



L-carnitine Improves Atherosclerotic Plaques in Hyperlipidemic Animal Model (Hyperlipidemic Rabbits)

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USE of L-carnitine (L-c.) bears potentials of decreasing hyperlipidemia following fat diet feeding. 25 Animal (rabbits) were divided randomly into five groups. Normal group were kept on a plain chow diet for 75 days. HC classes were on a diet (hypercholesterolemic diet) for seventy-five days. Atorvastatin group were fed the same diet and received atorvastatin orally (20mg/kg/day) from day forty-five for thirty days. L-carnitine group were fed the same diet and intraperitoneal L-c. (250 mg/kg/day). Treatment group were fed the same diet and atorvastatin orally (10 mg/kg/day) and L-carnitine intraperitoneally (125 mg/kg/day). Histology of aorta and biochemistry of serum were carried out. Foam cells with interrupted endothelial lining and transformed muscle fibers were observed in aorta wall in hypercholesterolaemic rabbits. Normal aorta wall was observed in rabbits of Atrovastatin and treatment groups. In rabbits of HC group triglyceride was significantly increased compared to that of other groups ($P < 0.05$). The animals that received L-carnitine showed significantly reduced triglyceride ($P < 0.05$). L-Carnitine reduces atherosclerotic plaques.

Keywords: L-carnitine, Atherosclerosis plaque, Cholesterol, Rabbits.

Introduction

Atherosclerosis is a condition in which the arteries narrow due to the accumulation of fatty plaque and lose their flexibility [1]. Genetics, metabolism, nutrition, hemodynamics, infectious-inflammatory factors are the intervening factors of this disease [2,3] The pathogenesis of the disease begins when the stores of low-density lipoprotein (LDL) increase and its stores in the tunica intima layer increase (Tunica intima) [4].

Different types of drugs are available for different risk factors, most of which aimed to prevent the formation of atheroma and plaques and prevent the growth of plaques. Statins, fibrous acid phosphate etc. are drugs used to treat hyperlipidemias [5]. A number of biological

compounds have been identified as new risk factors for atherosclerosis that cause abnormal coagulation and reduced fibrinolysis, infection, inflammation, cardiovascular regeneration, and cell adhesion [6]. Choline, phosphatidylcholine and carnitine are among the compounds that can play a role in atherosclerosis [7-9]. L-Carnitine (L.C.) is a quaternary amine. The body can produce L-carnitine from the amino acids lysine and methionine. When there are not enough essential amino acids or cofactors, getting enough LC status through diet should be done. About 75% of LC in the body is obtained from diet [10]. L-Carnitine is considered as a therapeutic agent for the treatment of hyperlipidemia due to its help in the metabolism of fatty acids. Others have suggested a possible physiological role for

carnitine, including involvement in long-chain β -oxidation of mitochondria in the mitochondria, buffering of mitochondrial acylCoA/CoA levels [11].

The protective effect of the heart by L.C. supplementation. Has been raised. LC treatment is available against ischemia-reperfusion injury and exercise tolerance and activity levels in patients with cardiovascular disease (CVD) have been demonstrated in studies [12-17]. To the best knowledge of the authors, the literature is poor regarding the effects of endogenous carnitine depletion and/or carnitine deficiency in atherogenesis. The present study was aimed at assessment of potential beneficial effects of L-carnitine on cholesterol-induced atherosclerosis in rabbits.

Materials and Methods

Study design and animals

Twenty five male white New Zealand rabbits approximately 3 months of age and weighing 2.0–2.5 kg were divided into 5 groups of 5 rabbits. Normal group were kept on a plain chow diet for 75 days; HC group on a hypercholesterolaemic diet for seventy five days; Atorvastatin fed the same diet and received atorvastatin orally (20mg/kg/day) from day forty five for thirty days; L-carnitine group fed the same diet and intraperitoneal L-carnitine (250 mg/kg/day) from day 45 for thirty days (end of treatment) and treatment group were fed the same diet and atorvastatin orally (10 mg/kg/day) and L-carnitine intraperitoneally (125 mg/kg/day) from day forty five for thirty days.

