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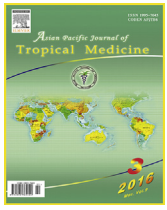

Original research http://dx.doi.org/10.1016/j.apjtm.2016.01.025

Effect of levocarnitine on TIMP-1, ICAM-1 expression of rats with coronary heart disease and its myocardial protection effect

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ARTICLE INFO

Article history:
Received 15 Dec 2015
Received in revised form 20 Dec 2015
Accepted 30 Dec 2015
Available online 11 Jan 2016

Keywords:
Levocarnitine
Coronary heart disease
TIMP-1
ICAM-1

ABSTRACT

Objective: To study the effect of levocarnitine (L-CN) on tissue inhibitor of metalloproteinase-1 (TIMP-1) and intercellular adhesion molecule-1 (ICAM-1) expression of rats with coronary heart disease and evaluate the protective effect of L-CN on myocardial cells.

Methods: High-fat diet feeding and intraperitoneal injection of pituitrin was performed on rats in model group and CHD Model of rats was built. Rats with successful model-building were selected and divided into L-CN group and Ctrl group randomly. Rats in L-CN group were given L-CN treatment, with intraperitoneal injection of 200 $mg \cdot kg^{-1} \cdot d^{-1}$ and successive administration for 3 d. Rats in Ctrl group were given equal volumes of normal saline. Blood was collected from carotid artery at different time and expression quantity of creatine kinase-MB (CK-MB) and Troponin I (TnI) in serum was detected. Rats in each group were put to death and were separated to obtain the myocardial tissue. Real-time PCR and Western Blotting hybridization were performed to detect the TIMP-1, ICAM-1 expression in myocardial tissue in each group. Statistical analysis was employed to explore the expression changes of TIMP-1 and ICAM-1, and ELISA test was used to analyze the expression changes of myocardial necrosis marker-CK-MB and TnIto learn the effect of L-CN and its myocardial protective effect.

Results: The total cholesterol, triglyceride and blood glucose levels of rats in model group were significantly higher than that in control group, which indicated that due to high-fat diet feeding, blood lipid of rats in model group was obviously higher than that in control group. In myocardial tissue of rats in model group, TIMP-1 level significantly reduced and ICAM-1 level significantly increased ($P < 0.01$). In model group, after L-CN treatment, TIMP-1 level had double increase, while ICAM-1 level had 43% of decrease in L-CN group compared with Ctrl group. After L-CN intervention treatment, CK-MB and TnI content in L-CN group relatively reduced compared with Ctrl group. The difference among groups was obvious ($P < 0.01$).

Conclusions: L-CN could increase the TIMP-1 expression level and inhibit the ICAM-1 expression level. L-CN has a certain myocardial protective effect.

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Peer review under responsibility of Hainan Medical College.
Foundation project: It was supported by Natural Science Foundation of China (81270238), Doctoral degree Scientific Research Foundation of Ministry of Education (20100131110059) and Shandong Science and Technology Development Plan (2012G0021850).

1. Introduction

Cardiovascular disease seriously threatens the health of people. At present, about 3.5 million people die of cardiovascular disease in our country each year. In coronary atherosclerotic heart disease [Coronary heart disease (CHD) for short], the lipids in the blood are deposited on the endarterium due to abnormal lipids metabolism, which result in the similar atheromatous lipids deposition on the endarterium. These lipids deposition would cause the arterial stenosis, block the blood flow, lead to heart ischemia and then result in clinical symptoms, like angina pectoris. Due to the higher morbidity rate and mortality rate, CHD has become the most serious cardiovascular disease threatening people's health [1,2]. CHD is a kind of disease induced by multiple factors. At present, studies show that some primary diseases, like primary hypertension, diabetes, dyslipidemia, are the risk factors inducing CHD. In addition, some habits, like smoking and drinking alcohol, could also induce the abnormal lipids metabolism [3]. Along with in-depth researches on the pathogenesis of CHD, we have achieved great progress for molecular mechanism researches of CHD. Reactive oxygen species accumulation, adhesion molecule increase [4], homocysteine increase in blood and the abnormal expression of blood fibrinogen and clotting factor could result in the occurrence of CHD [5].

