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Levo-Carnitine Reduces Oxidative Stress and Improves Contractile Functions of Fast Muscles in Type 2 Diabetic Rats

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ASTRACT

Background: Metabolic derangements in type 2 diabetes mellitus (T2DM) are likely to affect skeletal muscle contractile functions adversely. Levo-carnitine improves muscle contractile functions in healthy humans and rats and corrects metabolic derangements in T2DM. Therefore, it is likely to improve muscle contractile functions in T2DM as well. This study was designed to determine the effect of levo-carnitine on serum levo-carnitine levels, oxidative stress and contractile parameters of fast muscle in T2DM. **Methods:** Ninety Sprague-Dawley rats were randomly divided into three equal groups. Healthy rats served as the controls, while T2DM was induced in diabetic and carnitine groups. The carnitine group was administered levo-carnitine 200 mg/kg/day intraperitoneally for 6 days. At 28th day, extensor digitorum longus muscles were removed and their functions were assessed using iWorx data acquisition unit (AHK/214). Blood obtained by intra-cardiac sampling at 28th day was used for estimation of serum malondialdehyde (MDA) and levo-carnitine levels. **Results:** Maximum isometric twitch tension, time-to-peak twitch tension and time-to-relax to 50% of the peak twitch tension were not significantly different amongst the groups. Carnitine group showed significant improvement in maximum fused tetanic tension, maximum fused tetanic tension after fatigue protocol and recovery from fatigue after 5 minutes of rest period compared to the diabetic group. Serum MDA levels were reduced, while serum levo-carnitine levels were elevated significantly in carnitine group as compared to the diabetic group. **Conclusion:** Levo-carnitine supplementation increases serum levo-carnitine levels which decreases oxidative stress. This action improves contractile force but delays fatigue in fast muscles of diabetic rats. *Iran. Biomed. J. 17 (1): 29-35, 2013*

Keywords: Type 2 diabetes mellitus (T2DM), Levo-carnitine, Fast muscles, Contractile functions, Oxidative stress

INTRODUCTION

Insulin resistance developed in type 2 diabetes mellitus (T2DM) leads to significantly decreased content and rate of glycogen synthesis in fast (type II) skeletal muscle fibers [1]. Glycogen stores in sufficient quantities are required by fast muscle fibers for optimal adenosine triphosphate (ATP) generation during high intensity exercise [2]. Insulin-stimulated glucose oxidation is also reduced in T2DM patients, because of decreased pyruvate dehydrogenase (PDH) activity [3]. Excessive reactive oxygen species (ROS), such as hydroxyl radical, hydrogen peroxide and superoxide anion are produced in T2DM, which are cytotoxic and highly reactive in nature. This precipitates free-radical damage to the mitochondrial proteins and reduction in oxidative capacity of the skeletal muscles [4]. These metabolic derangements are likely to affect contractile functions of fast muscle fibers adversely.

Levo-carnitine (*L*-3-hydroxytrimethylammino-butanoate), an endogenous compound, transports long chain fatty acids inside the mitochondria for β-oxidation, stimulates pyruvate and branched-chain amino acid oxidative metabolism, helps in ketone body formation, ATP/adenosine diphosphate production and gluconeogenesis in liver and muscle [5]. Levo-carnitine-dependent metabolic processes provide skeletal muscle with the ability to gain and use energy. Skeletal muscles are dependent upon circulating free L-carnitine as it cannot be bio-synthesized there. Reduced levo-carnitine in blood leads to insufficient muscle levo-carnitine stores causing disturbed muscle metabolism, which is likely to reduce skeletal muscle performance [6]. It has been documented in healthy human athletes as well as animals that levo-carnitine supplementation enhances maintenance of skeletal muscle contractile force, adaptation to strenuous exercise and delays muscle fatigue [7, 8]. Studies in athletes have revealed a significantly improved

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Table 1. Body weight, plasma glucose, serum phosphocreatine and TG:HDL in three groups of rats at 1st, 21st and 28th days of the study.

