Combination of vitamin E and L-carnitine is superior in protection against Isoproterenol-induced cardiac affection: a histopathological evidence

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Combination of vitamin E and L-carnitine is superior in protection against Isoproterenol-induced cardiac affection: a histopathological evidence

Short running title: Vitamin E and L-carnitine relieve Isoproterenol-induced?

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Abstract

Background: L-carnitine and Vitamin E have antioxidant properties. This study aimed to assess the effectiveness of L-carnitine, Vitamin E and the combination of them in protection against isoproterenol (ISO)-induced biochemical and histopathological changes in rat heart.

Material and methods: Fifty male Wistar rats assigned to 5 groups; control, ISO-treated group (100 mg/kg), ISO+vitamin E-treated group (100 IU/kg), ISO+L-carnitine (100 mg/kg) and ISO+vitamin E+L-carnitine treated group. At the end of the experiment, serum cardiac enzyme as well as the cardiac level Malondialdehyde (MDA), antioxidant enzymes and inflammatory cytokines IL-6, TNF-alpha were assessed. Histopathological changes in the left ventricle wall was assessed using the light and electron microscopy.

Results: Treating rats with vitamin E and L-carnitine could alleviate ISO-induced changes as it significantly reduced the serum level cardiac enzymes, MDA and IL-6, TNF-alpha and improved the antioxidants enzymes (SOD, GSPxase and GSRase). Histopathological, they improved cardiac fibers atrophy, hemorrhages between cardiac fibers, lost striations, and disturbed sarcomere structure. The combined effect of vitamin E and L-carnitine was more superior compared to the other groups.

Conclusion: Combined administration of vitamin E, L-carnitine ameliorated the biochemical and histopathological cardiac affection induced by ISO. The effect seemed to be mediated
through the antioxidant and anti-inflammatory effect of vitamin E, L-carnitine. Administration of these two elements is recommended for patients at risk for myocardial infarction.

**Key words:** vitamin E, L-carnitine, Isoproterenol, heart, histology, antioxidant, anti-inflammatory

**Introduction**

L-carnitine, L-trimethyl 1-3-hydroxy ammoniobetanoate, is a natural endogenous water-soluble antioxidant located on the mitochondrial membrane and is found in all mammals. It reduces the intracellular buildup of toxic metabolites in ischemic conditions [25]. It was reported that "Carnitine plays a pivotal role in myocardial energy metabolism through transport of long-chain fatty acyl intermediates, across the inner mitochondrial membrane for subsequent oxidation, and regulation of carbohydrate metabolism by modulation of the intramitochondrial acetyl-CoA:CoA ratio" [26]. As such, it is used in the prevention and treatment of oxidative stress and related health problems [1]. It exerts its antioxidant potential by reducing reactive oxygen species (ROS) production and scavenging free radicals [17].

A link was reported between consumption of carnitine and cardiovascular diseases [27]. However, contradictory data were found regarding beneficial effects of L-carnitine. Liepinsh et al. reported that long-term decrease in L-carnitine is important for the energy metabolism regulation and treatment of atherosclerosis and heart diseases [18]. On the other hand, Zambrano et al. reported that arterial hypertension-related cardiac fibrosis could be inhibited by L-carnitine through modulation of Peroxisome Proliferator Activated Receptor-γ expression [35].

Vitamin E, a fat-soluble vitamin, possesses a powerful antioxidant activity [2]. Administration of Vitamin E was found to decrease the cardiovascular events in diabetic patients [20]. Contradictory data were found in literature regarding the role of Vitamin E in protection against cardiac disease. Hu et al. found that Vitamin E exerted harmful effect in young female mice, as it increased cardiomyocyte apoptosis after induction of myocardial infarction while it is protective in aged male mice [13].

Myocardial infarction (MI), an ischemic induced cardiac muscle necrosis, which resulted from "imbalance between coronary blood supply & myocardial demand" [32]. Induced
myocardial infarction using isoproterenol was adequately reproduced by many researchers [11]. It was used to test therapeutic or cardioprotective effect of any new medication or adjuvant natural supplementation [7]. Therefore, this study was designed to evaluate the effectiveness of L-carnitine, Vitamin E and the combination of both in protection against isoproterenol-induced biochemical histopathological changes in rat heart.

