Original Article

Does taking L-Carnitine and Cadmium have an impact on gene expression of the liver tissues from rat models?

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ABSTRACT

Objective

The levels of increased reactive oxygen species (ROS) in presence of liver disorder might enhance lipid membrane peroxidation as well as manufacturing of inflammatory molecules, concluding in cellular damage and death. Cadmium is known to promote body's ROS production leading to liver damage. L-Carnitine may lower oxidative stress, decrease inflammation, and increase antioxidant enzymes. ROS alters protein transcription and affects the *mTOR* network signaling. This study, therefore, investigated expression levels of important members of this pathway, *mTOR, Akt1,* and *Akt2,* in liver tissues obtained from rats treated with cadmium and L-Carnitine.

Materials and methods

Four distinct experimental groups were formed comprising of a total of 24 female rats that were administrated with the subsequent treatments orally for a period of 28 days. Cadmium was given to the first group at a dose of 2 mg/kg twice a week. The second group was provided with the same dose of cadmium as the previous group in conjunction with L-Carnitine 75 mg/kg daily. The third group was presented with L-Carnitine 75 mg/kg every day. Finally, the fourth group constituted the control group. Rats were euthanised to obtain liver samples and RNA was isolated to provide the template for cDNA synthesis.

Results and Discussion

The results displayed that there was no statistical significance in the difference between the expression levels of *mTOR, Akt1,* and *Akt2* among the control and study groups, respectively. This study showed that, administration of cadmium and L-Carnitine does not influence the activity levels of key mTOR pathway genes. Further studies will include expression analysis of other organs such as kidneys to investigate the interactions among cadmium and L-carnitine administration.

Keywords

Cadmium; Gene expression; L-carnitine; Liver; mTOR pathway

INTRODUCTION

Toxins can trigger cells to utilize existing signaling pathways crucial for maintaining normal organ function. When hazardous metals interact with lipid membranes, they can generate free oxygen or hydroxyl radicals within cells, exemplified by cadmium, a highly reactive metal restricted in various applications such

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as plastics, paints, enamels, and inks[1,2]. Cadmium toxicity is marked by its ability to deplete glutathione and antioxidant enzymes, disrupt the mitochondrial electron transport chain, and consequently elevate ROS production in the body[3]. At concentrations like 120 µmol/L and 10 or 20 M, cadmium induces neuronal cell death via the recruitment of *MAPK, mTOR, JNK,* as well as *PTEN-Akt-mTOR* networks, illustrating its detrimental impact on cellular signaling pathways[4]. NADPH oxidase activation by cadmium further amplifies ROS generation, underlining its role in oxidative stress pathways. Additionally, cadmium's association with lung cancer among exposed workers underscores its classification as a human pulmonary carcinogen[5]. In addition to its known adverse effects on neuronal and pulmonary system, cadmium can also be a hindrance for hematological and immunogenic cells[6]. Hepatotoxic effects of cadmium are evidenced by inflammatory responses involving polymorphonuclear neutrophils and activated Kupffer cells in the liver post-exposure, highlighting the liver's critical role in detoxification and defense against toxins[7]. L-Carnitine, recognized for its metal-chelating properties, functions as an antioxidant by reducing unbound iron ions in the body. It has documented benefits for multiple systems ranging from reproductorory health to cardiac wellbeing[8–11]. Moreover, it mitigates inflammation by facilitating the passage of oxidized long-chain fatty acids into mitochondria, thereby lowering ROS levels and promoting the excretion of harmful metabolites during fatty acid metabolism[12]. This compound's ability to scavenge free radicals is beneficial for patients suffering from liver disease, as it attenuates oxidative stress, reduces inflammation, and enhances enzymes vital for oxidative damage defense[12]. Clinical evidence supports L-Carnitine's efficacy in improving liver function in conditions like non-alcoholic steatohepatitis (NASH), evident in decreased histological steatosis and improved liver enzyme profiles[13]. Similarly, studies in broiler diets demonstrate L-Carnitine's antioxidant capacity, leading to reduced blood cholesterol levels potentially linked to enhanced lipid oxidation and metabolism[14]. Notwithstanding its benefits, oral supplementation of L-Carnitine is associated with occasional side effects including nausea, vomiting, diarrhea, and distinctive body odors in human trials[15,16]. In light of these findings, there is a promising avenue for utilizing L-Carnitine supplementation to mitigate ROS-induced oxidative stress and inflammatory responses in individuals exposed to cadmium. Given cadmium's hepatotoxic effects, this study focuses on elucidating how gene expression in liver tissues of mice treated with cadmium and/or L-Carnitine supplements is influenced. The study particularly explores the impact on the articulation levels of *mTOR, Akt1, and Akt2,* given their crucial roles in metabolic processes including ROS regulation and inflammatory responses[4].

