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Comparative analysis of two L-carnitine preparations and their concentration effects on CAT expression in healthy human peripheral blood lymphocyte cultures

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Abstract

CAT gene encodes catalase, a key antioxidant enzyme in the body against oxidative stress. This enzyme plays an important role in the molecular mechanisms of inflammation, apoptosis, mutagenesis and tumorigenesis. Anti-oxidant L-carnitine is used in food supplementation, medical co-treatment and bodyweight regulation. We aimed to investigate molecular basis of L-carnitine commercial preparations supplementation in reducing oxidative stress with customized *CAT* gene assay *in vitro*. Human lymphocytes cell culture was established using standard procedure and treated with range of concentrations of L-carnitine in two preparations. We tested two preparations: 500 mg tablets of L-carnitine and liquid L-carnitine with vitamin B6. L-carnitine significantly reduced the expression of *CAT* gene in cultured lymphocytes at concentrations of 50 μmol/l and 250 μmol/l compared to negative control, (p = 0,001; p = 0,001; respectively). The L-carnitine liquid supplement with vitamin B6 also reduced the transcription of *CAT* gene at concentrations of 50 μmol/l and 250 μmol/l as compared to the negative control (p = 0,018; p = 0,006; respectively). Selected L-carnitine preparations modulated the transcriptional activity of the antioxidant enzyme gene in human lymphocyte culture, indicating its possible effects in inhibition of pro-inflammatory processes that involve catalase activity.

Introduction

Many nutritional supplements have an overall positive influence on human health due to their antioxidant and anti-inflammatory effects. As a substance with known anti-oxidative effects, L-carnitine is being used for various purposes: as a food supplement, in medical co-treatment, or for body weight regulation based on its positive metabolic role and mitochondrial enhancement.

L-carnitine, (3R)-3-hydroxy-4-(trimethylazanyl) butanoate is a derivative of the essential amino acids lysine and methionine. It is responsible for the transport of long chains of fatty acids to mitochondria, in cells for the process of β-oxidation, i.e., the use of long-chain fatty acids as a source of energy (Müller et al., 2002). The antioxidant effects of certain commercially available preparations are based on their expression of genes involved in response to oxidative stress, inflammation, apoptosis, repair of DNA molecule damage as well as inhibition of tumor growth (Wang et al., 2014). In stressful situations, the need for L-carnitine can exceed an individual's ability to synthesize it, making it a conditionally essential nutrient. There is evidence that primary and secondary L-carnitine deficiencies may occur, and they are associated with inflammation, immune and neuromuscular dysfunction, endocrine dysregulation, and abnormalities in energy metabolism. Today, L-carnitine and its derivatives are widely used in genetic or primary disorders of L-carnitine metabolism, and some other disorders have resulted in secondary L-carnitine deficiency. L-carnitine supplementation is intended to remove toxic metabolites, which can lead to disruption of mitochondrial homeostasis and thus interfere with energy production (Kepka et al., 2020).

However, molecular mechanisms of its effects are not well studied so far. In this work we chose a targeted gene assay to test anti-oxidative activity of L-carnitine supplements *in vitro*. In general, increase of *CAT* expression is present in hypoxia and is recognized as a positive marker of treatment response (ischemic heart disease, myopathy, and peripheral arterial disease) and beneficial as treatment supplement in muscle recovery of athletes due to its role in oxidative stress. The aim of this study was to investigate differential effect of L-carnitine and L-carnitine supplemented with vitamin B6 on relative transcriptional activity of catalase gene as compared to those in non-treated cells.

Material and methods

Ethical aspects of the study were reviewed and approved by the Institutional Board of the Institute for Genetic Engineering and Biotechnology. Subjects signed informed consent following the oral explanation of the study objectives. The samples were treated as anonymous in all further study activities. To control for accidental individual variation in catalase activity effects on the study results, we performed a study in biological replicates: three males and three females with no self-reported acute or chronic illnesses.

Treatments and cell cultures

In this study, we tested two L-carnitine preparations commonly present on the market in Bosnia and Herzegovina: one containing L-carnitine in the form of a capsule at a dose of 500 mg and a pack of 30 capsules ("Shape up", manufactured by Essensa doo Serbia), and another preparation, a liquid preparation of L-carnitine with the addition of vitamin B6 (Ironmaxx Carnitin pro liquid 500 ml, manufactured by Ironmaxx nutrition GmbH, Germany). Testing was carried out in parallel series of cultures to examine similarities and differences in the effects of L-carnitine alone and L-carnitine in combination with vitamin B6.