At present, clinical treatments for CHD mainly include: blood lipid regulation treatment, anti-platelet aggregation treatment, thrombolytic and anticoagulant drug treatment, angiotensin converting enzyme inhibitors *etc* [6]. Therapeutic drugs for CHD mainly include: nitrates, statin antilipemic agents, anti-platelet agents, calcium channel blockers *etc*. Levocarnitine (L-CN) is a kind of small molecule amino acid derivatives and plays an important role in fatty acid oxidation and other major metabolic pathways. L-CN could adjust the balance between sugar and fatty acid oxidation through promoting sugar oxidation, reduce the toxic effect of long chain fatty acyl carnitine deposition at ischemic stage on myocardium, optimize the myocardial energy metabolism and then improve the diastolic and systolic function of heart [7–9]. At present, there are no in-depth specific mechanism researches on L-CN regulating blood lipid metabolism. In this paper, CHD model of rats is built to discuss the effect of L-CN on Tissue inhibitor of metalloproteinase-1 (TIMP-1) and Intercellular Adhesion Molecule-1 (ICAM-1) expression at mRNA and protein level. Besides, myocardial necrosis marker-creatine kinase-MB (CK-MB), Troponin I (TnI) expression changes are analyzed to discuss the effect of L-CN on TIMP-1, ICAM-1 expression of rats with CHD and its myocardial protective effect.

2. Materials and methods

2.1. Materials

2.1.1. Experimental animals

A total of 30 specific pathogen free (SPF) Wistar rats, with weight (250 ± 50) g, were bought from Beijing Vital River Experimental Animal Technology Co. Ltd. Rats were fed in standard animal cages, with 5 rats per cage. Free eating and drinking were allowed for rats during experimental period. Feeding room had excellent ventilation and natural day and night lighting, with indoor temperature maintained at (18–25) °C.

2.1.2. Reagent and instrument

Cholesterol was bought from Shanghai Biochemical Reagent Co. Ltd. Sodium cholate was bought from JK Chemical Science and Technology Ltd. Propylthiouracil was bought from SIGMA (America). Posterior pituitary injection was bought from Nanjing Xinbai Pharmaceutical Co. Ltd. RNA extraction kit was bought from QIAGEN (German). Inverse transcription kit was bought from Applied Biosystems (America). SYBR Green real-time PCR Master Mix was bought from Applied Biosystems (America). ReadyPrep protein extraction kit was bought from Bio-Rad (America). BCA protein quantitation kit was bought from Vazyme Biotech (China). TIMP-1, ICAM-1 and GAPDH monoclonal antibody was bought from Abcam (UK). HRP-Goat Anti-Mouse IgG was bought from Wuhan Boster Biotechnology Co. Ltd. PVDF membrane (0.22 μm) was bought from Millipore (America). ECL chemiluminescence detection kit was bought from Millipore (America). CK-MB, TnI ELISA detection kit was bought from Wuhan Uscn. AEROSET fully automatic biochemical analyzer: Abbott Laboratories. Nucleic acid quantitation analyzer: Qubit Fluorometer. Fluorescent quantitation PCR detecting system: Applied Biosystems 7500.

2.2. Methods

2.2.1. Building of CHD model of rats [6–10].

A total of 30 SPF Wistar rats were randomly divided into control group (n = 10) and model group (n = 20). High-fat diet feeding and intraperitoneal injection of pituitrin were performed on rats in model group and then CHD Model of rats was built. Rats in control group were fed with normal diet. High-fat diet formula consisted of yolk powder (10%), cholesterol (2%), lard oil (10%), propylthiouracil (0.2%), sodium cholate (0.5%), normal diet (77.3%). Each rat in model group was fed with 30 g of high-fat diet everyday, with continuous feeding for 10 weeks. Weight changes of rats were weighed.

Forty-eight hours before the final feeding, rats in model group were injected with pituitrin (30 U/kg), with once every day and continuous injection for 2 d. Before rats were put to death, blood was collected from carotid artery and serum was separated for storing at −20 °C. Blood liquid biochemical analyzer was used to detect the total cholesterol (TC), high-density lipoprotein (HDL), low density lipoprotein (LDL) cholesterin, triglyceride (TG) and blood glucose (BG) for confirming the successful building of model. The animal experiment was performed strictly according to the animal experiment projected that was approved by the animal ethics committee and it was inspected through the daily supervision of welfare ethics.