In L-carnitine group the animals were kept on a hypercholesterolaemic diet for 75 days and intraperitoneal L-carnitine (250 mg/kg/day) from day 45 for 30 days (completion of the intervention). In Treatment group, the animals were kept on a hypercholesterolaemic diet for 75 days, and atorvastatin orally (10mg/kg/day) and L-carnitine intraperitoneally (125 mg/kg/day) from day 45 for 30 days (end of the study).

Hypercholesterolaemia induction in animals

Two weeks before the study and within the experiments, the animals were separately caged in plastic cages at $23\pm3^{\circ}\text{C}$, under fixed air humidity and with a conventional nocturnal cycle. A standard rodent laboratory food and tap water were freely provided to rabbits. Two observers who were blind to grouping carried out all measurements. All studies were also carried out according to

the relevant ethical recommendations and the protocol of study was carefully revisited and ethic approval was obtained by Urmia University of Medical Sciences. L-carnitine and cholesterol were prepared by Sigma (St. Louis, USA).

Induction of hypercholesterolaemia in rabbits

Rabbit model (fed cholesterol in the diet) is valuable due to the quick formation of aortic lesions and low maintenance cost. The conventional diet for induction of atherosclerosis includes supplementation of 2% cholesterol per weight for around 75 days [13]. Hypercholesterolaemia induction was performed through 2% cholesterol-enriched fat diet for 75 days according to a previously described method [13].

The diet was prepared by spray coating the standard chow pellets containing cholesterol along with acetone, PVP, acetyl alcohol, ethyl alcohol, and carbowax to serve as solvent and binding.

This approach was used to prepare hypercholesterolaemic pellets with uniformly distributed cholesterol, and was welcomed by the rabbits without different taste and appearance in comparison with the regular chow pellets.

Apoptosis activity

End tagging of dUTP deoxynucleotidyl transferase was performed by the available model kit on cardiac sections in the infarct area (TUNEL staining). Groups were randomly selected. Tissue samples with 400% magnification were observed under a light microscope. At least 100 cells from the area around the infarct were counted for each field. A total of 10 fields were examined (Leica Q500MC). The area around the infarct was determined by staining hematoxylin and eosin on tissue slides.

Histopathological investigations of heart tissue, aorta and detection of macrophages and neutrophils in atherosclerotic plaques

Samples were taken from the aorta and heart, including the descending and ascending sections. Samples were immersed in formalin phosphate buffer for at least 24 hours to stabilize. Then, the samples were placed in paraffin (Sections of 5 μm). Hematoxylin and eosin staining was used for all samples and additional Mason trichrome for heart samples was used for staining. The maximum aortic wall thickness as well as the thickness of the intima (plate size) of each sample were measured using an image evaluation program (Optica, Italy) (compared to others). The presence

of macrophages and neutrophils in atherosclerotic plaques was also measured. NASDCE staining method was used to identify neutrophils in tissue.

Statistical analysis

Data analysis was done through SPSS version 16.0 and analysis of variance was used. Normality of data was investigated by Kolmogorov–Smirnov test ($P < 0.05$).

Results

Atherosclerotic plaque formation

Under the created (above-mentioned) conditions, rabbits quickly developed hypercholesterolemia (plasma cholesterol more than 1,000 mg/dl), with the lesions mainly including macrophage-derived foam cells (Fig. 1A).

Apoptosis activity findings

TUNEL staining was used to detect apoptosis of cardiomyocytes in each group. One-way ANOVA revealed a meaningful difference among the groups in respect of the mean \pm SEM of the number of apoptotic cells (with

TUNEL-positive arrows) in the peripheral and periinfarct area ($P < 0.05$). The rate of TUNEL positive cardiomyocytes in treatment group was significantly decreased in comparison with the HC group, but this decrease was not statically significant ($P < 0.05$) (Fig. 2, Table 1).