Human lymphocytes cell were established in a complete PB–MAX^{TM} Karyotyping medium (GIBCO-Invitrogen, Carlsbad, CA, USA) at 37 °C using standard procedures. Quantity of 3 ml of peripheral blood obtained with sterile venipunction from healthy donors was used for cultivation of peripheral blood lymphocytes. L-carnitine

preparations were diluted to make stocks containing 60 mg/ml of L-carnitine that were subsequently added to the cultures to achieve final treatment concentrations of 50, 250, 500 and 1000 µmol/l of L-carnitine for both tested preparations. After 72 hours of incubation, the cells were harvested for total RNA isolation.

We used total RNA from untreated cultures as a negative control of L-carnitine treatment on *CAT* expression. Total RNA was isolated from treated and non-treated cells. This RNA is a source of genetic information on gene expression and is a direct source of the transcriptional activity (mRNA) of the *CAT* gene. After treatment, the lymphocytes were precipitated by centrifugation at 16,000 rpm in lytic buffer to prepare for the extraction of total information RNA, which was performed using an extraction kit (NucleoSpin RNA extraction kit, Macherey-Nagel, Duren, Germany)

Relative gene expression analysis

Total RNA is translated into complementary DNA (cDNA) by two - step reverse transcription and subsequently used in standard PCR reaction with primers sequences as follows: (*CAT* Forward – GAACTGTCCCTACCGTGCTC and Reverse – GAATCTCCGCACTTCTCCAG (Kučirkova T. et al. 2018) and *GAPDH* Forward–TGAAGGTCGGAGTCAACGGA and Reverse-CCAAAATCAAGTGGGGCGATG (Zheng K.L. et. al. 2015). Real-Time PCR (7300 Real Time PCR System; Applied Biosystems, USA) was used to evaluate the expression ratio of *CAT* gene and *GAPDH* as the housekeeping gene for normalization. Relative gene expression value was calculated as ratio of Ct values of *CAT* and *GAPDH* genes in one sample. The chemistry for relative gene expression assay used in this study is Sybr green (KiCqStart® SYBR® Green Primers).

Statistical analysis

Differential gene expression between treatments that can be attributed to treatment was calculated using REST-384© software (Pfaffl et al., 2002)

with a statistical significance of p<0.05. To additionally explore the nature of variance in *CAT* gene expression in relation to different treatment and L-carnitine concentrations, we used an ANOVA test with P<0.05 considered as a threshold of significance. Monte Carlo and Exact permutation test with the rate of 9999 were used to explore the effect of small data set on obtained results (MedCalc® Statistical Software version 20.011 and PAST software version 4.07.).

Results and Discussion

Gene expression assay show high reproducibility in replicate measurements for each control (N = 6) and test samples (N = 6) for two treatment substances and serial concentrations (50 µmol/l, 250 µmol/l, 500 µmol/l and 1000 µmol/l). The average Ct values and their respective standard deviations with 95% C.I. are provided in Table 1.

The reproducibility of measurements in gene expression experiments is very important aspect in reliable assessments in relative changes in gene transcriptional activity due to treatment effects. The individual variation on *CAT* gene expression was normalized by deduction of Ct value for *GAPDH* from the Ct value of *CAT* gene. The *GAPDH* gene expression analysis was run in parallel with each *CAT* measurement, in two replicates for each treatment and control sample sets.

L-carnitine significantly reduced the transcription of *CAT* gene in human lymphocyte cultures at concentrations of 50 µmol/l and 250 µmol/l compared to negative control, (p = 0.001; p = 0.001; respectively).

The L-carnitine liquid supplement with vitamin B6 also reduced the transcription of *CAT* gene at concentrations of 50 µmol/l and 250 µmol/ as compared to negative control, (p = 0.018; p = 0.006; respectively) (Figure 1). Our results confirmed that supplementation of lymphocyte culture media with L-carnitine preparations caused a decrease in catalase transcriptional activity compared to untreated lymphocytes. The negative control of

Table 1. Average Ct values and Stdev for replicate measurements of *CAT* gene expression in non-treated cells (controls) and cells treated with two tested preparations of L-carnitine

Treatment conc.	L-carnitine (caps)		L-carnitine with B_6 (liquid)		Control (non-treated) cells	
50 µmol/l	27,63	0,08	26,40	0,01	27,79	0,29
250 µmol/l	27,13	0,01	27,83	0,15		
500 µmol/l	26,41	0,04	27,61	0,74		
1000 µmol/l	28,52	0,01	25,35	0,23		