2.2.2. Real-time PCR detection method

Rats with successful model building were selected and were randomly divided into L-CN group and Ctrl group (n = 10). Rats in L-CN group were given L-CN treatment, with intraperitoneal injection of 200 $mg \cdot kg^{-1} \cdot d^{-1}$ and successive administration for 3 d. Rats in Ctrl group were given equal volumes of normal saline. Rats in each group were put to death and myocardial tissues of rats were separated immediately. Precooling PBS (Rnase free) was used to wash the tissues. Tissue samples were ground in liquid nitrogen and total RNA was extracted from cells based on RNA extraction kit descriptions. Then Qubit

Fluorometer was used to detect its concentration and purity. According to reverse transcription kit descriptions, total RNA was inversely transcribed to cDNA and Real-time PCR was performed to detect the related gene. mRNA sequence of target gene was referred in NCBI database and Real-time PCR primers were designed, which were consigned to Beijing Liuhe BGI for gene synthesis. The specific sequence was as followed: *TIMP-1* (NM_011593.2): For: CGAGACCACCTTATACCAGCG; Rev: ATGACTGGGGTGTAGGCGTA, *ICAM-1* (NM_010493.2): For: GTGATGCTCAGGTATCCATCCA; Rev: CACAGTTCTCAAAGCACAGCG. Ct values of each gene amplification were detected and Ct values and DNA initial copy numbers showed negative correlation. Double ΔCt value method was used to calculate the relative expression levels of target gene: the average value of three parallel repeated experiments was calculated as the Ct value of each sample. ΔCt = Ct (Target Gene)-Ct (b-actin), ΔΔCt = ΔCt (sample) −ΔCt (contro1). Therefore, the relative expression levels of target gene = $2^{-\Delta\Delta Ct}$. The relative expression quantity in control group was $2^0 = 1$ [11]. Inverse transcription system (20 μL): 5 × SYBR Green Real-time PCR pre-mixed solution (4 μL); iScript reverse transcriptase (1 μL); RNA template (1 μg RNA) (1 μg). Primer concentration in the PCR reaction was 300–450 nM, with cDNA template (100 ng) and reaction parameters: pre-degeneration (95 °C, 10 min), degeneration (95 °C, 10 s), annealing (Tm-6 °C, 20 s), extension (72 °C, 33 s), cycle-index (n = 40).

2.2.3. Western blotting

Tissue samples were ground in liquid nitrogen and Ready-Prep Protein Extraction Kit was used to extract the tissue proteins. Proper frequency of short impact on the tissue proteins was performed on ice and mixture was pyrolyzed at 4 °C, with centrifuging for 20 min (13000 r/min). The supernatant was collected and placed into a new centrifuge tube and then BCA Protein Assay Kit was used to detect the protein concentration. SDS-PAGE electrophoresis was performed on protein samples. After electrophoresis, the gel was soaked in transfer buffer solution for 10 min balancing, with assembly shift 'Sandwich', voltage (100 V) and transmembrane (45–60 min). After transmembrane was finished, TBST was used to rinse the PVDF membrane for 15 min. Appropriate dilution of primary antibody [diluted by TBST containing 1% (w/v) skim milk] was added for 2 h incubation at room temperature. TBST was used to rinse the membrane for 3 times, with 5–10 min per time. TBST containing 0.05% (w/v) skim milk diluted second antibody (1:10000, HRP labeled) was used to incubate the membrane; TBST was used to rinse the membrane for 3 times, with 5–10 min per time. Experimental results were exposed and were stored by photo-taking. Quantity one v4.62 software molecular banding grey value was used for statistic analysis, with target protein/b-actin protein semi-quantitative value as the quantitative basis.

2.2.4. ELISA detection method

12, 24, 36, 48, 72 h after L-CN intervention treatment and before rats were put to death, blood was collected from carotid artery and serum was separated. ELISA detection kit was used to detect the myocardial necrosis marker-CK-MB, Tnl levels in the serum samples. Standard hole, sample hole (to be tested) and blank hole were set up. Standard substances with different concentrations and samples to be tested were put into standard hole for 2 h incubation at 37 °C; 100 μL of detection solution (biotinylated primary antibody) was added into each hole and tectorial membrane was used to cover the ELISA plate for 1 h incubation at 37 °C; liquid in the hole was poured out and 350 μL of cleaning solution was used for each hole washing, with soaking for 1–2 min and repeated plate-washing for 3 times; 100 μL of HRP-labeled second antibody was added into each hole and tectorial membrane was used to cover the ELISA plate for 30 min incubation at 37 °C; 90 μL of TMB substrate solution was added into each hole and tectorial membrane was used to cover the ELISA plate, with keeping out of the sun and coloration for 15–25 min at 37 °C; upon the front 3–4 holes of standard hole presented obvious gradient blue color, the reaction was terminated and 50 μL of 2M H_2SO_4 was added. ELIASA was used immediately to detect the OD value of each hole at 450 nm.