Materials and Methods
This study was conducted after obtaining the ethical approval of the biomedical research ethics committee, Faculty of Medicine, King Abdulaziz University (KAU), Jeddah Saudi Arabia and was done at the King Fahad Medical Research Center (KFMRC), King Abdulaziz University (KAU).

Drug
Isoproterenol hydrochloride was obtained from Sigma chemical company, St. Louis, MO, USA. Vitamin E and L-carnitine were obtained from GNC Store at Jeddah, Saudi Arabia.

Animals
Fifty male Westar albino rats weighing from 150 to 200 grams and were purchased from the experiment animal unit at the KFMRC. They were kept in large cages at 25 °C with a 12-hour dark/light cycle with free mobility and allowed free access to water and standard rat diet ad libitum. They were left to acclimatize for two weeks in their cages before start the experiment.

Experimental protocols
Rats were randomly assigned to 5 groups (n= 10 rats). GI: served as a control group. Rats of this group were injected subcutaneously with 2 ml of saline daily for 4 weeks. GII: in which rats were subcutaneously injected with isoproterenol (100 mg/kg body weight (BW)) in 2 ml of saline daily for 3 successive days according to Panda and Kar [23]. GIII: in which rats were subcutaneously injected with isoproterenol with same dose and duration plus vitamin E (100 IU/kg BW) intraperitoneal injection daily for 4 weeks according to Gayathri et al., [8]. GIV: in
which rats were subcutaneously injected with isoproterenol plus intraperitoneal daily injection of L-carnitine (100 mg/kg BW) for 4 weeks according to Wong et al. [33].

GV: in which rats were subcutaneously injected with isoproterenol plus vitamin E and L-carnitine at the same dose for 4 weeks.

**Biochemical assessment**

At the end of the experiment, blood sample was collected from the retro-orbital vein under after thiopental anesthesia. Blood was centrifuged at 4000 g at 4°C for 15 minutes and the serum was stored at -80°C till the time of the biochemical assessment of serum aspartate transaminase (AST), lactate dehydrogenase (LDH), and creatine kinase (CK-MB) by colorimetric method using commercial KIT from Biomedical [30]. Rats were then sacrificed with cervical dislocation, the chest was open and the heart was immediately dissected out.

Part of the heart wall was homogenized in 9 volumes of ice-cold saline and centrifuged at 8000 g at 4°C for 20 min. Malondialdehyde (MDA) level was assessed in the homogenate as was described according to Moran [2]. SOD activity was assessed in the homogenate by the nitro blue tetrazolium reduction method according to Habig et al. [12]. Glutathione peroxidase (GSPlaxe) was assessed as described by Pagila and Valentine [22] and glutathione reductase (GSRase) was estimated according to Deore et al. [6]. Levels of inflammatory cytokines IL-6, TNF-α were measured by ELISA kits from BIORAD (England).

**Histopathological assessment**

The dissected heart was washed with saline solution, cut transversely and longitudinally and fixed in 10% buffered neutral formalin solution, and processed for obtaining paraffin blocks. The latters were sectioned serially at 4-6 µ and routinely stained with hematoxylin and eosin [3].

Specimens measured about 2x2 mm from left ventricle were fixed in 3% glutaraldehyde in phosphate buffer at pH 7.4 for 24 hours, and post fixed in 1% osmic acid for 1 hour and further processed to be examined transmission electron microscopy (TEM) (JEM-100 Cx11; Jeol) at Assuit university, Assuit, Egypt. In order to determine the orientation of the ultrathin sections, semithin ones with thickness 1 mm were prepared and stained using toluidine blue to be examined by the light microscope. The ultrathin sections (prepared at the thickness of 500–800 Å) were stained with uranyl acetate and lead citrate.
Results

Biochemical findings

In this study, rats received ISO showed a significant increase in the serum level of cardiac enzymes included; AST, LDH and CK-MB compared to the control rats. Treating rats with vitamin E, L-carnitine or both of them along with ISO resulted in a significant reduction in these enzymes levels compared to the untreated rats. Although the level of these enzymes did not reach that of the control group. The combined effect of both vitamin E and L-carnitine was more significant on the level of CK-MB compared with LDH and AST Figure (1A). The level of MDA, a marker of oxidative stress, was assessed in the cardiac tissue homogenate. It was observed that ISO significantly elevated MDA level in the cardiac homogenate compared to the control group. Administration of vitamin E, L-carnitine or both of them along with ISO significantly decreased in MDA in the cardiac tissue compared to the untreated group Figure (1B).