transcriptional activity may be through reduction of oxidative stress and need for catalase cellular activity. The primary and secondary lacks of L-carnitine are found to be associated with inflammation, immune and neurological dysfunction, endocrine deregulation and energy metabolism abnormalities (Sies, 2014). However, the molecular mechanism of action of supplemental L-carnitine is not fully understood. Some forms of non-pharmacological treatments such as hyperbaric oxygenation or ethnobotanical preparations are thoroughly investigated and their positive treatment effects are associated with their effects on the expression of genes involved in oxidative stress response, apoptosis, inflammation and anti-inflammatory response, DNA repair and even tumor growth inhibition (Lisanti et al., 2011; Góth et al., 2016).
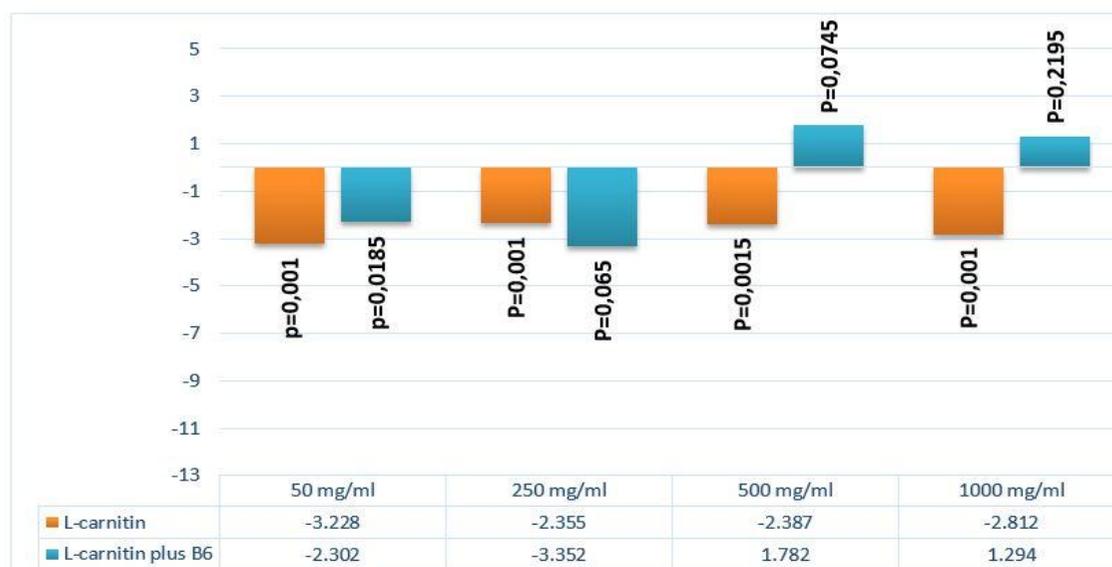

	50 mg/ml	250 mg/ml	500 mg/ml	1000 mg/ml
L-carnitin	-3.228	-2.355	-2.387	-2.812
L-carnitin plus B6	-2.302	-3.352	1.782	1.294

Figure 1. Relative gene expression of *CAT* gene in cultured lymphocytes treated with two concentrations of L-carnitine and L-carnitine plus B6. Blocks are representing fold change in relation to control (untreated cells); negative value represent decreased regulations; P-values next to each block represent level of statistical significance in observed gene expression differences.
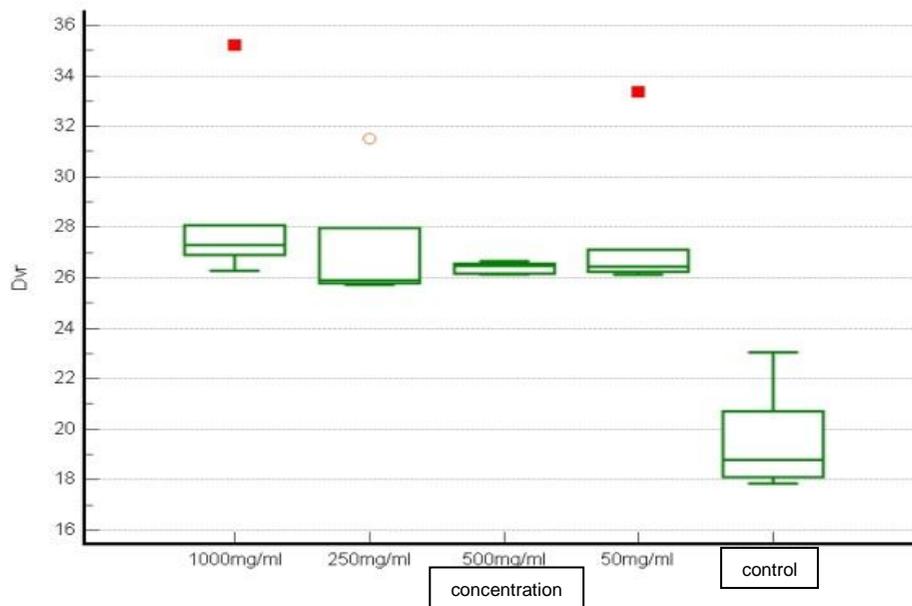
Figure 2. Comparison of differences in normalized Ct values for *CAT* gene between controls (far right) and treatment concentrations of L-carnitine in capsule preparation.