MATERIALS AND METHODS

Ethical approval was obtained from the IRB of Near East University. The Wistar Albino female rats were bred and maintained in the Near East University Experimental Animals Facility. Study design overall followed a well known pattern of tissue harvesting, RNA extraction, cDNA synthesis and qRT-PCR employed by multiple studies[17,18].

A grand sum of 24 prepubertal Wistar Albino female rats (60-70 gr, each) were randomly separated into four groups; control group, the second group (treated with cadmium chloride, $CdCl_2$), the third group (treated with L-carnitine) and the final group (treated with $CdCl_2$ + L-carnitine). The control group was given physiological saline solution of 1ml intraperitoneally. The second group was administered 75mg/kg L-carnitine (Sigma Aldrich- US) by oral gavage. The third group was prescribed with 2mg/kg $CdCl_2$ (Sigma-Aldrich, USA) intraperitoneally and forth group was treated with $CdCl_2$ and L-carnitine *via* oral gavage daily for 4 weeks. The administration of the drugs were performed at the same time in the morning each day. The samples were obtained from the right lobe of the liver and the samples were processed in the DESAM Research Institute Molecular Medicine laboratory.

RNA Extraction was accomplished by utilising the Hibrigen total nucleic acid isolation kit (Hibrigen, Turkey) adhering to the manufacturer guidelines. In brief, initially PBS washes were performed followed by splitting of the samples into smaller pieces and centrifugation. Samples were degraded by the addition of lysis buffer with the aid of vortexing. Samples were incubated on ice to prevent RNA degradation at which point addition of chloroform ensued. The samples were vortexed and subsequently centrifuged again before ethanol was added. Finally, spin columns were employed to elute RNA in subsequent centrifugations.

RNA purity and quantity was assessed using the Nano-drop Spectrophotometer (Thermo-scientific, Pittsburg,

USA). Three different kits were utilized for cDNA synthesis. The first one was the Norgen TruScript First Strand cDNA Synthesis kit for mRNA (Norgen, Canada, product no. 54400), the second was Transcriptor First Strand cDNA Synthesis Kit (Roche, Switzerland, cat. No. 04 896 866 001), and the last one was Hibrigen cDNA synthesis kit (Hibrigen, Turkey).

The LightCycler® 480 SYBR Green I Master kit (cat. No. 04 707 516 001) was used in qRT-PCR reactions without altering the manufacturer guidelines. The final concentration for forward and reverse primers were 0.2 µM. Primer sequences are listed in Table 1.

Table 1. List of primer sequences and their melting temperatures.

Genes	Forward Primer	Reverse Primer	Tm C°
mTOR	CGCTTCTATGACCAGCTGAA	TGACAACTGGATCGCTTGAG	56 C°
AKT1	GGCAGGAAGAGACGATG	CCTGTGGCCTTCTCTTTCAC	55 C°
AKT2	GAAGACTGAGAGGCCACGAC	CTTGTAATCCATGGCGTCCT	56.6 C°

Real-time PCR Rotor-Gene Q (Qiagen) and relevant software was carry out qRT-PCR. Real-time PCR was set up with an initial denaturation step at 95˚C for 10 minutes, followed immediately by 40 cycles of amplification reactionswhere annealing temperatures varied between 55-56˚C depending on the primer under investigation. A negative control sample lacking any cDNA was conducted for each PCR.

Statistical analysis was conducted using GraphPad prism v8.4.2. One-way ANOVA was performed to analyse gene expression levels following administration of drug among the animal models and the controls. Furthermore, unpaired student's T-test analyses were also performed to analyse the significance of drug administration in each group.