2.2.5. Statistical analysis

Statistical software SPSS 17.0 was used for statistical analysis. Results were expressed by mean ± SD. Comparison between groups was performed by t-test, with significance testing level: $\alpha = 0.05$.

3. Results

3.1. Identification of CHD model of rats

After the continuous feeding for 10 weeks, weight of rats in control group was (326 ± 12) g and weight of rats in model group was (519 ± 17) g. Compared with control group, weight of rats in model group was significantly increased ($P < 0.01$).

Blood lipid biochemical analyzer was used to detect the TC, HDL, LDL, TG, BG levels for confirming the successful building of model. Drooping spirits, less activity and dull reaction were observable in model group. Blood liquid was further detected by biochemical analysis and the results were as shown in Table 1. TC, TG, BG levels in model group were significantly higher than that in control group, which indicated that because of high-fat diet feeding, blood liquid in model group was significantly higher than that in control group and the model building was successful.

3.2. Effect of L-CN on TIMP-1, ICAM-1 expression of rats with CHD

Compared with control group, *TIMP-1* level significantly decreased and *ICAM-1* level significantly increased ($P < 0.01$) in myocardial tissues of rats in model group. In model group, after L-CN treatment, *TIMP-1* level in L-CN group had double increase, while *ICAM-1* level had 43% of decrease compared with Ctrl group.

In model group, TIMP-1 level decreased and ICAM-1 level increased, which was in accordance with Real-time PCR experimental results. Quantity one v4.62 software molecular banding grey value was used for statistic analysis, with target protein/b-actin protein semi-quantitative value as the quantitative basis, compared with control group, $P < 0.05$. After L-CN intervention treatment for rats in model group, TIMP-1 level increased and ICAM-1 level decreased in a certain degree, compared with Ctrl group.

Table 1
Blood liquid changes of biochemical detection.

Groups	*n*	TC (mM)	HDL (mM)	LDL (mM)	TG (mM)	BG (mM)
Control group	10	0.39 ± 0.06	0.10 ± 0.12	0.53 ± 0.06	1.65 ± 0.14	5.43 ± 0.31
Model group	20	0.81 ± 0.03*	0.39 ± 0.19*	0.86 ± 0.09	3.88 ± 0.16**	12.00 ± 0.46**

vs. control group. $^{*}P < 0.05$;$^{**}P < 0.01$.

Table 2
CK-MB, TnI detection in serum using ELISA test.

Group	*n*	CK-MB (IU/l)	TnI (ng/mL)
Control	10	7.36 ± 1.06	6.21 ± 1.07
Model (Ctrl)	10	13.26 ± 1.61*	10.07 ± 1.62*
Model (L-CN)	10	8.47 ± 0.39$^{\Delta\Delta}$	7.06 ± 0.18$^{\Delta}$

vs. control group $^{*}P < 0.05$;$^{**}P < 0.01$, *vs*. model group (Ctrl)$^{\Delta}P < 0.05$; $^{\Delta\Delta}P < 0.01$.

3.3. Protective effect of L-CN on myocardial tissues of rats with CHD

CK-MB, TnI expression levels were at a lower level in control group. 12 h after L-CN intervention treatment in model group, CK-MB and TnI expression levels had a certain decrease. Along with the continued treatment, CK-MB and TnI expression levels decreased gradually. 3 d after drug treatment, CK-MB and TnI expression levels had no obvious difference with control group. CK-MB and TnI content changes in serum before rats in each group were put to death were shown in Table 2. Myocardial necrosis marker-CK-MB, TnI expression levels in serum significantly increased in model group ($P < 0.05$). After L-CN intervention treatment in model group, CK-MB and TnI content in L-CN group reduced, compared with Ctrl group. The difference among groups was obvious ($P < 0.01$).

4. Discussion

Abnormal lipids metabolism is an important inducing factor resulting in cardiovascular disease. The lipids in the blood are deposited on the endarterium due to abnormal lipids metabolism, which results in the similar atheromatous lipid material deposition on the endarterium. These lipid material depositions would cause the arterial stenosis, block the blood flow, lead to heart ischemia and then result in clinical symptoms, like angina pectoris. At present, clinical treatments for CHD mainly include: blood lipid regulation treatment, anti-platelet aggregation treatment, thrombolytic and anticoagulant drug treatment, angiotensin converting enzyme inhibitors *etc* [6], among which, blood lipid regulation treatment is suitable for all patients with CHD [12].