The level of SOD, GSPxase and GSRase; the cardiac antioxidant enzymes was assessed in the homogenate of the cardiac tissue. It was observed that administration of ISO induced a significant increase in the level of SOD and a significant decrease in the level of GSPxase and GSRase respectively compared with the control group. Administration of vitamin E, L-carnitine or both of them along with ISO significantly reduce the level of SOD and significantly increased in the levels of GSPxase and GSRase when compared with the untreated group. Although none of these antioxidants reached that of the control group, the combined effect of both vitamin E and L-carnitine was more significant on the level of GSRase compared with other enzymes Figure (1C). When it came to the levels of the inflammatory cytokines, it was found that the levels of IL-6 and TNF-α showed a significant increase in the cardiac tissue after administration of ISO while their levels significantly reduced in rats received both vitamin E and L-carnitine compared with the Untreated rats Figure (1D).

Histopathological results

Light microscopic findings
The light microscopic inspection of the left ventricle of the control rat revealed intact cylindrical cardiac muscle fibers, with acidophilic cytoplasm and ill-defined transverse striations. The nuclei of the cardiac fibers were vesicular, oval or rounded and central in position. Connective tissue between the fibers was scanty and contained thin wall blood capillaries and few fibroblasts identified by their flat nuclei. The blood vessels, which were branches of the coronary vessels between the cardiac muscle fibers had intact wall with normal structure and perivascular tissue (Figure 2 A, B).

Isoproterenol administration induced histological alterations included atrophy of some cardiac fibers, and degeneration of some fibers, which recognized by the dark cytoplasm and small pyknotic nuclei. Some capillaries appeared congested or ruptured resulting in hemorrhagic foci between the fibers. The blood vessel between the fibers appeared damaged with perivascular inflammatory cells around them (Figure 2 C, D). Sections in the heart wall of groups treated with vitamin E, L-carnitine or the combination of them showed that most of the cardiac muscle were intact while few fibers were degenerated and the blood vessels appeared normal similar to control (Figure 2 E, J).

Semithin sections prepared from the same samples confirmed what was observed by routine histological stains. The cardiac muscle fibers of the control rats appeared intact with preserved striation and intact capillaries in between. Evident focal damage of cardiac fibers was observed in rats received ISO as some fibers lost their striation compared to the control. Most of blood capillaries were dilated and congested. On the other hand, most of the cardiac muscle fibers in the hearts of the treated groups showed preserved striations while few fibers had lost them. The rats received both vitamin E, L-carnitine showed the best effect on preserving the structure of the cardiac muscle Figure (3).

**Electron microscopic findings**

When the ultrastructure of the cardiac muscle fibers was assessed using TEM, it was noticed that the control rats showed euchromatic nuclei, intact, well-defined sarcomeres and intact mitochondria with normal size and shape. On the other hand, cardiac fibers of rats received ISO appeared atrophied with occasional ill-defined sarcomeres, smaller mitochondria with disrupted cristae. Although the cardiac fibers of rats treated with vitamin E, L-carnitine or
combination of both of them possessed preserved sarcomeres, the mitochondria still appeared smaller in size and less frequently seen in the fibers compared to the control rats Figure (4).

Discussion

"Myocardial infarction is a condition that results from the interruption of coronary blood supply needed to satisfy myocardial demand, leading to oxygen and nutrient deprivation of the heart, eventually destroying cardiac tissues" [5]. "Isoproterenol, a synthetic catecholamine and adrenergic agonist that is documented to produce in large dose due to generation of highly cytotoxic free radicals through its auto-oxidation". These free radicals initiate lipid peroxidation and induce irreversible damage of the myocardial membrane [9]. This study was planned to assess the possible protective effect of vitamin E, L-carnitine, and the combination of both of them on isoproterenol-induced changes in rat cardiac muscle.