Groups	Days	Control	Diabetic	Carnitine
Body weight (g)	01	215.55 ± 5.04	248.62 ± 6.47	251.54 ± 5.32
	21	256.63 ± 7.40	269.70 ± 8.35	268.70 ± 7.70
	28	265.71 ± 7.15	278.70 ± 7.61	269.86 ± 8.34
Plasma glucose (mmol/l)	01	5.85 ± 0.34	5.84 ± 0.30	5.87 ± 0.34
	21	5.83 ± 0.31	23.13 ± 0.40	22.90 ± 0.41
	28	5.91 ± 0.32	23.90 ± 0.47	10.55 ± 0.45
Serum phosphocreatine (IU/L)	01	180.24 ± 7.84	180.77 ± 5.79	179.23 ± 6.81
TG : HDL	21	1.37 ± 0.55	2.17 ± 0.78	2.16 ± 0.92
	28	1.38 ± 0.38	2.40 ± 0.84	1.56 ± 0.24

All values have been expressed as mean ± SD, plasma glucose ≤ 16.65 mmol/l is considered normal, TG:HDL≤1.8 is considered normal

recovery from fatigue and lesser post exercise muscle damage and pain with levo-carnitine supplement [8]. Studies have shown a significant reduction in the exercise-induced oxidative stress in rats receiving levo-carnitine [7, 9].

In T2DM patients, plasma levo-carnitine levels decrease and its supplementation significantly improves insulin sensitivity, glucose uptake and glucose oxidation by skeletal muscles [10]. Levo-carnitine supplementation also corrects an inappropriate shift in substrate use from carbohydrates to lipids, which is common in type 2 diabetic patients through modulating the expression of glycolytic and gluconeogenic enzymes. [10]. Levo-carnitine also decreases the oxidative stress in T2DM [11]. These findings manifest the ability of levo-carnitine to normalize metabolic derangements in T2DM.

This study was designed in rodent model of insulin-resistant T2DM to determine the effect of levo-carnitine supplementation on serum levo-carnitine levels, oxidative stress and contractile functions of fast muscles.

MATERIALS AND METHODS

This randomized control trial was carried out in Physiology Department of Army Medical College, Pakistan, for a total duration of 28 days after approval by the Ethical Committee of the Institution. Healthy Sprague-Dawley rats (n = 90, 80 ± 5 days old) with average weight of 250 ± 50 grams were taken from National Institute of Health (NIH), Islamabad, Pakistan. Plasma glucose and serum creatine phosphokinase levels were measured to exclude any rat with disturbed glucose metabolism and skeletal muscle disease (Table 1). Rats were kept in 2 × 3 feet steel cages placed in a well-ventilated room with exposure to the cycles of 12 hours light and 12 hours dark. Room temperature was kept at 20-22˚C. Rats had free access to the clean water through bottles fitted over the cages.

Rats were randomly divided into control, diabetic and carnitine groups each having 30 rats. Control rats were fed on normal diet, while rats of diabetic and carnitine groups were fed high fat diet (Table 2) for 14 days *ad libitum*. On 15th day, streptozocin was administered (35 mg/kg body weight) intraperitoneally in the lower-right quadrant of the abdomen [12] to induce diabetes mellitus in diabetic and carnitine groups, while intraperitoneal normal saline was injected in control group. On 21st day, body weight, plasma glucose and triglyceride to high density lipoproteins (TG:HDL) ratio of all rats were measured by tail vein sampling, after an overnight fast, to confirm the development of T2DM in diabetic and carnitine groups [13]. Sprague-Dawley rats with plasma glucose level >16.65 mmol/l [14] and TG:HDL ratio >1.8 [14] were considered as having developed insulin-resistant T2DM.

Table 2. Composition of feed given to various groups of rats.

Control group		Diabetic and Carnitine groups	
Ingredients	**Weight (g/kg)**	**Ingredients**	**Weight (g/kg)**
Wheat flour	285	Powdered NPD	365
Wheat brawn	285	Lard	310
Salt (common)	5	Casein	250
Mollasen	15	Cholesterol	10
Soybean oil (ml/kg)	50	Vitamin & mineral mix	60
Fish meat	150	DL-Methionine/L-cystine	3
Vitamins/ Minerals	10	Yeast powder	01
Dried skimmed milk	200	Sodium chloride	01

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Levo-carnitine (L-carnitine hydrochloride, Sigma-Aldrich, USA) was administered intra-peritoneally (200 mg/kg/day) to the carnitine group from 22nd to 27th day for 6 days [7], while the other two groups were administered normal saline. Levo-carnitine has high first-pass metabolism, because it relies on active transport system within the epithelial cell membranes of the gastrointestinal tract for its absorption, which gets saturated quickly [6]. Therefore, levo-carnitine was injected intraperitoneally to ensure its optimum levels in plasma and bioavailability.

