raloksifene may be safe drugs to use in osteoporosis complicated with fractures, since they do not seem to affect negatively the early stages of fracture healing.

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Nitric Oxide and Oxidative Stress Properties of L-Carnitine in Diabetic Hypertensive Rats Biochemical & Histological Study

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ABSTRACT

Background: Diabetes mellitus and hypertension are a global health problem due to their serious complications, which along with oxidative stress have been shown to contribute to endothelial dysfunction.

Aim: The aim of the present study was to investigate the role of the beneficial effect of L-carnitine in healthy and streptozotocin (STZ) N$^{\omega}$-nitro-L-arginine methyl ester (L-NAME) induced diabetes mellitus and hypertension in rats.

Results: Results showed that diabetic hypertensive (DH) rats had significant increase in the level of plasma glucose, malondialdhyde (MAD), cholesterol (CH), triglycerides (TG), urea, creatinine, and the activity of serum liver enzymes (AST, ALT) compared to normal control rats. Blood glutathione (GSH) content and erythrocyte superoxide dismutase (SOD) activity and nitric oxide level (NO) were significantly lowered. Supplementation of L- carnitine for 6 weeks improved plasma glucose, lipids, liver and kidney functions. In addition, both normal healthy rats and DH rats treated with L-carnitine showed increase in blood GSH and SOD activity and serum nitrate level (stable product of nitric oxide NO) as compared with healthy controls and DH respectively. Histopathological and immunochemical study of heart confirmed the biochemical results.

Conclusion: It was concluded that administration of L-carnitine reduces or delays oxidative stress in diabetic hypertensive rats.

Key words: L-carnitine - L-NAME- Nitric Oxide, Oxidative Stress, Diabetic rats, Hypertensive rats, Histological Study.

Introduction

Diabetes results in a state of increased reactive oxygen species (ROS) production, and oxidative stress is implicated in the development and progression of various diabetic complications (Baynes, 1991;Yao et al. 2009). Increased oxidative stress is thought to play an important role in the etiology and pathogenesis of chronic complications of diabetes (Scott and King, 2004; Yao et al., 2009).

The antidiabetic actions of individual free amino acids are of great interest. Intervention of glycation may prove to be beneficial to patients suffering from diabetes mellitus. Free amino acids are known to mitigate the glycation of lens protein, delay cataractogenesis and bring down blood sugar levels in diabetic rats and promote tissue sensitivity towards insulin. Further, amino acids inhibit the binding of glucose with proteins, the first step in the pathway of glycation cascade by competitive inhibition, thereby offering protection (Anuradha, 2009).

Arterial hypertension is associated with a high production of reactive oxygen species and a decrease in the antioxidant defence systems. Since oxidative stress has gained importance in the last few years as one of the mechanisms involved in the origin and development of hypertension, and considering that L-carnitine (LC) is a useful compound in different pathologies characterized by increased oxidative status, the aim of this work was to test the hypothesis that LC might protect the heart against hypertension-induced oxidative damage. In spite of a wide range of drugs being available in the market, treatment of arterial hypertension still remains a challenge, and new therapeutic strategies could be developed in order to improve the rate of success in controlling this disease (Zambrano et al., 2013).

The importance of L-carnitine (b-hydroxy-y-N-trimethyammonium butyric acid) for the lipid and carbohydrate metabolism has been long established. Carnitine is required to transport long -chain fatty acids from the cytoplasm to the mitochondrial matrix where their oxidation occurs, and on the other hand, carnitine increases the sensitivity of the cells to insulin and the use of glucose by the peripheral tissues (Mamoulakis et al., 2004).

Some other effects of carnitine on cellular metabolism as protection against oxygen free radicals and of mitochondrial biogenesis in aged rats were demonstrated. Carnitine influence membrane fluidity, ion channel function, and smooth muscle contractility. It was suggested that membrane effects are implicated in the mechanism by which carnitine derivatives protect the heart from ischemia or oxidative stress. This might be in concert with findings on the changes of cardiac carnitine metabolism in various hypertensive models (Rauchova, 1998).

Nitric oxide (NO) is the most pivotal molecule secreted by endothelium and thus is a major mediator of endothelial function. The production of NO is catalyzed by a family of enzymes called nitric oxide synthases (NOS), which convert the amino acid L-arginine to L-citrulline and NO, apart from playing an important role in vasodilation. Evidence suggests that NO plays a major role in regulating blood pressure and glucose levels, and thus impaired NO bioactivity forms an important component of hypertension and diabetes. The physiological importance of NO in the regulation of blood pressure is evidenced by the fact that pharmacological inhibition of NO synthases leads to severe hypertension, vascular injury, and glomerulosclerosis in experimental animals (Shiekh et al., 2011).

Material and Methods

Experimental design and animal grouping

Design: 40 white male albino rats weighing 150-200 g. were used for this study. All animals were housed in stainless steel cages, 10 per cage under controlled environmental conditions. Diabetes was induced in male Wister albino rats by single intraperitoneal injection of 50 mg/kg streptozotocin (STZ) (Heo et al.,2002). Four days after STZ injection, rats received N$^{\omega}$-nitro-L-arginine methylester (L-NAME) (0.5 mg/mL in drinking water for four weeks) for induction of hypertension (Zambrano et. al., 2013). L-carnitine (0.5 g/100 gm diet) was given daily to DH rats for six weeks, respectively (Oka et al., 2008).

Our work was carried out in accordance with the guidelines of Faculty of Applied Medical Science at Taif University in K.S.A. for animal use.

Handling of the animal was the same for all groups and did not affect weight gain.

Groups:

1- ***Group 1:*** Normal control: 10 rats fed the balanced diet during the entire study (10 weeks).

2- ***Group 2: L-carnitine:*** 10 rats fed the balanced diet supplemented with L-carnitine during the entire study (10 weeks).

3- ***Group 3: Diabetic hypertensive group (DH):*** this group contains 10 diabetic hypertensive rats.

4- ***Group 4: Diabetic hypertensive group treated with L-carnitine (DH+L-car):*** 10 diabetic hypertensive rats receive L-carnitine as treatment for 6 weeks.

Our goal is to achieve a diabetic hypertensive model in 4 weeks following treatment period for 6 weeks. This model provided us with a reliable method and resembles the clinical cases of diabetic hypertensive rats and their treatment. After six weeks treatment, heart rate and blood pressure of all animals were recorded by the tail cuff method using the Harvard Blood Pressure Monitor. Blood samples were collected from the tail vein of lightly anesthetized overnight fasted animals from all the groups.

