Beneficial Effects of L-carnitine on Cholesterol-Induced Atherosclerosis in Rabbits: An Animal Model Study

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Twenty five rabbits were randomized into five groups (n = 5): Normal Control group (NC) were kept on a plain chow diet for 75 days. AT group were kept on a hypercholesterolaemic diet for 75 days. ATO (atorvastatin) group were fed the same diet and received atorvastatin orally (20mg/kg/day) from day 45 for 30 days. L group were fed the same diet and intraperitoneal L-carnitine (250mg/kg/day) from day 45 for 30 days (completion of treatment). ATO/L group were fed the same diet and atorvastatin orally (10 mg/kg/day) and L-carnitine intraperitoneally (125 mg/kg/day) from day 45 for 30 days. Histopathological investigations of aorta and assessment of serum profile were carried out to determine Blood urea nitrogen (BUN), creatinine (Cr), Sodium (Na), potassium (K), Phosphorus (P), triglycerides (TG), total cholesterol, high-density lipoprotein cholesterol (HDL), low-density lipoprotein cholesterol (LDL), alanine aminotransferase (ALT), aspartate aminotransferase (AST) and Alkaline phosphatase(ALK). The aorta wall of hypercholesterolaemic rabbits showed foam cells with disrupted endothelial lining and changed muscle fibers. The animals of ATO and ATO/L groups had normal aorta wall. TG significantly increased in hypercholesterolaemic rabbits. L-carnitine treatment significantly reduced TG (P<0.05). Serum BUN, Cr, Na, K, and P values did not significantly change in experimental groups (P>0.05) except for L group, where BUN level was significantly higher than those in ATO and ATO/L groups (P<0.05). Serum AST, ALT and ALK levels were significantly reduced in L-carnitine treated animals compared to ATO group (P<0.05). L-carnitine prevented progression of atherosclerotic lesions.

Keywords: L-carnitine, Cholesterol-induced atherosclerosis, Rabbits, Atorvastatin.

Introduction

Atherosclerosis is triggered by sequences of extremely specific molecular and cellular responses of the endothelium to atherogenic factors. Hypercholesterolaemia, smoking, vascular endothelial injury, diabetes, hypertension, increased platelet aggregation, reduction in nitric oxide bioactivity and/or synthesis, lipid peroxidation, inflammation and genetic alterations are predisposing to atherosclerosis [1,2]. Formation and progression of atherosclerosis is a time-consuming process which begins with accumulation of fat inside the vessels walls. Fat accumulation triggers an inflammatory process as well as platelet activation and accumulation which subsequently lead to formation of an atherosclerotic plaque [2,3]. Some traditional risk factors for initiation or progression of plaques include hypertension, smoking, diabetes mellitus, obesity, hyperlipidemia and infections.

Although different types of medications are available for various risk factors, most of them are aimed at preventing atheromas and plaques from establishing and preventing the established plaques from growing.
These risk factors induce endothelium adhesiveness, accumulation of mononuclear cells, as well as migration and proliferation of smooth muscle cells, leading ultimately to vessel wall thickening.[2,3]. In a phenomenon namely remodeling, the vessel wall compensates by gradual dilatation so that the lumen can remain unchanged. In some points, the vessel wall discontinues compensation through dilatation and as a result the lesion may bulge into the lumen, culminating in a decline in the blood flow and obstruction [3]. For controlling or preventing atherosclerosis from progressing, and the resulting pathological consequences, the initial therapeutic strategy is to reduce the atherogenic agents. For this purpose, a variety of agents have been suggested, such as L-carnitine and its natural short chain derivative propionyl-L-carnitine.[4]. L-carnitine, a natural quaternary ammonium, is essential to transport activated long-chain fatty acids from cytoplasmic compartment into mitochondria where beta-oxidation enzymes are present [5].