On 28th day, after an overnight fast, rats were weighed and anaesthetized. Blood (2 ml) was obtained by intra-cardiac sampling to assess biochemical parameters.

Biochemical parameters. Estimation of serum malondialdehyde (MDA) levels by using thiobarbituric acid reactive substances assay (enzymatic calorimetric method) [15]. ROS cause lipid peroxidation of polyunsaturated fatty acids releasing MDA as a byproduct which is an adequate indicator of oxidative stress [16]. Plasma glucose was estimated by Trinder's method [17] and serum TG:HDL ratio was calculated after measuring TG by glycerol phosphate oxidase method [18] and HDL by direct method using homogenous enzymatic test [19].

Skeletal muscle functions. On 28th day, after intracardiac sampling, the femoral compartment of distal limb was opened and the proximal tendon of extensor digitorum longus muscle (EDL) was dissected. Anterior crural compartment was exposed by removing the biceps femoris muscle. The distal tendons of EDL were dissected out intact from the foot through distal fasciotomy along with the removal of connective tissue and ligaments. Whole EDL was mounted in a 25-ml organ bath system (Harvard Apparatus, USA), containing Krebs-Ringer bicarbonate buffer that was continuously bubbled with a mixture of 95% O_2 and 5% CO_2. Temperature was maintained at 30˚C with the help of a thermostat [20]. The four distal EDL tendons were tied together by non-absorbable surgical silk and fixed to a support, while proximal tendon was tied to the force transducer (100) connected to iWorx advanced animal/human physiology data acquisition unit AHK/214 (Harvard Apparatus, USA). Muscles were evoked by supramaximal stimulation via platinum electrodes placed directly on the muscle aligned in a parallel direction to the muscles longitudinal axis [20].

Measurement of isometric contractions. Length of the muscle was adjusted via a micromanipulator to obtain maximum twitch tension for single muscle twitch using 1 Hz electrical stimulations with one minute rest period in between. Maximum isometric twitch tension, time-to-peak twitch tension and time taken to relax to 50% of the peak twitch tension were calculated. The optimum length was also noted and maintained for all subsequent stimulations. [20].

The maximum fused tetanic tension. Muscle was evoked at frequencies of 10, 30, 50, 70, 90 and 110 Hz for 1 second with 3 minutes rest period after each stimulation and response was recorded. The maximum fused tetanic tension was calculated and termed as the initial maximum fused tension. The optimum frequency that produced this tension was noted [20]**.**

Muscle fatigue. Muscle was stimulated at optimum frequency for 1 second with 5 seconds rest period in between, for the total period of 5 minutes. The muscle response was recorded for every subsequent minute after the initial maximum fused tension, for a total period of 5 minutes and expressed as a decline in force from the initial maximum fused tetanic tension. The recovery from fatigue was estimated by recording the tetanic tension after 5 minutes of rest period following the fatigue protocol [20]. All the muscle tensions were normalized to muscle mass and expressed as Newton per gram (N/g) wet muscle mass. SPSS 15 was used to calculate the mean with standard deviation of all parameters and statistical significance of differences across the groups by applying ANOVA followed by Tukey's honestly significant difference (HSD) where required. $P \leq 0.05$ was considered significant.

RESULTS

The initial body weight, plasma glucose and serum creatine phosphokinase levels of the rats were within normal range. Rats of the diabetic and carnitine groups successfully developed insulin-resistant T2DM which was confirmed on 21st day of the study. The body weight of diabetic and carnitine groups was also found to be increased as compared to the controls. Plasma glucose, TG:HDL ratio and body weight of each rat was measured at 28th day. These parameters remained significantly deranged in the diabetic group, while the carnitine group showed normalization of plasma glucose and TG:HDL ratio with minimal increase in the body weight despite feeding high fat diet (Table 1).

Serum* levo-*carnitine levels. Serum levo-carnitine levels were found significantly different among the groups ($P<0.001$) after applying one-way ANOVA. Application of Tukey's HSD revealed reduced the levels in diabetic rats, while the levels were very significantly ($P<0.001$) increased in the carnitine group as compared to the other two groups (Fig. 1A).