Carnitine is a kind of native compound and there are three kinds of isomer in nature: L-carnitine, R-carnitine and D-carnitine. Studies showed that only L-CN had physiological and pharmacological effects. L-CN is mainly distributed in the cardiac muscle and skeletal muscle. It could bring the long-chain fatty acids into mitochondrial matrix, promote the fatty acid oxidation and decomposition, provide the ATP for cells and export the short-chain fatty acyl, which is generated in mitochondria. L-CN itself is not the nutrient of organism, but a kind of necessary cofactor of fatty acid entering into mitochondria for β-oxidation [13,14]. Researches showed that L-CN played an important role during the process of myocardial cell fatty acid metabolism. At present, L-CN is used in the treatment of CHD clinically. L-CN is used to adjust the lipid metabolism, reduce the myocardial necrocytosis and fibrosis induced by ischemic hypoxemia, repair the damaged myocardial cells and finally recover the normal function of heart [15].

Pathogenesis of CHD is very complex. Except for abnormal lipids metabolism, inflammation involving in the process of atherosclerosis is also generally recognized by scholars. Studies found that inflammatory reaction ran through the whole process from lipid formation to plaque rupture [16,17]. The reconstruction of extracellular matrix plays an important role during the process of atherosclerosis development. Researches found that matrix metalloproteinases (MMPs) played an important regulating effect during the process of extracellular matrix formation [18]. The abnormal MMPs expression or enzymatic activity could result in the epimatrix deposition and tissue inhibitor of matrix metalloproteinases (TIMPs) could inhibit the MMPs activity. The abnormal MMPs expression promotes the excessive proliferation of smooth muscle cells, which results in various inflammatory cell infiltration, causes intravascular stenosis and then promotes the atherosclerotic formation. There is evidence that TIMPs plays an effect of regulating blood lipid metabolism through regulating the MMPs activity [19]. However, there are no in-depth researches on the action mechanism of TIMPs during the process of CHD development. In this paper, CHD model of rats was built to discuss the effect of L-CN on TIMP-1, ICAM-1 expression and its myocardial protective effect, aiming to provide some theoretical basis for CHD clinical treatment.

The building of CHD model of rats: model rats were randomly divided into L-CN group and Ctrl group. Rats in L-CN group were given L-CN treatment, with intraperitoneal injection of 200 $mg \cdot kg^{-1} \cdot d^{-1}$ and successive administration for 3 d. Rats in Ctrl group were given equal volumes of normal saline. After L-CN intervention treatment, Real-time PCR detection was used to analyze the effect of L-CN on TIMP-1, ICAM-1 expression. The results showed that in myocardial tissues of rats in model group, TIMP-1 level was significantly reduced and ICAM-1 level was significantly increased ($P < 0.01$). In model group, after L-CN treatment, TIMP-1 level had double increase, while ICAM-1 level had 43% of decrease in L-CN group, compared with Ctrl group, which were in accordance with the western blotting hybridization results. After L-CN intervention treatment for rats in model group, TIMP-1 level in myocardial tissues was increased and ICAM-1 level was decreased in a certain degree, compared with Ctrl group, which indicated that L-CN, as a kind of regulatory factor of lipid metabolism, the biological effects of which were showed at multiple levels. L-CN could inhibit the MMPs through increasing the TIMP-1 expression. In addition, L-CN could reduce the immune cell (monocyte *etc*) infiltration, inhibit the inflammatory process and finally slow down the process from lipid formation to plaque rupture.

CK-MB and TnI were the most common biochemical indexes for myocardial damage diagnosis [20]. In order to explore the protective effect of L-CN on myocardial cells, we discussed the protective effect of L-CN on myocardial tissues from the perspective of myocardial damage. Serum of rats in model group was separated and ELISA test was used to detect the myocardial necrosis marker-CK-MB, TnI expression changes in serum. The results showed that myocardial necrosis marker-CK-MB, TnI expression levels in serum were significantly increased in model group ($P < 0.05$). After L-CN intervention treatment for rats in model group, CK-MB, TnI content were relatively reduced, compared with Ctrl group. Difference among groups was obvious ($P < 0.01$). Through myocardial damage markers, we could verify that L-CN had a certain repairing and protective effect on myocardial tissues of rats with CHD. As for this protection mechanism, we thought that myocardial cells could obtain the metabolism required L-CN in a normal physiological state. When ischemic hypoxemia occurred, myocardial cell could not obtain adequate ATP. Therefore, exogenous supplement of L-CN could relieve the myocardial damages induced by endogenous L-CN synthesis disturbance.

Conflict of interest statement

We declare that we have no conflict of interest.

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