L-carnitine is considered to be a therapeutic agent for the treatment of hyperlipidaemia due to its contribution to fatty acid metabolism. In a hyperlipidaemic-rat model, L-carnitine was found to lower the synthesis of very-low-density lipoprotein VLDL by promoting beta-oxidation and increasing the hepatic level of fatty acid binding proteins[6,7]. It has also been reported that the hypolipidemic impact of a 4-weeks L-carnitine treatment in rabbits fed with a fat-enriched diet for this duration, was partly because of reduced transport of VLDL-TG and not because of a change in its fractional catabolic rates[8]. Other studies have shown that L-carnitine affects the cholesterol metabolism by inhibiting 3-hydroxy-3-methyl-glutaryl-CoA reductase activity along with increasing LDL binding[9]. Carnitine deficiency has been reported to play a substantial part in the pathophysiology of many cardiovascular diseases [10].

As part of the innate immune response, neutrophils are extremely efficacious effector cells, which can rapidly be mobilized and recruited to sites of inflammation, carrying multiple weapons that are either primarily directed against an enormous variety of pathogens or serve as signal molecules for the subsequent recruitment of secondary immune cells. Furthermore, the neutrophils are known to be the main protagonist in the first line of defense under acute inflammatory conditions. However, in recent years, the neutrophils have drawn more attention with respect to chronic inflammatory processes, such as atherosclerosis, adipose tissue inflammation, and arthritis [11].

Macrophages in atherosclerotic lesions actively participate in lipoprotein ingestion and accumulation, leading to the creation of foam cells filled with lipid droplets. Accumulation of foam cells contributes to lipid storage and atherosclerotic plaque growth. Popular of macrophages the atherosclerotic plaque have a reduced ability to migrate, which leads to failure of inflammation resolution and furthered progression of the lesion into complicated atherosclerotic plaque. In this stage, macrophages contribute to the maintenance of the local inflammatory response by secreting proinflammatory cytokines, chemokines and producing reactive oxygen species. Dying macrophages are responsible for necrotic core formation in progressing plaques[12]. To the best knowledge of the authors, the evidence is scant regarding the effects of endogenous carnitine depletion and/or carnitine deficiency in athrogenesis.

Given this, this study aimed to examine the potential beneficial effects of L-carnitine on cholesterol-induced atherosclerosis in rabbits. To this end, our assessments were mainly histopathological investigations and analyses of serum and urine.

Materials and Methods

Study design and animals

A total of 25 male white New Zealand rabbits aged 3–4 months and weighing 2.0–2.5 kg were randomly assigned to four experimental groups of five each. In Normal Control (NC) group, the animals were kept on a plain chow diet for 75 days. In AT group, the animals were fed with a hypercholesterolaemic diet for 75 days. In Atorvastatin (ATO) (as a standard medication) group, the animals were kept on a hypercholesterolaemic diet for 75 days and received atorvastatin orally (20mg/kg/day) from day 45 for 30 days [13].

In L 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) [14]. In ATO/L group, the animals were kept on
a hypercholesterolaemic diet for 75 days, and atorvastatin orally (10 mg/kg/day) and L-carnitine intraperitoneally (125 mg/kg/day) from day 45 for 30 days (end of the study) [15].

Induction of hypercholesterolaemia in rabbits

Since two weeks before the study and during the experiment, the animals were separately caged in plastic cages at 23 ± 3°C, under natural relative 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 procedures performed on the animals were in full compliance with the Guide for the Care and Use of Laboratory Animals (NIH US publication no. 85–23 revised 1985).

All experiments were also carried out according to the relevant ethical recommendations of the Pasteur Institute of Iran; The study protocol was carefully revised and approved by the Ethics Committee of Urmia University of Medical Sciences. L-carnitine and cholesterol were procured from Sigma (St. Louis, USA). All other chemicals used in this study had the highest analytical grade, as well.

Induction of hypercholesterolaemia in rabbits

The cholesterol-fed rabbit model is valuable because of the quick development of aortic lesions and low cost of maintenance. The conventional diet for induction of atherosclerosis includes supplementation of 2% cholesterol per weight (cholesterol enriched fat diet) for around 75 days [16]. The diet was prepared by spray coating the standard chow pellets containing cholesterol along with acetone, ethyl alcohol, PVP, acetyl alcohol and carbowax to serve as solvent and binding.

This method was used to prepare hypercholesterolaemic pellets with uniformly distributed cholesterol, and was welcomed by the rabbits with no different appearance and taste compared to the regular chow pellets.