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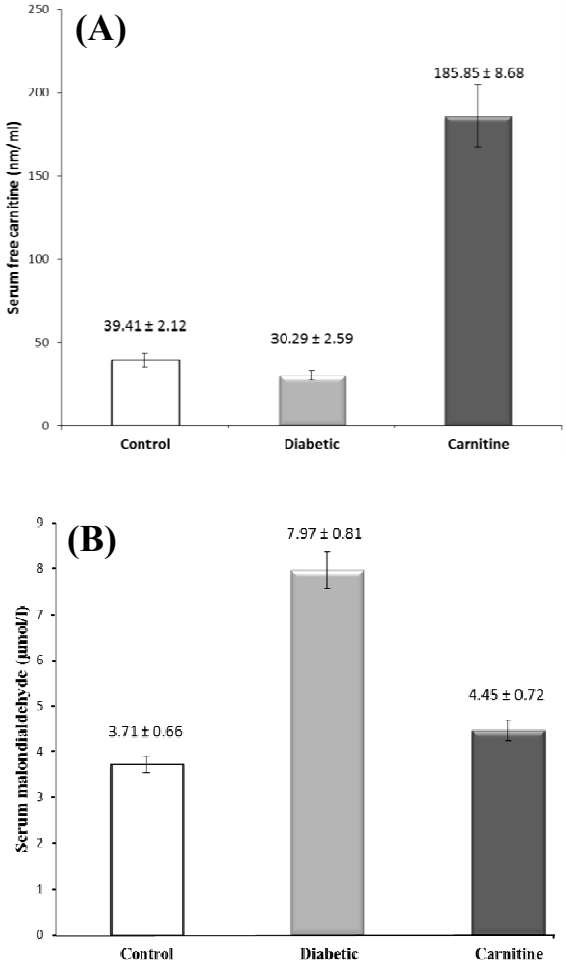

Fig. 1. Serum-free carnitine **(A)** and serum malondialdehyde **(B)** levels in three study groups of rats on 28th day. All values have been expressed as mean ± SD. Comparison between any two groups is highly significant (P<0.001).

Serum malondialdehyde levels. Serum MDA levels were found significantly different among the groups (P<0.001) after applying one-way ANOVA. Application of Tukey's HSD showed that levels were significantly (P<0.001) high in diabetic rats as compared to the other two groups, while the levels were significantly lower in carnitine group than diabetic group (P<0.001) but still higher than control group (P<0.001) (Fig. 1B).

Skeletal muscle function. There was no statistically significant difference among the groups in maximum isometric twitch tension (P>0.15), time to peak twitch tension ($p > 0.38$) and time taken to relax from maximum twitch tension to its 50% (P>0.29) (Table 3). Statistically significant difference was found among the groups in maximum fused tetanic tension (P<0.02), maximum fused tetanic tension after fatigue protocol (P<0.01) and tetanic tension measured after the period of 5 minutes of rest following the fatigue protocol (P<0.03), when one-way ANOVA was applied (Table 3). After application of Tukey's HSD, the diabetic group was found significantly different from other groups, while control and carnitine groups had insignificant differences (Table 4).

DISCUSSION

The rodent T2DM model developed by Srinivasan *et al.* [13] was used, as it closely replicated the natural history and metabolic characteristics of human T2DM. The rodent models based on feeding high sucrose or fructose diet for induction of T2DM had low resemblance with human T2DM. Insulin resistance was induced with the help of high fat diet (58% fat calories), while frank hyperglycemia was produced by β-cell destruction with low-dose streptozocin [13]. This procedure was simple and cost effective and took short period to induce T2DM in rats. Weight gain was greater in these rats compared to controls. Increased body weight, plasma glucose and serum triglycerides made it an ideal rodent model of obesity-induced T2DM similar to the human T2DM. The dose of levo-carnitine (200 mg/kg body weight) was used in this study, as it was found safe and did not produce untoward effects even when administered for the duration of one month. In healthy rats, this dose of levo-carnitine produced significant increase in forced muscular activity and endurance time [7].

Table 3. Comparison of muscle contractile parameters on 28th day of the study using one-way ANOVA.