Sample collection and analytical methods

By the end of the experimental periods, blood samples were collected in two centrifuge tubes. One tube contained

heparinized blood used for determination of SOD and GSH activity. In the second tube serum was separated by leaving blood sample for 15 minutes at a temperature of 25 oC then it was centrifuged for 20 minutes at 4000 r.p.m., using clean dry disposable plastic syringes and stored at -20°C for subsequent biochemical measurements as follows: lipid profile (TC, TG) (Fossati and Prencipe, 1982), liver enzyme activities related to its function (ALT, AST) (Reitman and Frankel, 1957), kidney function (urea and creatinine) (Li, 1996), heart biomarkers (LDH, CK) (Würzburg et al., 1977), glucose (Trinder,1969) Lipid peroxidation and oxidative stress were measured through malondialdehyde (MDA), nitric oxide (NO), oxidative stress markers, reduced glutathione (GSH) and superoxide dismutase activity (SOD) according to Mahesh et al. (2009). Tissues were analyzed using commercial kits routine (Bancroft and Gamble, 2002), histological methods H&E stain.

Histological Examination: Heart specimens from each group were examined histopathologically. Heart tissues were fixed in 10% formalin solution for 48 hours and embedded in paraffin wax, sectioned (4 um), and then stained with hematoxylin and eosin. Pathological changes were evaluated in the tissues as previously described (Helin HO 2006), De Rossi A 2007).

Immunohistochemical staining:

Immunohistochemistry was conducted on paraffin embedded heart sacrifices from control and treated rats. Slides were deparaffinized, dehydrated, washed in phosphate buffer saline then covered with peroxide block staining (PBS) to block off endogenous staining and incubated for 10 minutes at room temperature in a humidity chamber. Monoclonal mouse caspase-3 antibody (Lab vision, USA) (Cai, L 2002) was applied on the tissue sections then incubated horizontally in a humidity chamber for an hour, at room temperature. After removal of excess buffer, the sections were incubated in preformed strept avidin peroxidase. DAB substrate- chromogen (3.3- Diaminobenzidine tetrahydrochloride) was applied on slides for 5-15 minutes until the desired brown color was obtained. Counter - staining was done by using Mayer's haematoxylin.

Image Analysis

The data were obtained using Lecia Qwin 500 image analyzer computer system (England). The image analyzer consisted of a colored video camera, colored monitor, hard disc of IBM personal computer connected to the microscope, and controlled by Lecia Qwin 500 software. The image analyzer was first calibrated automatically to convert the measurement units (pixels) produced by the image analyzer program into actual micrometer units. The area and area % of caspase-3 immunoexpression in myocardial sections of the animals of all groups were measured using an objective lens of magnification 10, i.e. of total magnification of 100. Ten random fields were measured for each specimen. In each chosen field, the areas of caspase-3 immunoactivity were enclosed inside a standard measuring frame (Figure 1 - below).

Statistical Analysis

Statistical analysis was carried out using Graph Pad Instat software (version 3, ISS-Rome, Italy). Unless otherwise specified, groups of data were compared with an unpaired t-test one-way analysis of variance (ANOVA). Values of $P < 0.05$ were regarded as significant. Data were expressed in tables as mean ± standard error (SE) (Altman and Bland 1996).

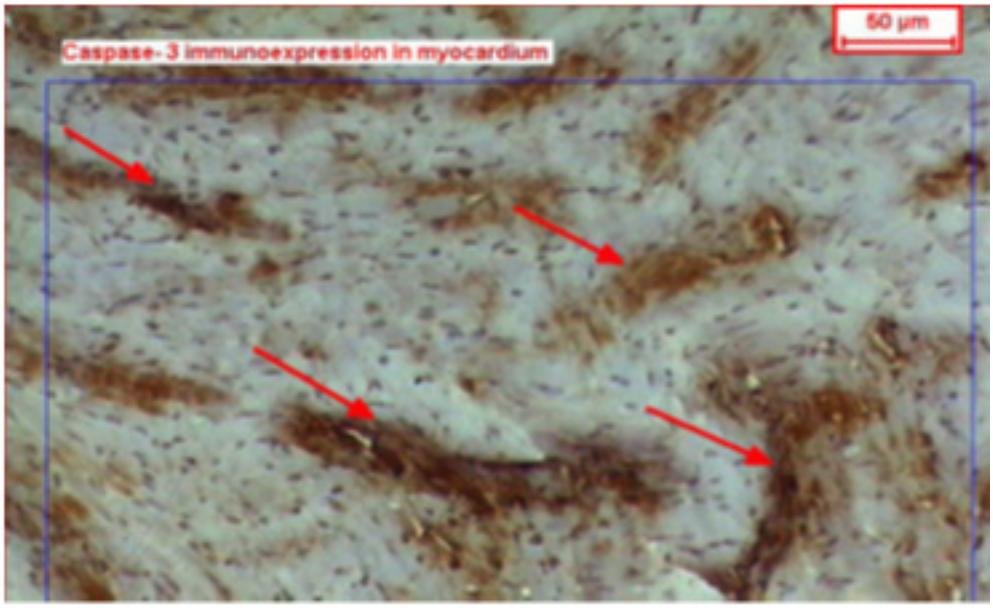


Figure 1: A copy of display seen on the monitor's screen of the image analyzer, showing +ve caspase-3 immunoreactivity in the myocardium

Biochemical Results

Effect of L- carnitine on hypertension, heart rates and heart weight:

The current results tabulated in Table 1 administration of L-carnitine by healthy and DH rats showed that there is a significant decrease in hypertension in the diabetic hypertensive L- carnitine treated group (STZ+L-NAME +L-car) compared to the untreated group(STZ+L-NAME) , heart rates also showed that there is a significant amelioration after treatment with L-carnitine ($P < 0.05$).

Table 1: Effect of L-carnitine (L-car) on the blood pressure , heart rates and mean heart weight in control and diabetic hypertensive (STZ+L-NAME) rats

Treatment / Group	Mean blood pressure mmHg Mean ± SE	Heart rates beats/minutes Mean ± SE	Heart weight Gm Mean ± SE
Control	107.0 ± 5.0	314 ± 20	0.89 ± 0.09
L-car[a]	90.0 ± 3.0[a]	331 ± 25[a]	0.87 ± 0.06
STZ+L-NAME[a]	139.0 ± 5.0[a]	270 ± 24[a]	0.95 ± 0.03[a]
STZ+L-NAME +L-car[a,b]	118.0 ± 4.0[a,b]	293 ± 27[a,b]	0.92 ± 0.04[a,]

Data are presented as mean ± SE, n= 10 a and b indicate significant change from control, STZ+L-NAME, respectively at $p \leq 0.05$ using ANOVA test.