Histopathological findings

Histopathological results regarding aorta in the Normal group are illustrated in Fig. 1B. The aorta wall appeared to have a uniform thickness without any bulging in the lumen and the endothelial lining appeared intact with no interruption. Muscle fibers and elastic lumina were normal as well.

Furthermore, rabbits exposed to 75-day exposure to 2% cholesterol-enriched diet showed atherosclerotic lesions that were seen as pronounced changes in the aortic wall, which are observed by three intimal plaques indicated using arrows.

Plaques' magnification images revealed extremely stained foam cells and endothelial gaps in the sub-endothelial layer. Foam cells formation already fulfilled with cholesterol ester

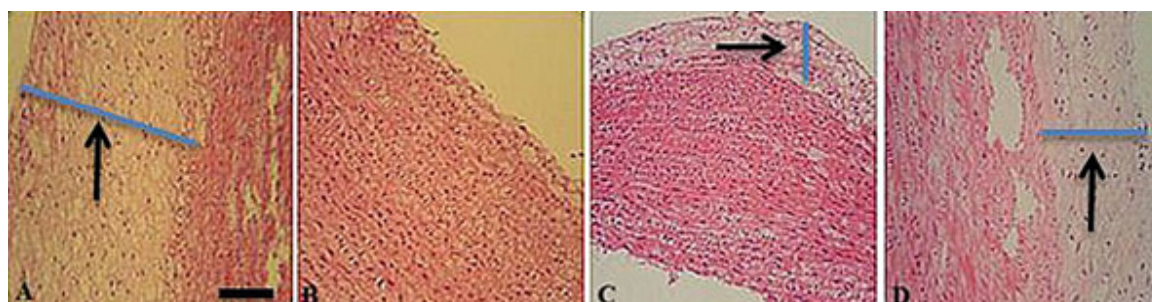


Fig. 1. Profile from aorta of (A) hypercholesterolaemic animal, (B) normal rabbit, (C) animals of Atorvastatin group and (D) animals of Treatment. In normal rabbit (B) the aorta wall showed a uniform thickness without bulging in the lumen and the endothelial lining was intact without any interruption. In hypercholesterolaemic rabbit (A) the aorta wall displayed foam cells (arrow and blue bar) with disturbance in the endothelial lining and muscle fibers change. In animals placed in Atorvastatin and Treatment (C and D) the aorta wall appeared normal, no foam cells (arrow and blue bar) without disturbance in the endothelial lining and muscle fibers change. Scale bar: 500 μ m.

TABLE 1. Detection of apoptotic cells by TUNEL staining in experimental groups

Experimental Groups	Number of TUNEL positive cells (Mean \pm SEM)
Normal	4.5 \pm 0.5
hypercholesterolemic diet (HC)	47.5 \pm 3.5
L-carnitine	23.5 \pm 3.5
Atorvastatin	25.5 \pm 3.5
Treatment	15.2 \pm 2.5*