Variables	Control group (n = 30)	Diabetic group (n = 30)	Carnitine group (n = 30)	*P* value
Max isometric twitch tension (N/g)	0.34 ± 0.06	0.31 ± 0.07	0.32 ± 0.05	0.15
Time to peak twitch tension (ms)	20.7 ± 3.70	22.1 ± 4.10	21.5 ± 3.90	0.38
Time taken to relax from max twitch tension to its 50% (ms)	21.2 ± 3.50	22.8 ± 4.30	21.9 ± 4.00	0.29
Max fused tetanic tension (N/g)	3.98 ± 0.07	3.93 ± 0.09	3.97 ± 0.06	0.02
Max fused tetanic tension after fatigue protocol (N/g)	1.83 ± 0.05	1.79 ± 0.05	1.82 ± 0.06	0.01
Tetanic tension after 5 min of rest period following fatigue protocol (N/g)	3.88 ± 0.07	3.84 ± 0.05	3.87 ± 0.06	0.03

All values have been expressed as mean ± SD; max, maximum

Table 4. Comparison of control, diabetic and carnitine groups using Tukey's honestly significant difference.

Contractile parameters	*P* value		
	Control vs diabetic	Control vs carnitine	Diabetic vs carnitine
Maximum fused tetanic tension	0.01	0.55	0.04
Maximum fused tetanic tension after fatigue protocol	0.00	0.48	0.03
Tetanic tension after 5 minutes of rest period following fatigue protocol	0.01	0.55	0.03

Serum levo-carnitine levels were found to be reduced significantly in the diabetic rats compared to controls. It has been documented earlier that total, free and esterified carnitines are decreased in diabetic patients.

Free levo-carnitine content was especially lower in T2DM, which is associated with impaired the fatty acid oxidation. This impairment leads to elevated triglyceride and free fatty acid concentrations, reduced ketogenesis and lipid infiltration in liver and muscles. In response to decreased plasma levo-carnitine levels, skeletal muscles release levo-carnitine which leads to its depletion in the muscles. This issue reduces muscle's ability to use long chain fatty acids as metabolic fuel results in weakness, muscle aches, asthenia, malaise and early fatigue [6].

Serum MDA level was used as marker of oxidative stress that were markedly increased in the diabetic group, but decreased significantly in the carnitine group, which shows the antioxidant property of levo-carnitine. This finding suggests that the skeletal muscles of the diabetic group suffered from damaging effects of the ROS more than the control and carnitine groups, which led to decline in their contractile functions. ROS decrease the oxidative ability of the muscle cells by damaging the mitochondrial enzymes [4] and also reduce the myofibrillar Ca^{2+} sensitivity, which leads to decline in force of contraction and early fatigue [21].

Levo-carnitine (250 mg/kg per day) was administered orally [22] for 28 days to obese diabetic rats. Serum MDA levels were reduced significantly in treated diabetic rats (7.96 ± 0.08 µmol/l) as compared to the untreated ones (9.72 ± 0.08 µmol/L). However, serum MDA levels of treated rats were significantly higher than control rats (4.69 ± 0.06 µmol/l). The most probable cause is because of oral administration of levo-carnitine that caused the serum level becomes lower than the required levels [5]. At low serum levels, levo-carnitine could not play its role optimally as an anti-oxidant. Failure to suppress ROS production effectively resulted in higher than normal MDA levels.

EDL muscle was selected for the measurement of contractile functions because it had abundant type II fibers which depended largely on glycogenolysis for ATP production [23]. Reduction in performance due to decreased glycogen content and decreased activity of PDH was expected to be more evident in this muscle compared to muscles having type I fibers such as soleus or mixed fibers like gastrocnemius [1].

There was no significant difference in maximum isometric twitch tension among the groups. The maximum force produced by the muscle depends on the number of myosin heads bound with the actin filaments at a given instance which, in turn, depends upon the availability of the ATP and calcium ions (Ca^{++}) in the sarcoplasm [23]. Adequate ATP phosphocreatine and free adenosine diphosphate, along with optimal pH are available in the sarcoplasm of the diabetic muscle for a single muscle twitch [24]; therefore, strength of contraction after single electrical stimulus did not differ statistically among the groups.

The time to peak twitch tension depicts the rapidity of release of Ca^{++} from the sarcoplasmic reticulum and time for relaxation to half of the maximum twitch tension signifies the rapidity of Ca^{++} pump to transport the Ca^{++} from the sarcoplasm into sarcoplasmic reticulum [20]. Both remained unaffected in this study probably due to adequate availability of ATP in type 2 diabetic muscles [24].