Diabetic hypertensive rats (STZ+L-NAME), showed decreased heart weights that was statistically significant as compared with controls (group I). On the other hand, in animals of (STZ+L-NAME +L-car), the loss of heart weights was statistically insignificant as compared with controls (Table 1).

Effect of L- carnitine on blood glucose and lipid profile:

Results tabulated in Table 2 revealed that administration of L-carnitine at tested doses by healthy rats showed a non significant decrease in serum glucose level. While administration of L-carnitine at the same doses by DH rats showed a significant decrease in glucose level $p \leq 0.05$. Regarding serum CH,TG and LDL-C levels our results showed a significant decrease in the DH+L-carnitine group than the diabetic hypertensive group, while HDL-C showed a significant increase $P \leq 0.05$.

Table 2: Effect of L-carnitine (L-car) on the levels of blood glucose, triglycerides, total cholesterol, high-density lipoprotein-cholesterol (HDL-C) and low-density lipoprotein-cholesterol (LDL-C) in control normal and diabetic hypertensive (STZ+L-NAME) rat serum

Treatment / Group	Glucose (mg/dl)	Triglycerides (mg/dl)	Total Cholesterol (mg/dl)	HDL-C (md/dl)	LDL-C (mg/dl)
Control	85.3±0.5	79.7±0.5	82.3±0.9	40.6±0.4	38.6±0.8
L-car[a]	86.9±0.5	77.8±0.7	83.5±1.7	42.1±0.6	37.9±0.9
STZ+L-NAME[a]	215.0±1.0[a]	126.9±0.9[a]	150.2±1.8[a]	18.6±0.5[a]	101.1±1.9[a]
STZ+L-NAME +L-car[a,b]	150.5±0.8[a,b]	84.3±0.5[b]	93.3±1.4[a,b]	32.7±1.1[a,b]	64.2±. [a,b]

Data are presented as mean ± SE, n= 10 a and b indicate significant change from control, STZ+L-NAME, respectively at $p \leq 0.05$ using ANOVA test.

Effect of L-carnitine on ALT, AST, LDH, CK, urea and creatinine, in healthy and the corresponding diabetic hypertensive rats:

The results tabulated in Table 3 illustrate the effect of L-carnitine on serum ALT, AST, LDH, CK, urea and creatinine, in healthy and diabetic hypertensive rats. The results revealed that L-carnitine supplementation to the healthy rats resulted in a non significant change compared to untreated healthy rats. While administration of L-carnitine by DH rats showed a significant decrease ($P \leq 0.05$) in the levels of ALT, AST, LDH, CK, urea and creatinine as compared with the diabetic hypertensive control group.

Table 3: Effect of L-carnitine (L-car) on the levels of Liver enzyme activities (ALT, AST), cardiac enzyme activities (LDH, CK) and kidney functions (urea, creatinine), in control normal and diabetic hypertensive (STZ+L-NAME) rat serum

Treatment / Group	ALT (u/l)	AST (u/l)	LDH (u/l)	C.K-NAC (u/l)	Urea (mg/dl)	Creatinine (mg/dl)
Control	17.1 ± 0. 9	24.9 ± 0.9	258.5 ± 10.8	250.2 ± 12.6	28.6 ± 0.9	0.59 ± 0.05
L-car[a]	15.3 ± 0.9[a]	22.8 ± 1.1	241.7 ± 10.6	242.2 ± 10.6	26.5 ± 1.3	0.55 ± 0.06
STZ+L-NAME[a]	24.6 ± 0.6[a]	41.18 ± 1.11[a]	472.8 ± 6.4[a]	407.4 ± 8.9[a]	52.8 ± 0.71[a]	1. 6 ± 0.04[a,]
STZ+L-NAME +L-car[a,b]	20.6 ± 0.9[a,b]	34.2 ± 1.1[a,b]	397.9 ± 8.2[a,b]	340.4 ± 8.9[a,b]	37.3 ± 0.7[a,b]	0.82 ± 0.03[a,b]

Data are presented as mean ± SE, n= 10 a and b indicate significant change from control, STZ+L-NAME, respectively at $p \leq 0.05$ using ANOVA test.

Effect of L-Carnitine on GSH, SOD, NO and MDA in healthy and their corresponding diabetic hypertensive rats:

Regarding tblood (GSH) and erythrocyte (SOD) activity, serum NO results tabulated in Table 4 revealed that consuming L-carnitine either by healthy and diabetic hypertensive groups showed a significant increase in these values as compared with their corresponding groups. Our results showed also administration of L-carnitine by the healthy rats (group 2) (L-car) caused a non significant reduction in the serum level of MDA as compared with control (group one). On the other hand, administration of L-carnitine by DH rats resulted in a significant reduction ($P < 0.05$) on serum MDA compared with diabetic hypertensive untreated rats.

Table 4: Effect of L-Carnitine on Glutathion reductase (GSH), superoxide dismutase (SOD), nitric oxide (NO), and malondialdehyde (MDA) in control normal and diabetic hypertensive (STZ+L-NAME) rat (mean ±SE).

Treatment / Group	GSH (mg/gHb)	SOD (units/ml)	NO (µmol/L)	MDA (nmol/ml)
Control	68.7± 0.8	334.7±25.8	98.7±12.2	4.7 ± 0.1
L-car[a]	72.6 ± 0.7	360.4±29.3[a]	102.3±14.2	4.4 ± 0.1
STZ+L-NAME[a]	37.3 ± 0.7[a]	197.1±41.3[a]	40.7±12.3[a]	9.9 ± 0.3[a]
STZ+L-NAME +L-car[a,b]	54.4 ± 0.7[b]	303.7±34.5[a,b]	78.4±22.3[a,b]	5.2 ± 0.1[a,b]

Data are presented as mean ± SE, n= 10 a and b indicate significant change from control, STZ+L-NAME, respectively at $p \leq 0.05$ using ANOVA test.

Effect of L-Carnitine on mean area % of caspase-3 immunoreactivity in the myocardium in control normal and diabetic hypertensive rats: Table 5 shows mean area % of caspase-3 immunoreactivity in the myocardium of control and treated groups:

Table 5: Effect of L-Carnitine on mean area % of caspase-3 immunoreactivity in the myocardium in control normal and diabetic hypertensive rats.

Treatment / Group	Mean area % of caspase-3 immunoreactivity in the myocardium
Control	6.04 ± 1.9
L-car[a]	6.08 ±2.01
STZ+L-NAME[a]	26.33± 15.17[a]
STZ+L-NAME +L-car[a,b]	8.79 ± 4.86[b]

Data are presented as mean ± SE, n= 10 a and b indicate significant change from control, STZ+L-NAME, respectively at $p \leq 0.05$ using ANOVA test.