*The mean difference is significant at the .05 level vs. other groups

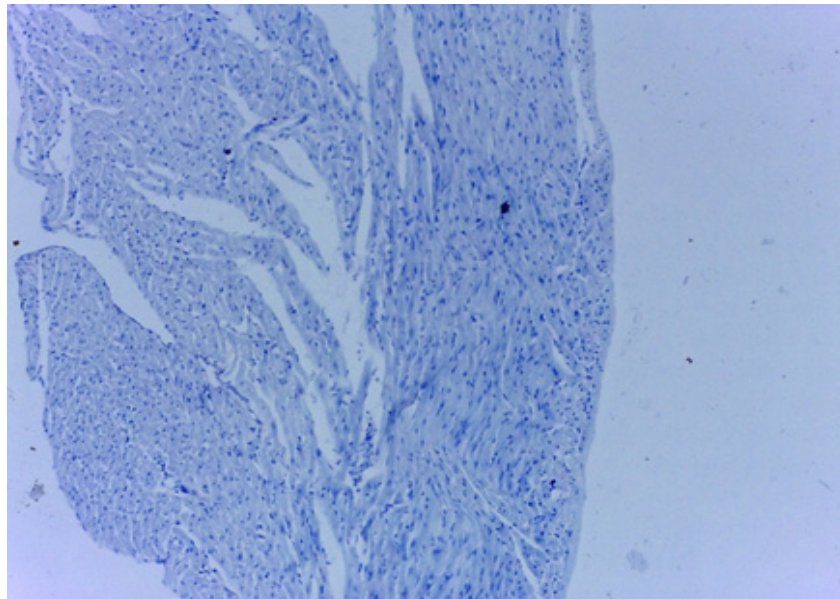


Fig. 2. Efficacy of L-carnitine in treatment group on the heart apoptosis rate. Cell death was determined by the TUNEL method at 10X magnification (black nuclei are the apoptotic cells)

and disturbed endothelial integrity are the main characteristics in atherosclerosis.

Hypercholesterolaemia induced disruption and splitting of the superficial elastic membranes at intimal plaques. Treatment with L-carnitine along with atorvastatin in treatment group significantly improved the aortic architecture compared to the group treated with atorvastatin, as a standard medication, which was intensively disturbed using hypercholesterolaemia ($P < 0.05$). The aorta wall appeared normal, without any disturbed endothelial lining, muscle fiber change, or foam cells (Fig 1C and D). The mean number of inflammatory cells, neutrophils and macrophages, indicated a meaningful difference between atorvastatin and treatment groups ($P < 0.05$) (Table 2). In Normal group myocardial fibers were organized and showed normal striation. The cardiomyocytes were either mononucleated or binucleated which were blue in color and centrally located. Masson's Trichrome staining of myocardium in Normal group showed that the cardiac muscle was bright red and there was no collagen content (blue staining) in between the myocardium fibers. H&E staining of HC group showed that myocardial fibers were disorganized and showed irregular striation. The cardiomyocytes nuclei were acentric and variable in size. Masson's trichrome staining showed the diffuse pattern of collagen

deposition in the atherosclerotic heart. Cardiac myofibers showed loss of striation around area of collagen deposition. The size of nuclei in the cardiomyocytes was variable (Fig 3).

Discussion

Atherosclerosis is a deadly disease [19]. Hypercholesterolemia is one of the most important risk factors for this disease, which causes functional and structural disorders of blood vessels due to deposition in blood vessels [20]. Accumulation of LDL causes monocytes to adhere to the endothelium and ultimately facilitates their differentiation into macrophages, which ultimately leads to the formation of fat streaks/fatty streak [21]. The endothelium regulates the production the extracellular matrix [22,23]

Rabbits have various similarities to humans in terms of lipoprotein metabolism, except for hepatic lipase deficiency [26-28]. Therefore, change in carnitine amounts in such tissues may lead to adverse impacts. Accumulating evidence has revealed a close link of carnitine deficiency to many types of cardiovascular diseases such as arrhythmia, congestive heart failure, acute ischemia, angina pectoris, and peripheral vascular disease [29-31]. Although some clinical and experimental research has shown the effectiveness of L-carnitine treatment in peripheral vascular

TABLE 2. Comparison of mean number of inflammatory cells, macrophages and neutrophils, in experimental groups

Experimental Groups	Number of TUNEL positive cells (Mean \pm SEM)
Normal	84.7 \pm 3.5
hypercholesterolemic diet (HC)	91.5 \pm 3.8
L-carnitine	89.3 \pm 2.9
Atorvastatin	48.5 \pm 3.9
Treatment	40.2 \pm 2.4*