Ethical clearence

The study was conducted with full adherence to universal and national regulations for animal experiments. Ethical approval was granted by the Institutional Review Board of Near East University prior to the initiation of experiments.

RESULTS

The comparative ΔΔCT method was utilised to examine the relative transcription levels of genes in a total of 24 liver samples collected from female rats, which were then split into four groups.

As shown in figure 1, levels of gene expression in female rat liver tissues were assessed using one-way ANOVA (Table 2). The *P* value was 0.327 and *F*= 1.22 hence the results were non-significant (*P*>0.05).

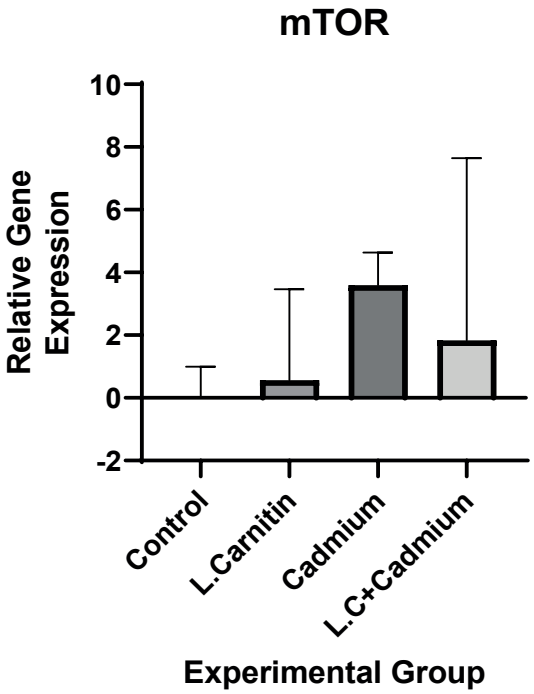

Figure 1 One-way ANOVA results evaluating the *mTOR* gene expression in different study groups

Figure 2 represents one-way ANOVA results evaluating the *Akt1* gene expression. With a *P* value of 0.113 and *F*=2.25, the results were non-significant and subsequent comparison between the control group to the other groups individually using t-test concurrently yielded non-significant results. However, a statistically significant difference (*P* <0.0001, *t*=6.58) was observed in the expression of Akt1 between the liver tissues treated with a combination of cadmium group with L-carnitine and cadmium only group.

DISCUSSION

Cadmium, a highly toxic metallic element, poses significant health risks to all organisms. Exposure to cadmium has been associated with various cancers including breast, prostate, pancreatic, and kidney malignancies. Cells respond to cadmium exposure by producing metallothionein (MT), proteins that bind tightly to cadmium ions, thereby protecting cells. The synthesis of these proteins relies on the cellular capacity for protein synthesis[19].

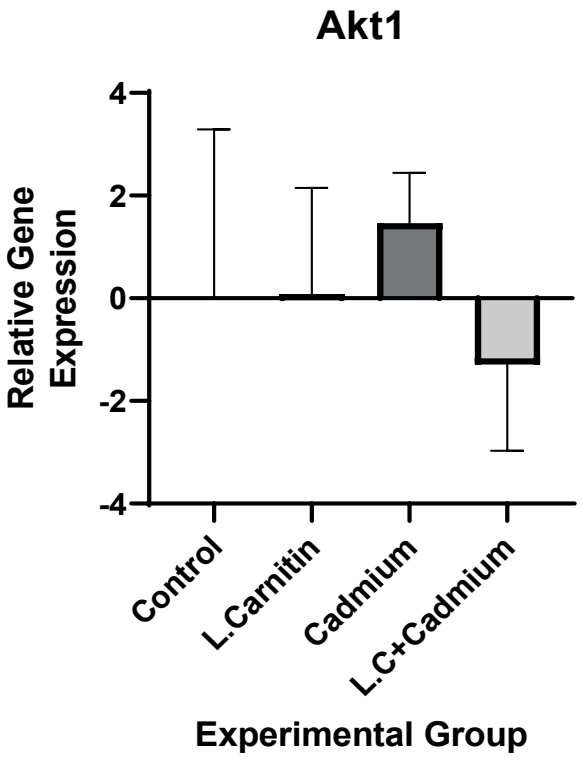

Figure 2 One-way ANOVA results evaluating the *Akt1* gene expression in different study groups