Histological Results

Diabetic hypertensive treated animals of (group III), showed increased heart weights that was statistically highly significant as compared with controls (group I). On the other hand, in animals of (group IV), the loss of heart weights was statistically insignificant as compared with controls (Table 1). Mean area % of caspase-3 immunoreactivity in the myocardium of control and treated groups shown in Table 5.

Light microscopic results of control heart sections (group I) stained with H&E, the myocardium showed branching and anastomosing muscle fibers with centrally located oval basophilic euchromatic nuclei and connected together by intercalated discs (Figure 2). In L-carnitine treated control animals (group II), the myocardium appeared nearly with normal structure as control rats (fig.3). Infiltrating inflammatory cells were observed in the left ventricle sections of D H rats, and these cells were usually found in clusters of cells located throughout the interstitium with congested blood vessels (Figure 4). Treatment with L-carnitine in (group IV) showed markedly reduced inflammatory cell infiltration in diabetic hypertensive rats however, some inflammatory cells were still found within the interstitial component of the left ventricle (Figure 5).

Figure 2: A photomicrograph of a longitudinal section of the myocardium of a control adult rat (group I) showing branching muscle fibers with centrally located oval nuclei (arrows). Note: intercalated discs (lines) and flat dark nuclei of the fibroblasts (white arrow) of the connective tissue endomysium. (Hx. & E. X 400)

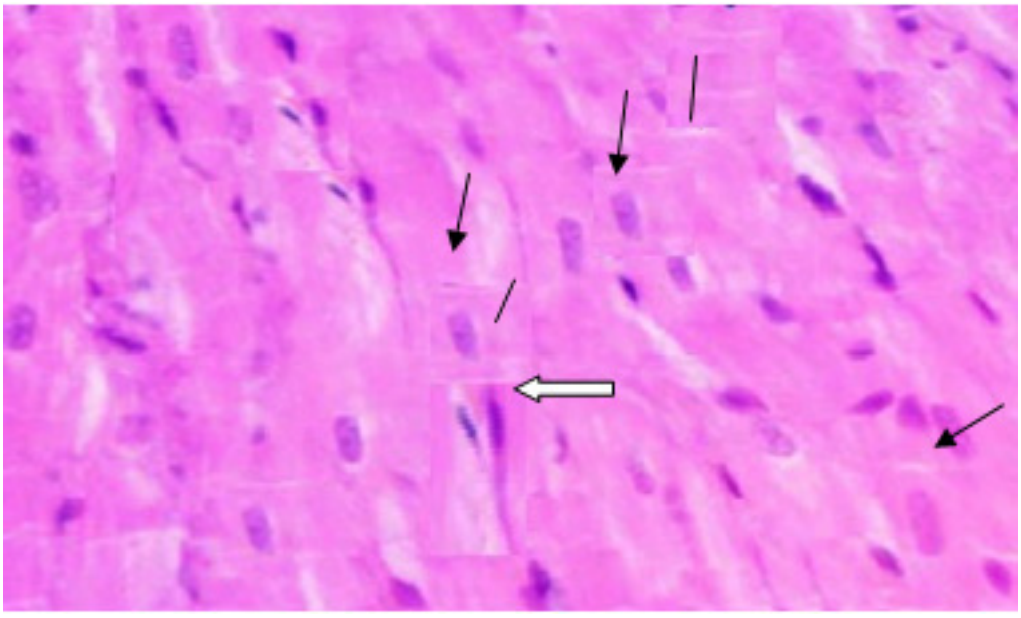


Figure 3: A photomicrograph of a longitudinal section in the myocardium of an adult rat from group II (L carnitine control rats), showing normal myocardium which appeared nearly the same as control rats. (Hx. & E. X 400)

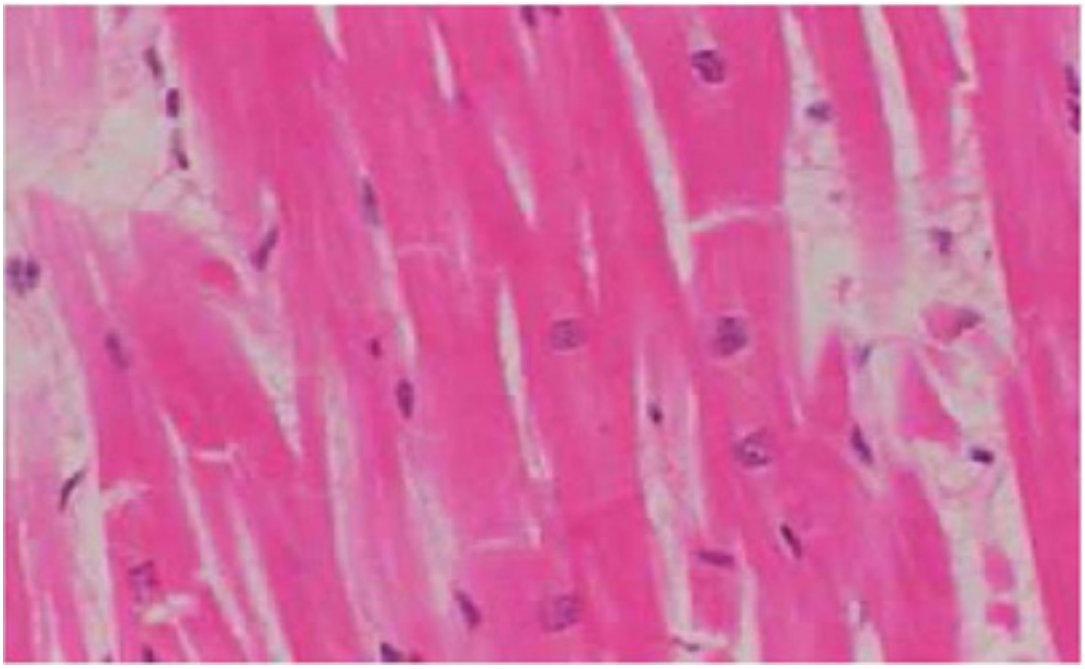


Figure 4: A photomicrograph of a longitudinal section in the myocardium of an adult rat from group III (diabetic hypertensive rats), showing inflammatory cell infiltration (arrow) & congestion of blood vessels (2 heads arrow) with some degeneration in myocardium rats (white arrow). (Hx. & E. X 400)