*The mean difference is significant at the .05 level vs other groups

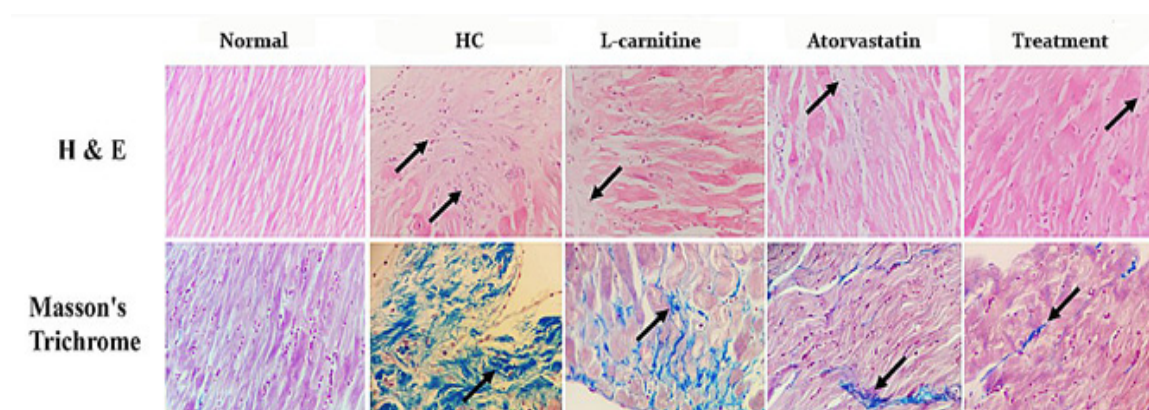


Fig. 3. H&E staining: Myocardium showed areas with faint cross striations (arrows). In treatment group the cardiac muscles showed focal muscle disruption and little irregular striation, which were more evident in HC group (arrow). Masson's trichrome staining: The diffused pattern of collagen deposition in the area of myocardial destruction was observed (arrow).

diseases and atherosclerosis, the relationship of carnitine deficiency to atherogenesis has not yet been examined, and is therefore difficult to study because there is no satisfactory experimental carnitine deficiency–atherosclerotic model [32–39].

Fat-lowering activity by L-carnitine helps in the treatment of peripheral vascular diseases and atherosclerosis [33, 40, 41]. In the present study could be due to another mechanism plus its lipid-lowering activity. Notably, differences in the diet and other research could lead to different pathogenic patterns and effects on cellular machinery of carnitine [40, 42–44]. In the hyperlipidemic-rabbit model, others have observed increased plasma carnitine (free, acyl and total), increased long-chain acyl-carnitine accumulation in tissue, and decreased its tissue content after feeding on a high-fat diet [32].

L-carnitine increases tissue carnitine and also decreases plasma levels to normal levels (before diet) [32]. In our research, L-carnitine supplementation was able to reverse atherosclerotic damage. Decreased endogenous L-carnitine can inhibit long-chain beta oxidation of fatty acids, which in turn reduces ATP supply as well as long-term highly toxic intracellular accumulation.

L-carnitine treatment could stimulate beta-oxidation of long-chain fatty acids, culminating in increased ATP supply and prevention of the accumulation of intermediates of toxic long-chain fatty acids. The results of this study suggest that detailed mechanistic studies should be conducted to examine the levels of total, free and esterified-carnitine under various conditions, and to determine the precise mechanism of prevention of progression of atherosclerotic lesions by L-carnitine.

Inflammation is a factor that both indicates and contributes to atherosclerosis [45]. Macrophages and leukocytes, including polymorphonuclear cells and T-cells, have been reported as the primary cellular components that are essential for the formation of inflammatory plaques [46]. These inflammatory cells accumulate in the plaques of the circulating blood and produce proinflammatory cytokines, recruit inflammatory cells and induce the release of acute-phase reactants on the local and systemic scale, which results in the growth of atheromatous plaques and subsequent plaque rupture [47,48]. Herbal and mineral compounds are used in wound healing [49, 50] and herbal products have healing properties due to their antioxidants, flavonoids, flavonoid compounds and tannins [51-61].

We did not study the potential mechanisms of prevention of atherosclerosis that could be considered as a limitation of the present study. Studies regarding the underlying signal transduction pathways leading to decrease and inhibition of atherosclerosis are recommended.

Conclusion

It can be concluded that L-carnitine can prevent the atherosclerotic lesions' progression, in addition to lipid-lowering impacts of atorvastatin; carnitine deficiency and endogenous carnitine depletion should also be regarded as risk factors for atherogenesis.

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Conflict of interests

There are no conflicts of interests to declare.

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