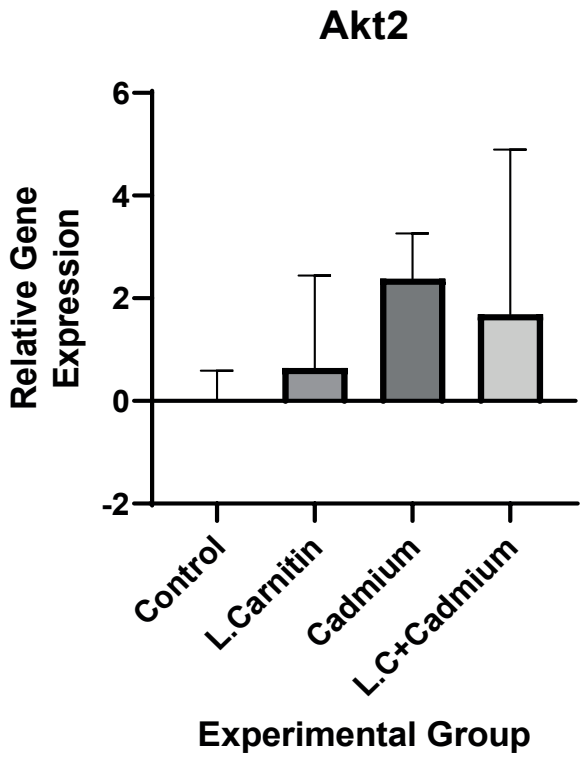

Figure 3 One-way ANOVA results evaluating the *Akt2* gene expression in different study groups

For *Akt2* expression, the statistical test parameters were recorded as P=0.544 and F=0.73, due to that, it was concluded that no significant differences between the groups was observed (Figure 3, table 2).

L-Carnitine is a cornerstone in the final stages of glucose metabolism and has implications for mitochondrial function, potentially influencing the mitochondrial respiratory chain[20]. Supplementing with L-Carnitine has shown promise in preventing hepatitis and hepatocellular carcinoma in rats, in addition to mitigating liver damage caused by too much alcohol intake[13]. The *mTOR* pathway integrates diverse signals including energy levels, stress, growth factors, and nutrients to regulate cellular processes. Activation of *mTOR* is influenced by the *TSC2-Rheb* signaling pathway in response to energy and growth factor stimulation. The *PI3K-AKT* pathway, primarily regulated by growth factors like insulin and *IGF1*, also plays a crucial role in *mTOR* signaling. Overeactive *PI3K* or *AKT* leads to an elevation in phosphorylation of translational regulators such as *S6K1* and *4EBP-1*[21]. Cadmium has been found to alter granulosa cells' cell cycle regulation, proliferation, and apoptosis in by modulating pathways including *MAPK, AKT/FoxO3a,* and *mTOR*[22]. Current study, analyzed L-Carnitine and cadmium treated liver samples to assess the relative expression levels of genes involved in the *mTOR* pathway, specifically *mTOR, Akt1,* and *Akt2*. Statistical analyses using one-way ANOVA and t-tests did not yield significant results. Interestingly, contrary to these findings, increased expression of the *mTOR* gene was observed in the liver following dietary supplementation with L-Carnitine, suggesting a potential positive impact on protein synthesis. However, previous research by Jang et al. indicated no direct association between L-Carnitine and the *Akt/mTOR* pathway[23]. Furthermore, the study found statistically significant differences in Akt1 expression between liver tissues treated with the combination of L-Carnitine and cadmium compared to those treated with cadmium alone. This observation is supported by Keller et al.'s findings demonstrating elevated phosphorylation of *Akt1* and *mTOR* proteins in

Table 2. Multiple comparisons one-Way ANOVA results on mTOR, Akt1 and Akt2 gene expression analyses between control group animals and treatment groups.