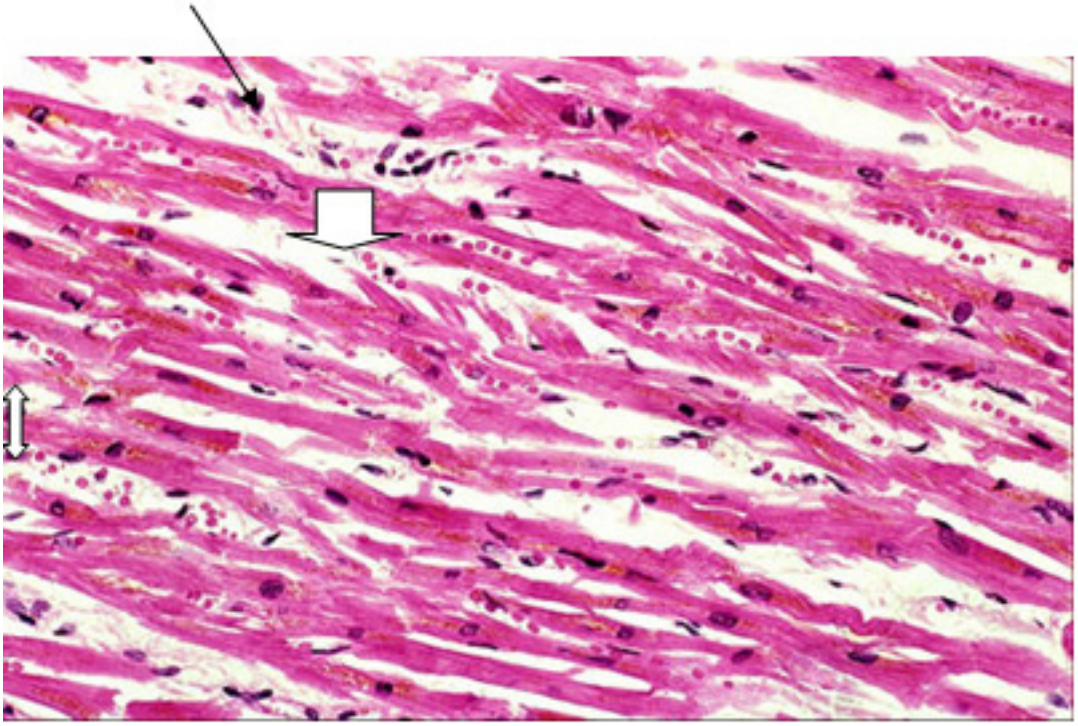
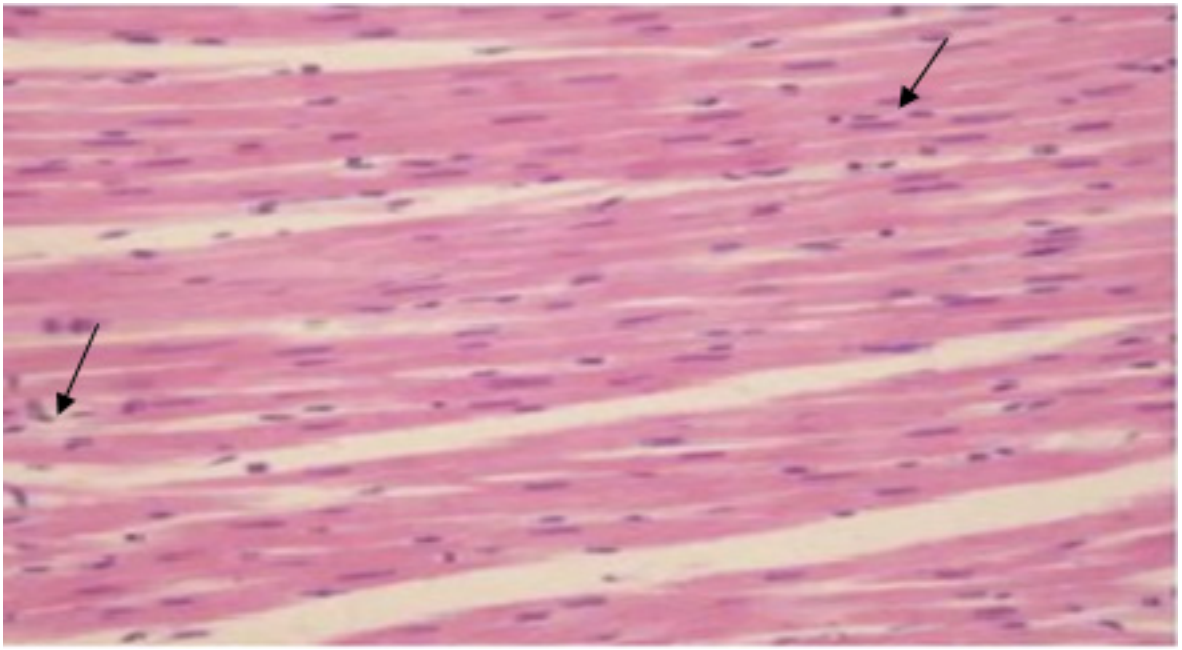


Figure 5: A photomicrograph of a longitudinal section in the myocardium of an adult rat from group IV (l-carnitine treated diabetic hypertensive rats), showing myocardium with some inflammatory cells (arrows).(Hx. & E. X 400)



Immunohistochemical and image analysis results:

Myocardial sections of control animals (group I) showed a negative immune reaction to caspase - 3 (Figure 6) i.e. no brownish color in the cytoplasm (apoptosis). Group II (LC treated animals) like controls showed a negative immune reaction to caspase - 3 (Figure 7).

Figure 6: A photomicrograph of a longitudinal section of the myocardium of a control adult rat (group I) showing negative immune reaction to caspase - 3. (Caspase-3 Immunoreactivity, X 400)