There is a growing interest lately to develop molecular markers for studying non-pharmacological treatment effects of known food supplements on cellular level processes (Hampton and Orrenius, 1997; Li et al., 2018). The catalase gene (HGNC: HGNC: 1516), encodes catalase, a key antioxidant enzyme in the body against oxidative stress. Its main role is to deactivate hydrogen peroxide by converting it into oxygen and water (Boehm, 2006). It is the most abundant enzyme in nature. It is found in most aerobic organisms, but also in anaerobic bacteria. In mammals, it is found in all tissues. Its greatest activity is in erythrocytes and liver. Also, the activity of this enzyme in the brain, heart, skeletal muscle, and spleen has been demonstrated (Dröge, 2002).Within cells, it is found in peroxisomes, where it occurs in free form and is bound to the membrane, and in mitochondria. In erythrocytes, almost the entire amount of this enzyme is found in the cytosol (Glorieux et al., 2015). This enzyme plays an important role in the molecular mechanisms of inflammation, apoptosis, mutagenesis, and tumorigenesis. Altered activity has also been demonstrated in diabetes, regenerating tissues,

Down syndrome, hyponutric status, hemolytic anemia, liver damage, pancreatitis, muscular dystrophy, and neonatal sepsis. Catalase expression in hypoxia is recognized as a positive marker of treatment response (ischemic heart disease, myopathy, and peripheral arterial disease) and beneficial as a treatment supplement in muscle recovery of athletes due to its role in oxidative stress (Glorieux et al., 2015; Sies, 2017). As described in animal studies *in vitro* before (Glorieux et al., 2015), L-carnitine supplementation significantly reduced the reactive oxygen species level which may lead to negative control of catalase gene expression.

ANOVA test of relative gene expression analysis for *CAT* after treatment with serial dilutions of L-carnitine (capsule) revealed statistically significant differences in normalized Ct values between different treatment concentrations (F-ratio = 13,556; P<0,001, *Monte Carlo* permutation test p = 0,00003, Figure 2).Same method of analysis of variance between normalized Ct values for *CAT* gene between treatments with different dilution concentrations of L-carnitine (liquid

preparation) showed (F-ratio = 12,444; P<0,001, *Monte Carlo* permutation test p = 0,00002, Figure 3) with a

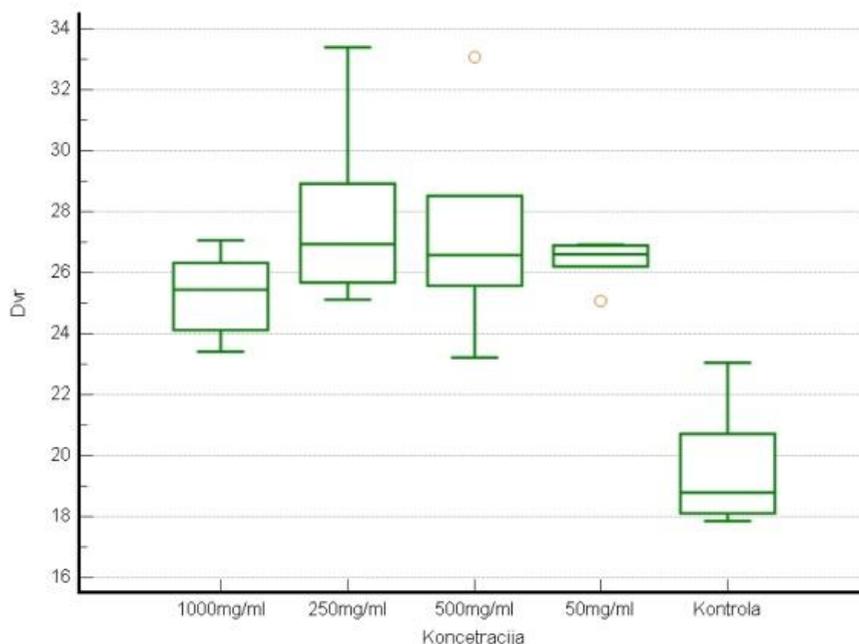statistically significant difference observed between control and all four treatment concentration effects.

Figure 3. Comparison of differences in normalized Ct values for *CAT* gene between controls (far right) and treatment concentrations of L-carnitine in liquid preparation.

Conclusion

The administration of L-carnitine or its analogs has been extensively studied in order to improve metabolic abnormalities associated with mitochondrial dysfunction. Application of selected L-carnitine preparations reduced the expression of the antioxidant enzyme gene in human lymphocyte culture compared to untreated cells, suggesting catalase transcription level as a sensitive marker of antioxidative L-carnitine effects *in vitro* on inhibition of pro-inflammatory processes that inversely reduced catalase expression.

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Conflict of interest

Authors declare no conflict of interest.

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