The diagnostic cardiac enzymes CK-MB, LDH and AST are considered sensitive markers that being used to assess the severity of myocardial infarction [32]. ISO, in this study, significantly increase the diagnostic cardiac enzymes levels (LDH, AST and CK-MB) in the serum and MDA level in the cardiac tissue compared with the control rats. In addition, it significantly increased SOD activity and significantly decrease GSPxase and GSRase activity indicating its harmful effect on the cardiac muscle. These findings were supported by those of Panda et al. observed in rats after administration of ISO [24].

Free radicals generated by ISO stimulate lipid peroxidation of polyunsaturated fatty acid of the membrane, with subsequent loss of the structure and function of the myocardium. When the metabolic function of the myocardium was disturbed, it releases its lysosomal enzymes into the blood. Therefore, the serum levels of AST, LDH and CK-MB were found to be significantly increased [19].

Isoproterenol induced structural alterations in cardiac muscle. Among these alternations were the appearance of some muscle fibers with dark cytoplasm and small pyknotic nuclei indicating apoptosis and degeneration of these fibers. These finding was supported by those previous reported by Shukla et al. who stated that ISP initiates apoptosis evidenced by up-regulation of TUNEL and Bax expression and down regulation of Bcl-2 expression [28]. Atrophy of cardiac muscle fibers as well as hemorrhage and congestion of some blood vessel were observed following ISO administration, in this study. These finding were in agreement with
what was reported by Panda et al. following administration of ISO [24]. Gayathri et al. reported that ISO administration was associated with vascular changes, edema and leucocyte infiltration [8]. Marked focal myonecrosis where among the histopathological changes induced by ISP in rats as described by Shukla et al. [28]. In this study, ISO administration affected also the sarcomere, the functional unit of muscle fiber, and mitochondria, the power house of the fiber. Affection of the membranous organelles like mitochondria is attributed to lipid peroxidation induced by ISO [10]. Tappia et al. reported that "oxidative stress increases cAMP levels by exhausting ATP, depresses sarcolemmal Ca\(^+\) transport resulting in intracellular calcium overload, leading to ventricular dysfunction and contractile failure in rat heart" [29].

Administration of L-carnitine was found, in the present study, to protect rat heart from ISO-induced changes observed in untreated group. It improved the oxidative stress induced by ISO as evident by the significant reduction in MDA. In addition, L-carnitine increased antioxidant capacity of the cardiac muscle evident by increased GSPxase and GSRase activity. In another model of myocardial infarction induced by amethopterin, L-carnitine was found to improve the biochemical, histopathological, and immunohistochemical alterations followed amethopterin administration [31]. Previously L-carnitine administration was reported to have a cardio-protective role in cardio-myopathy and prevention of myocardial infarction [14]. Lee et al. found that L-carnitine supplementation increased antioxidant enzymes improved lipid profile and decreased oxidative stress in patients with coronary artery diseases [15].

Vitamin E in the present study was also found to ameliorate degenerative changes and other cardiac fiber alterations induced by ISO administration. This protection was observed at both light and electron microscopic level. In addition, Vitamin E reduced ISO-induced oxidative stress as evidenced by reduction in MDA. Vitamin E also increased cardiac muscle antioxidant capacity evidenced by increased GSPxase and GSRase activity. The ant-inflammatory effect of vitamin E was evident as it significantly reduced the elevated inflammatory cytokines IL-6 and TNF-\(\alpha\) in the cardiac tissue after administration of ISO. These finding were supported by the study of Boaz et al., [4] and Lee et al. who stated that vitamin E was known to be a potent antioxidant with effective prevention of cardiovascular diseases [16]. It has been proposed that the cardio protective effect of L-carnitine and vitamin E is due to anti-inflammatory and antioxidant activities on coronary endothelial function and vasoreactivity [34]. This could
explain the better effect induced by the combination of L-carnitine and vitamin E in protection against ISO-induced cardiac affection that was documented in this study.