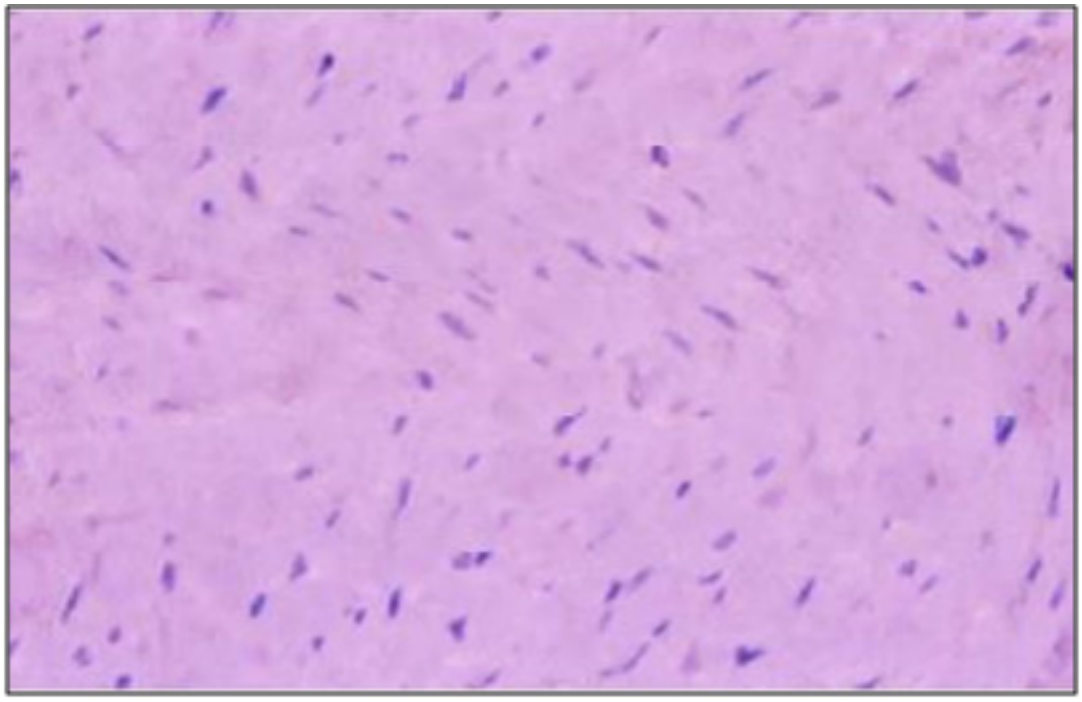
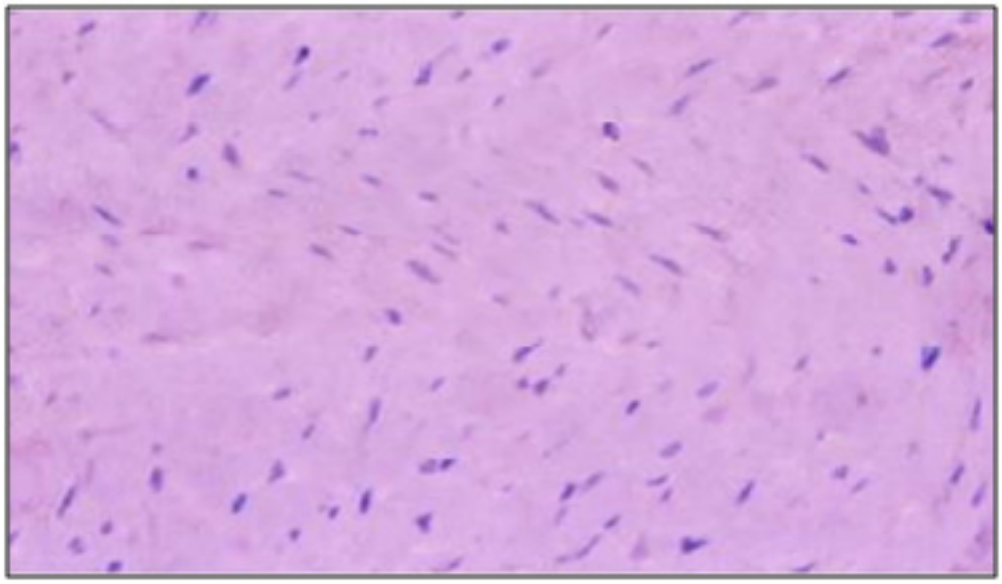


Figure 7: A photomicrograph of a longitudinal section of the myocardium of an adult rat from group II, (l carnitine treated animals) showing negative immune reaction to caspase-3 (arrows). (Caspase-3 Immunoreactivity, X 400).



Diabetic hypertensive treated animals (group III), showed increased area of positive immune reaction to caspase- 3 (Figure 8). In group IV, there were few areas with positive immune reaction to caspase - 3 (Figure 9). Diabetic hypertensive treated animals (group III) showed a highly significant increase in the mean area % of caspase-3 immunoreactivity in the myocardium as compared with controls. However the (Group VI) showed a statistically significant decrease in the mean area % of caspase-3 expressions (Table 5).

Figure 8: A photomicrograph of a longitudinal section of the myocardium of an adult rat from group III, (diabetic hypertensive animals) showing very strong positive immune reaction to caspase-3 (arrows). (Caspase-3 Immunoreactivity, X 400)