Histopathological investigations of aorta and detection of macrophages and neutrophils in atherosclerotic plaques

The aorta including ascending and descending parts was excised and totally immersed in phosphate buffered formalin (PBF) for at least 24 hours for fixation. Following fixation, the samples were embedded in paraffin. Then 5 µm sections were obtained from each paraffin block and stained by Hematoxylin and Eosin (H&E) method. Maximum wall thickness of aorta as well as intima thickness (plaque size) of each sample was measured using an image evaluation program (Optika, Italy) and compared with others. The presence of macrophages and neutrophils in atherosclerotic plaques were also measured. NASDCE staining, a common method to identify neutrophils in tissue was adopted [13].

Assessment of Serum profile

At the completion of the experiment, the rabbits were fasted for 12 hours before anesthesia and peripheral blood samples were obtained from the marginal vein of their ears. Then, serum samples were isolated and analyzed for the levels of blood urea nitrogen (BUN), Cr, Na, K, P, TG, TC, HDL, LDL, AST, ALT and ALK.

Statistical analysis

The data were reported as mean ± standard error of the mean (SEM). Data analysis was performed by SPSS 16.0 (SPSS Inc., Chicago, IL, USA) using analysis of variance (ANOVA). Normality of data was investigated by Kolmogorov–Smirnov test, and P ≤ 0.05 was considered significance level.

Results

Atherosclerotic plaque formation

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

Histopathological findings

Histopathological results regarding aorta in the NC 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 shown by three intimal plaques indicated by arrows.

Magnification of these plaques images revealed extremely stained foam cells and endothelial gaps in the sub-endothelial layer.

Formation of foam cells already filled with cholesterol ester and disturbed endothelial integrity are the main characteristics in atherosclerosis.

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Moreover, hypercholesterolaemia induced disruption and splitting of the superficial elastic membranes at intimal plaques. Treatment with L-carnitine along with atorvastatin in ATO/L group significantly improved the aortic architecture compared to the group treated with ATO, as a standard medication, which was severely disturbed by 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, showed a significant difference between ATO/L and ATO groups ($P<0.05$) (Table 1).

**Serum profile findings**

The 75-day exposure of rabbits to 2% cholesterol-enriched diet produced a markedly significant (5.3-fold) increase in serum cholesterol in comparison to the NC group ($P<0.05$). This increase was mainly notable in the atherogenic particle LDL. The serum TG significantly was increased in hypercholesterolaemic rabbits ($P<0.05$). Besides that, daily administration of L-carnitine and atorvastatin significantly reduced TG when compared to the group treated with ATO, as a standard medication, and produced insignificant change in cholesterol (Fig 2).

Serum BUN, Cr, Na, K, and P levels were significantly different among experimental groups ($P>0.05$) (Fig 3). Serum AST, ALT and ALK levels significantly was reduced in ATO/L treated animals compared to the animals treated with ATO, as a standard medication ($P<0.05$) (Fig 4).

![Fig. 1. Section from aorta of (A) hypercholesterolaemic rabbit, (B) normal rabbit, (C) animals of ATO group and (D) animals of ATO/L. In normal rabbit (B) the aorta wall showed a uniform thickness with no 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 change in the muscle fibers. In animals of ATO and ATO/L (C and D) the aorta wall appeared normal, no foam cells (arrow and blue bar) with no disturbance in the endothelial lining and change in the muscle fibers. Scale bar: 500µm](image)

**TABLE 1. Comparison of mean number of inflammatory cells, in experimental groups. . (mean ± SEM).**

| Experimental Groups | Mean ± SEM |
|---------------------|------------|
| NC                  | 84.7±3.5   |
| AT                  | 91.5±3.8   |
| L                   | 89.3±2.9   |
| ATO/L               | 40.2±2.4*  |
| ATO                 | 48.5±3.9   |

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

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Fig. 2. Serum concentration of TG, TC, HDL and LDL in experimental groups. Values are presented as mean ± SEM. * P< 0.05 vs. AT group.

Fig. 3. Serum concentration of BUN (Blood Urea Nitrogen), Cr (Creatinine), Na (Sodium), K (Potassium), P (Phosphorus) in experimental groups. Values are presented as mean ± SEM. * P< 0.05 vs. ATO group.