Time to peak twitch tension and half relaxation time in single muscle twitches of EDL from genetically obese (*ob/ob*) mice were increased in the study by Warmington and coworkers [20] due to increased number of slow twitch fibers in the EDL of the *ob/ob* mice. Furthermore, probability of reduced Ca^{++} cycling ability of the sarcoplasmic reticulum in the *ob/ob* EDL was also mentioned. As obesity was induced by feeding high fat diet in this study, without any genetic manipulation, any change in the fiber type of obese diabetic rats as compared to the controls was unlikely. In a study by Howarth and colleagues [25], no effect on Ca^{++} ATPase activity was observed in rat cardiomyocytes during early stages of T2DM. It is suggested that after 1 week of the development of diabetes in our study, the Ca^{++} ATPase activity remained undisturbed in EDL which maintained the half relaxation time in normal limits.

When EDL of the control group were stimulated to produce tetanic contraction, higher magnitude of the maximum force of contraction was generated as compared to the diabetic group. Large amount of ATP by fast muscle are required fibers for tetanic contraction, which are provided by the stored muscle glycogen [23]. About 20% reduction occurs in

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glycogen stores of fast muscles because of the impaired glycogen synthase activity in T2DM due to the insulin resistance and relative insulin deficiency [1]. Moreover, PDH activity is reduced in T2DM, which impairs utilization of muscle glycogen and causes increase in lactate levels [3]. Excessive ROS generation in diabetic group, as manifested by high MDA levels, has been documented to decrease the muscle performance during exercise by reducing myofibrillar Ca^{++} sensitivity and damaging muscle proteins [7, 21]. Therefore, diabetic muscles generated less tension than controls due to their poor fuel supply and its utilization along with high oxidative stress and lactate levels.

Increased maximum force of contraction at tetanizing frequency was recorded in the *ob/ob* group by Warmington and coworkers [20]. It was attributed to morphological changes in muscle fibers coupled with reduced Ca^{++} cycling ability of the sarcoplasmic reticulum in the *ob/ob* EDL. This led to availability of excessive Ca^{++} in sarcoplasm generating higher tension in the *ob/ob* muscles. In our study, no morphological change took place in EDL of obese diabetic rats. Therefore, the maximum tension at tetanizing frequency was decreased in diabetic group as compared to the controls.

The maximum force of contraction by EDL of the carnitine group was statistically similar to the healthy control group. It is suggested that high serum levo-carnitine levels improved insulin sensitivity leading to the increased glucose uptake, increased storage of glycogen [10], restoration of PDH activity, which stimulates the oxidative utilization of glucose and correction of an inappropriate shift in substrate use from carbohydrates to lipids [10]. These effects led to generation of ATP which is sufficient enough to produce tension by these muscles comparable to the controls. Reduced ROS produced due to levo-carnitine supplementation also contributed positively to improve force of contraction by improving myofibrillar Ca^{++} sensitivity and reducing damage to muscle proteins [21].

Muscle tension was significantly decreased in the diabetic group of this study as compared to the control group during fatigue protocol because of a number of factors like higher rate of ATP exhaustion due to reduced glycogen stores, increased lactate levels and higher oxidative stress. Moreover, in diabetic muscles, there is rapid phosphocreatine depletion, diminished ability to utilize glycogen and quick reduction in pH as result of repeated contractions [26]. Warmington and coworkers noticed reduction in the fatigue of *ob/ob* EDL which was attributed to excessive slow fibers which are fatigue resistance [20].

EDL of the carnitine group manifested statistically similar decline in muscle tension following fatigue protocol to that of control which point towards ample regeneration of ATP in levo-carnitine treated diabetic muscles. This may be attributed to the replenishment of glycogen stores, appropriate shift in substrate use in muscles and reduction in the ROS because of high levo-carnitine levels [10, 12].

The tetanic tension recorded after the rest period of 5 minutes was significantly less in diabetic group while insignificant difference was observed between control and carnitine groups. The diabetic skeletal muscles are known to have reduced capability of glucose uptake [3]. On the other hand, muscles of the control and carnitine groups were able to take up glucose from the buffer medium in which they were placed and utilized it adequately leading to better replenishment of ATP stores. This helped them recover from fatigue better than diabetic muscles.

In T2DM, serum-free levo-carnitine levels decrease while oxidative stress increase leading to derangement of underlying metabolic processes resulting in decreased maximum tetanic tension in fast muscles and early fatigability. Levo-carnitine supplementation, in T2DM, increases serum-free levo-carnitine levels, reduces the oxidative stress which most likely corrects the underlying metabolic processes in fast muscles resulting in maximum tetanic tension and fatigue equivalent to health fast muscles.

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