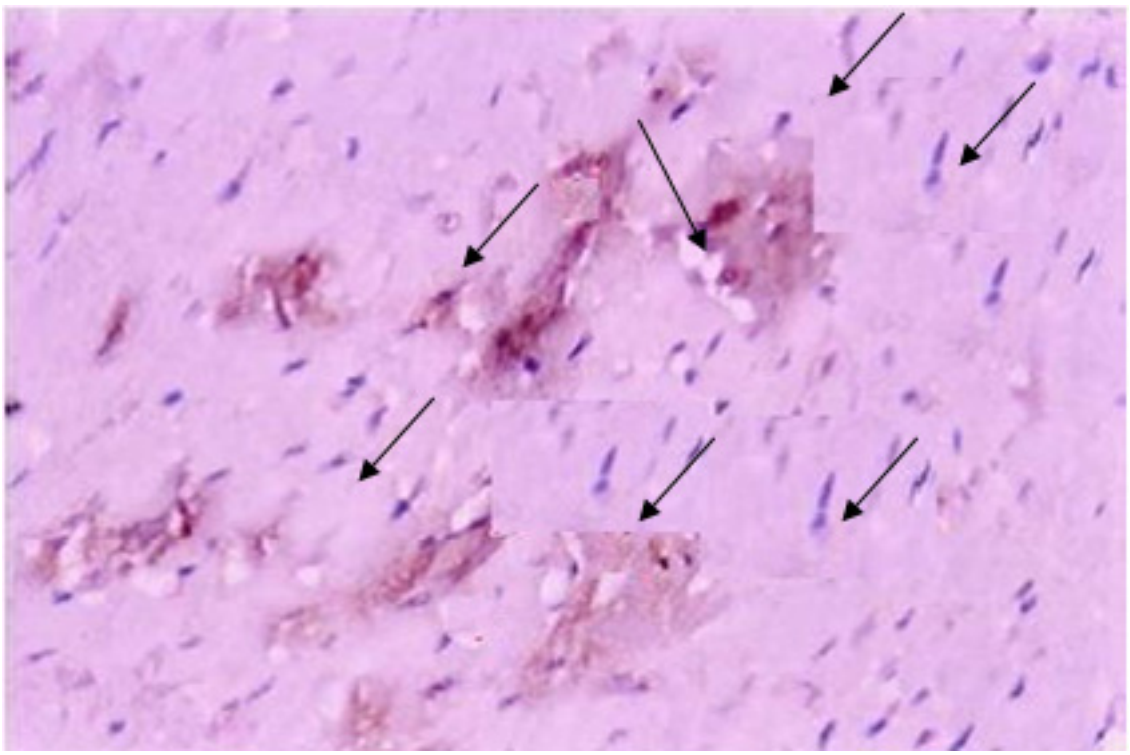
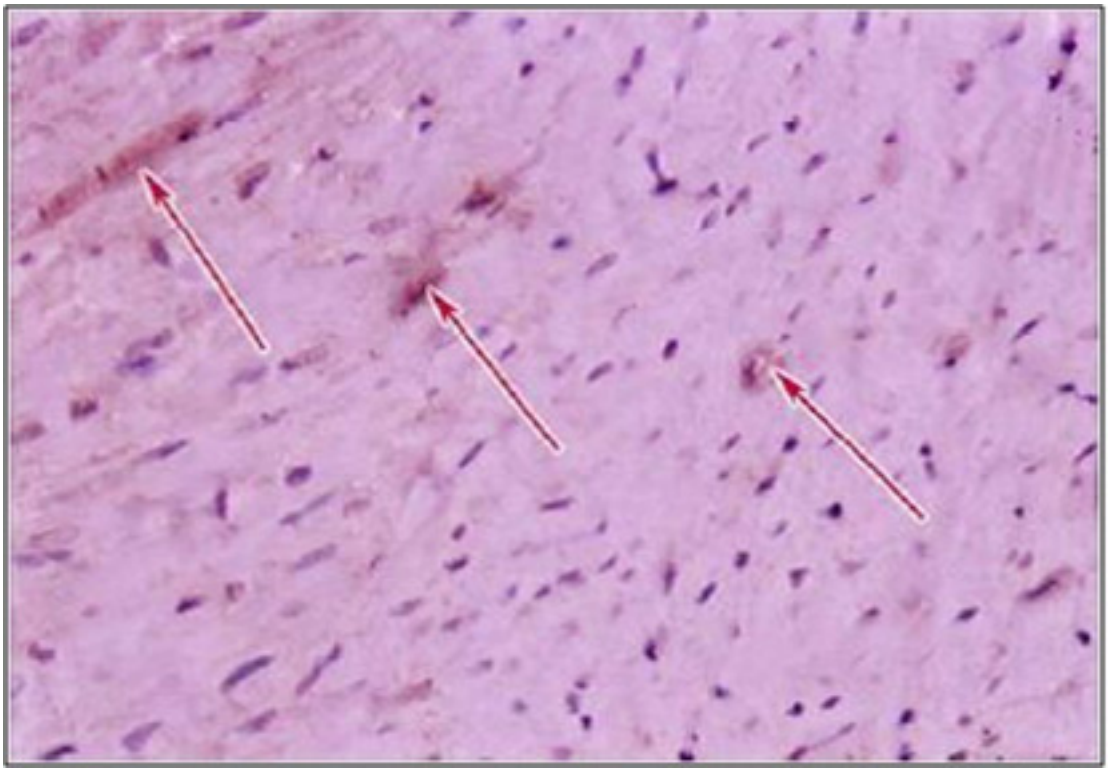


Figure 9: A photomicrograph of a longitudinal section of the myocardium of an adult rat from group IV, (diabetic hypertensive treated animals) showing weak positive immune reaction to caspase-3 (arrows). (Caspase-3 Immunoreactivity, X 400)



Discussion

Reduced NO availability in diabetes mellitus and hypertension, underlines its relevance to the development of secondary complications in these clinical conditions. Alteration of NO metabolism and increased oxidant stress, was demonstrated to be involved in the pathogenesis of macrovascular events, which are increased in hypertensives as well as diabetics. Results of the present study revealed that administration of L-carnitine by healthy and diabetic hypertensive rats caused a significant decrease in hypertension, while, resting heart rates were increased as compared to their corresponding controls. These results are in agreement with Ruggenenti et al (2009) and Malton et al (2006). They suggest, that a deficiency of free carnitine can manifest as elevated free fatty acids, suggesting a reduction of inter-cellular transport. Elevating free carnitine levels in diabetic animals with a fixed and relatively inadequate availability of glucose as a myocardial fuel apparently corrected the defects in myocardial function by providing more intracellular fatty acids as an energy substrate.

In the present study diabetic untreated rats showed a significant elevation of serum glucose level. Our results were confirmed by (Malton et al., 2006) who illustrated that the blood glucose level of diabetic rats increased compared to control animals. Administration of L-carnitine at the tested doses by diabetic rats only showed a significant decrease in serum glucose level compared to the untreated group. These results are in agreement with Mamoulakis et al (2004) who reported that carnitine increases the sensitivity of the cells to insulin and the use of glucose by the peripheral tissues. Carnitine administration improved whole body insulin sensitivity, glucose tolerance and prevents oxidative stress.

L-carnitine supplementation produced significant decreases in serum TG, and T-cholesterol, LDL-C while there was a significant increase in HDL-C in diabetic hypertensive rats. These results are in agreement with those of González-Ortiz et al (2008) and El-Metwally et al (2003), who reported that oral L-carnitine increases plasma free carnitine levels, improves dyslipidemia and decreases oxidative stress, with reduction of cardiac parameters. L-carnitine administration to diabetic hypertensive rats reduces significantly hypertriglyceridemia (Table 2) via decreased synthesis of triglycerides by the liver or by inhibition of triglyceride release from the liver.

L-carnitine suppressed hydroxyl radical production in the Fenton system, probably by chelating their iron, is required for the generation of hydroxyl radicals. Thus, the reduction in lipid peroxidation in the present study might be due to the iron-chelating property of L-carnitine. This hypothesis is consistent with the previous study which has demonstrated that L-carnitine showed a strong antioxidant activity against irradiation-induced lipid peroxidation and has free radical scavenging effects (Mansour, 2013).

In the present work diabetic hypertensive animals showed a significant increase in the AST, ALT, CK and LDH activities (P ?0.05), also serum urea and creatinine levels. This may be due to increase in oxidative damage and decrease in antioxidant capacity of the liver which suggests that oxidative stress has an impaction of liver disorders. These results are in harmony with that of Khalil (2009).