Among the limitations of this study was the inability to assess changes in gene expression of the antioxidants profile in the cardiac muscle in all studied groups in order to prove the mechanism behind the augmentation of action between vitamin E, L-carnitine.

Conclusions and clinical implications

This biochemical and histological studies demonstrated that administration of vitamin E, L-carnitine ameliorated cardiac alteration induced by ISO with the superior effect of the combination of both of them. Based on these findings it is likely that administration of these two element is recommended for patient at risk for myocardial infarction as they exerts a cardioprotective effect by stabilizing the myocardial membrane. The membrane stabilizing activity may be due to an increase in endogenous antioxidants, which may increase the myocardial antioxidant reserve and strengthen the defense mechanism(s) operating in the myocardium.

References:


FIGURE LEGENDS

**Figure 1.** Changes in cardiac enzyme (LDH, AST, CK-MB) (A), MDA (B), Antioxidants in cardiac (C), TNF-α (D) and IL-6 levels in the studied groups. GI (control), GII (Isoproterenol), GIII (Isoproterenol+vitamin E), GIV (Isoproterenol+L-carnitin) and GV (Isoproterenol+vitamin E+L-carnitin). AST: aspartate transaminase, LDH: lactate dehydrogenase, CK-MB: creatine kinase, MDA: Malondialdehyde, SOD: Superoxide Dismutase, TNF-α: Tumor necrosis factor-α, IL-6: Interleukin-6.

**Figure 2.** Transvers (A) and longitudinal (B) sections in control rat heart (GI) showed intact cardiac muscle fibers (black arrow) and intact blood vessels (white arrow). Sections in heart of GII rats (C, D) show atrophy and decrease size of cardiac fibers (thick arrows) with some hemorrhages in between them (stars). Some muscle fibers appear degenerated with dark cytoplasm and small pyknotic nuclei (interrupted arrow). The blood vessel (white arrow) appear damaged with perivascular inflammatory cells around. Sections in the heart of GIII rats (E, F)
and those of GIV rats (G, H) showing few degenerated muscle fibers (interrupted arrows) and the blood vessels appear intact. Sections in the heart of GV rats (I, J) showing almost intact muscle fibers and blood vessels (H&E X400). GI (control), GII (Isoproterenol), GIII (Isoproterenol+vitamin E), GIV (Isoproterenol+L-carnitin) and GV (Isoproterenol+vitamin E+L-carnitin).

**Figure 3.** Semithin sections in the heart of GI (A) showing intact cardiac muscle fibers with vesicular central nuclei (arrows) and intact striation. Note, the presence of thin-walled capillaries (white arrow). Section in the heart of GII (B) showing reduced diameter of muscle fibers with some of them have dark small nuclei (thick arrow) and some fibers lost their striations (star). Note most of blood capillaries are dilated and congested (white arrow). Sections in hearts of GIII (C), GIV (D) and GV (E) show that most of muscle fibers are intact with vesicular nuclei (arrow). Striations are preserved in most of the muscle fibers while few lost them (Toluidine blue stainx600). GI (control), GII (Isoproterenol), GIII (Isoproterenol+vitamin E), GIV (Isoproterenol+L-carnitin) and GV (Isoproterenol+vitamin E+L-carnitin)

**Figure 4.** EM micrograph of rat cardiac muscle from the left ventricle of control group (GI) (A, B) showing normal structure of the cardiac fibers with open face nuclei (N), well defined sarcomeres (black arrows) and normal size and site of mitochondria (thick white arrows). Cardiac muscle of GII (C, D) showing reduced diameter (atrophy) of cardiac muscles (interrupted arrow) with occasional ill-defined sarcomeres (arrows). Mitochondria appear smaller in size with disrupted cristae (thick white arrows). GI (control) and GII (Isoproterenol-treated group).

**Figure 5.** EM micrograph of rat cardiac muscle from the left ventricle of GIII (A, B), GIV (C, D) and GV (E, F) showing increased diameter of most of the cardiac fibers, defined sarcomeres (black arrows) while the mitochondria (thick white arrows) still have small size and fewer number compared to the control group. GIII (Isoproterenol+vitamin E), GIV (Isoproterenol+L-carnitin) and GV (Isoproterenol+vitamin E+L-carnitin)