Fig. 4. Serum concentration of AST (Aspartate Aminotransferase), ALT (Alanine Aminotransferase) and ALK (Alkaline Phosphatase) in experimental groups. Values are presented as mean ± SEM. * P< 0.05 vs. AT group.
Discussion

Rabbits have various similarities to humans in terms of lipoprotein metabolism, except for hepatic lipase deficiency [17-19].

Some characteristics of rabbits make them an excellent model for the study of impacts of human transgenes on predisposition to atherosclerosis and lipoprotein metabolism. For example, apoB-containing lipoproteins in rabbits and humans are similar; the liver of both produces apoB-100-containing VLDL, and ester-transfer protein is ample in the plasma of both [20-22].

In rabbits used to examine the development of atherosclerosis in humans, lesions are quickly induced by cholesterol supplementation of their diet with cholesterol (<0.5%); Therefore, a moderate hypercholesterolemia is induced, with plasma cholesterol levels of 200-800 mg/dl [23]. As a result, the lesions produced are different from those in humans from topographical and morphological perspective [24]. This difference can be, to some extent, attributed to humans not usually having large amounts of cholesterol: generally their plasma cholesterol levels do not exceed 800 mg/dl, and they process and tolerate cholesterol intake better than rabbits.

In addition, long-term experiments in rabbits on diets with large contents of cholesterol are not favourable because the hepatotoxicity and the likelihood of animal’s thriving lack [25-28].

In our study, histopathological investigations during the treatment, revealed that the cholesterol level increased quickly and the atheroma was formed in the aorta.

It has been established that L-carnitine is a vital agent, in addition to other factors, for mitochondrial transport and oxidation of long-chain fatty acids which are the preferable substrate for production of ATP in the cells of vascular endothelial and smooth muscles and myocardium [29,30]. 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 [11,12,31]. Carnitine treatment has been frequently found to enhance the mechanical function of such hearts.

Although some clinical and experimental researches have shown the effectiveness of L-carnitine treatment in peripheral vascular 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 [4,5,32-36].

To examine this relationship, the present study examined whether L-carnitine supplementation would prevent the progression of atherogenesis in rabbits feeding with a 2% cholesterol-enriched diet. Under our experimental conditions, 35 days treatment with L-carnitine completely prevented the progression of atherosclerotic lesions which were clearly shown in aorta hypercholesterolaemic rabbits. In rabbits as a model of diet induced hyperlipoproteinaemia, it has been reported that L-carnitine administration obviously prevents the formation of early atherosclerotic lesions by reducing the extent of liver steatosis and decreasing plasma triglyceride and cholesterol values [4].

Although the lipid-lowering property of L-carnitine contributes greatly to its therapeutic potential in peripheral vascular diseases and atherosclerosis [5,7,8,33], the protection achieved by L-carnitine against atherosclerosis in the present study could be due to another mechanism in addition to its lipid-lowering activity.

Notably, differences in the diet lipid composition in our study and other research could lead to different pathogenic patterns and different effects on cellular machinery of carnitine [6,9]. In the hyperlipidemic-rabbit model, others have observed increased plasma carnitine (free, acyl and total), increased accumulation of long-chain acyl-carnitine in tissue, and decreased its tissue content (free, short-chain and total) after feeding on a high fat diet [4]. They also reported that, supplementation with L-carnitine raised tissue carnitine content and reduced its plasma content to the normal values before the diet [4]. In the present study, L-carnitine supplementation could reverse the atherosclerotic damage. A likely explanation for this is that, the depletion of endogenous carnitine
L-carnitine could cause the beta-oxidation of long-chain fatty acids to be inhibited, leading to a decrease in ATP supply and an increase in the buildup of intracellular highly toxic long-chain fatty acid intermediates, such as long-chain acyl-carnitine and long-chain acyl-CoA. On the other hand, 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[36]. 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[38]. These inflammatory cells accumulate in the plaques of the circulating blood and induce the production of proinflammatory cytokines, recruit inflammatory cells and induce the production of acute-phase reactants on the local and systemic scale, which results in the growth of atheromatous plaques and subsequent plaque rupture[38].

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 which lead to reduction and inhibition of atherosclerosis are recommended.

Conclusion

It can be concluded that L-carnitine prevents the progression of atherosclerotic lesions by another mechanism, 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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