The oral administration of L-carnitine (Table 3) shows that serum concentration of urea and creatinine were significantly decreased. The effect of L-carnitine on renal lipid metabolism

could serve as a new therapeutic approach, as it counters the renal changes associated with metabolic syndrome. Hence, L-carnitine has beneficial effects on renal function.

Anuradha (2009) reported that L-carnitine protects against liver, kidney and heart disease. L-carnitine improves heart function in diabetics and hypertensives and increases the level of glucose oxidation, a process that helps cells make use of glucose. McMackin (2007) and Ruggenenti, et.al. (2009) reported that, Acetyl-l-carnitine safely ameliorated arterial hypertension, insulin resistance, impaired glucose tolerance, and hypoadiponectinemia in subjects at increased cardiovascular risk. Whether these effects may translate into long-term cardioprotection is worth investigating.

In the present work, the increased serum MDA value in diabetic hypertensive rats may be attributed to the increased level of oxygen free radicals which could be due to their increased production and/or decreased destruction by non-enzymatic and enzymatic antioxidants. Our results are in agreement with Sailaja-Devi and Das (2005), who reported a significant increase in the plasma level of MDA, as well as a significant decrease in serum level of NO(x). GSH and the activity of SOD in RBCs lysate in diabetic animals compared to healthy controls. Also Barakat (2006) illustrated that the decrease in GSH levels during diabetes is probably due to its increased utilization by the hepatic cells. This may be due to an attempt by the hepatocytes to counteract the increased formation of lipid peroxides. Tas et.al. (2007) illustrate both plasma and tissue MDA levels were significantly reduced in the diabetic group treated with individual free amino acids compared to those of the diabetic untreated group. These alternations might be related to hypolipidemic, hypoglycemic and direct oxidative effects of free amino acids. The antioxidative and hypoglycemic effect of L-carnitine might also be involved in the changes in antioxidative enzyme activities. Our results showed that erythrocyte SOD activity was significantly increased in the diabetic hypertensive control group and the diabetic hypertensive group treated with L-carnitine compared to control group; the elevation might be due to the protective mechanism against oxidative stress.

L-carnitine effectively protects and improves mitochondrial function in vivo: it acts as an antioxidant, so by inhibiting ROS and RNS it protects the vascular endothelial tissues against oxidative damage in hypertension Gómez-Amores et al (2007). Thus, L-carnitine treatment effectively protected the liver tissue against oxidative damage and showed marked improvement in its antioxidant status.

Nitric oxide level showed a significantly reduced level ($P < 0.5$) in diabetic hypertensive rats (Table 4) than control ones. Various studies have reported a significant decrease of plasma nitric oxides in diabetes mellitus and hypertension; our results coincide with these reports. Shiekh et.al. (2011), presumed that the cascade of NO bioactivity and availability on smooth muscle cells was impaired in the early affected stage of diabetes mellitus and followed the decrease of endothelial NO production.

In the present study, the decreased serum NO value in diabetic hypertensive rats was ameliorated by administration of L-carnitine. Shiekh et.al.(2011), reported that, Nitric oxide (NO) turnover is vital for proper endothelial function to maintain a healthy vascular system. Various risk factors responsible for hypertension and diabetes may disrupt this homeostasis, leading to decreased bioavailability and/or bioactivity of NO, which potentiates endothelial dysfunction. Plasma NO is a useful indicator of NO homeostasis and vascular endothelial function which plays a key role in the development and progression of diseases like diabetes and hypertension.

Cardiovascular remodeling includes hypertension, endothelial damage, cardiac hypertrophy, inflammation, ventricular contractile dysfunction and fibrosis (Weber KT et al 2001). L-Carnitine plays a major role, as a cofactor, in the transportation of free fatty acids from the cytosol to the mitochondria for adenosine triphosphate synthesis. An altered metabolic substrate used in the failing heart also contributes to the dysfunction of the mitochondrial electron transport chain, resulting in enhanced production of superoxide (Rosca MGetal 2008). Mitochondrial dysfunction and increased mitochondrial superoxide production, preceding endothelial dysfunction, might favour the development of hypertension (Puddu P et. al. 2008).

Free radicals also potentiate mitochondrial dysfunction by further damaging mitochondrial DNA, with resultant impairment in the synthesis of some components of the respiratory chain and further increases in superoxide production (Puddu P et. al. 2008, Esposito LA et. al. 1999 Shibutani S 1991, Zorov DB 2006). The current experiment was designed to estimate the effect of L-carnitine on nitric oxide and oxidative stress in normal and in diabetic hypertensive rats. In this study Infiltrating inflammatory cells were observed in the left ventricle sections of D H rats, and these cells were usually found in clusters of cells located throughout the interstitium with congested blood vessels. Treatment with L-carnitine in (group IV) showed markedly reduced inflammatory cell infiltration in diabetic hypertensive rats, however, some inflammatory cells were still found within the interstitial component of the left ventricle. The results of the current investigation were strongly supported by (Ferrari R, 2003, Malone JI,2003) who found that L-Carnitine treatment improved heart function after ischaemia and reperfusion injury, and also improved heart rate regulation and ventricular size in streptozotocindiabetic rats. In addition, the anti-hypertensive effects of L-carnitine in this study may result from inhibition of inflammation, as inflammation is an integral part of the cardiovascular remodeling observed in L-NAME hypertensive rats. Furthermore, plasma concentrations and cardiac expression of inflammatory markers such as IL-6 and TNFa were reduced after L-carnitine treatment in L-NAME

Several other studies have also proved the efficiency of L-carnitine in reducing blood pressure in patients with pulmonary hypertension (El-Beshlawy A 2008), in rats with L-NAME-induced hypertension (Miguel 2008) and in fructose-fed hypertensive rats (Rajasekar P 2007). The mechanisms by which L-carnitine can decrease blood pressure include its role

in enhancing fatty acid oxidation (El-Beshlawy A 2008) and the consequent role to reduce the production of superoxide (G_lÅin I 2006), and further increasing the availability of nitric oxide (Rajasekar P 2007). L-carnitine controls oxidative stress by improving mitochondrial function (G_lÅin I 2006), (Calvani M, 2000). Diabetic hypertensive treated animals (group III) showed a highly significant increase in the mean area % of caspase-3 immunoreactivity in the myocardium as compared with controls. However the (Group VI) showed decrease in the mean area % of caspase-3 expressions in the present study. These results were in agreement with (Abdel Baky N, 2011), who found that l-carnitine significantly reduced the level of heart-type fatty acid binding protein, capase-3 activity, as well as myocardial DNA damage in diabetic hypertensive rats.

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