	Control vs. L.Carnitin	1.455	-11.67 to 14.58	No	0.9844				
	Control vs. Cadmium	6.732	-6.389 to 19.85	No	0.4379				
	Control vs. L.C+Cadmium	8.358	-4.763 to 21.48	No	0.2749				
	Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	n1	n2	q	DF
	Control vs. L.Carnitin	33.99	32.54	1.455	5.165	6	6	0.2817	20
	Control vs. Cadmium	33.99	27.26	6.732	5.165	6	6	1.303	20
	Control vs. L.C+Cadmium	33.99	25.63	8.358	5.165	6	6	1.618	20
Akt1	Dunnett's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Adjusted P Value				
	Control vs. L.Carnitin	-5.493	-21.55 to 10.56	No	0.7198				
	Control vs. Cadmium	-9.958	-26.01 to 6.095	No	0.294				
	Control vs. L.C+Cadmium	-15.82	-31.87 to 0.2352	No	0.054				
	Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	n1	n2	q	DF
	Control vs. L.Carnitin	19.31	24.8	-5.493	6.319	6	6	0.8694	20
	Control vs. Cadmium	19.31	29.27	-9.958	6.319	6	6	1.576	20
	Control vs. L.C+Cadmium	19.31	35.13	-15.82	6.319	6	6	2.503	20
Akt2	Dunnett's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Adjusted P Value				
	Control vs. L.Carnitin	6.69	-7.087 to 20.47	No	0.4802				
	Control vs. Cadmium	2.693	-11.08 to 16.47	No	0.9254				
	Control vs. L.C+Cadmium	6.732	-7.046 to 20.51	No	0.4753				
	Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	n1	n2	q	DF
	Control vs. L.Carnitin	35.67	28.98	6.69	5.423	6	6	1.234	20
	Control vs. Cadmium	35.67	32.98	2.693	5.423	6	6	0.4967	20
	Control vs. L.C+Cadmium	35.67	28.94	6.732	5.423	6	6	1.241	20

Abbreviations used in the table are as follow; L.C: L. Carnitine, Diff.: Difference, CI: Confidence

Interval, SE: Standard Error, n: Number of samples, DF: Degree of Freedom

Table 2. Multiple comparisons one-Way ANOVA results on mTOR, Akt1 and Akt2 gene expression analyses between control group animals and treatment groups.

Abbreviations used in the table are as follow; L.C: L. Carnitine, Diff.: Difference, CI: Confidence Interval, SE: Standard Error, n: Number of samples, DF: Degree of Freedom

rats' quadriceps femoris muscle following L-Carnitine supplementation[24]. Earlier studies have also linked L-Carnitine supplementation to increased levels of *IGF1*, which could potentially explain the observed differences, as high *IGF1* levels activate *Akt1*, subsequently influencing the *mTOR* pathway. Several limitations were identified in this study. One major constraint was the sample size, which was limited due to ethical considerations in animal research. Individual variations in rat body weight could have led to discrepancies in drug concentrations administered, potentially affecting the study outcomes. Improving drug administration methods to minimize errors and considering adjustments for weight-based dosing could enhance experimental accuracy. Additionally, extending the duration of the study beyond 28 days might allow for better drug absorption and more accurate evaluation of longer lasting effects.

These limitations likely impacted the study's outcomes, contributing to the absence of statistical significance in our findings. Addressing these concerns in future research could lead to more conclusive results, advancing our understanding of how L-Carnitine interacts with cadmium within the *mTOR* pathway in liver tissues.

CONCLUSION

Current work investigated the relative expression of genes belonging to the *mTOR* signaling pathway. Four distinct experimental groups were formed from the liver samples obtained from 24 female rats that had specific drugs administered as assigned prior to the initiation of the experiments. It was suspected, the drugs L-Carnitine and cadmium would have opposing effects on the liver, as one is beneficial and the other is toxic. Limitations are the sample size, the inaccuracy of the drug concentrations caused by the inherent differences in rats such as body weight in addition to lost portions of the drugs due to the sudden movements of the rats while the drug delivery, and the duration of the rats' drug intake. To our knowledge, no other studies have been conducted on rat liver samples to determine the involvement of cadmium and L-Carnitine in *mTOR* pathway-related genes (*mTOR, Akt1,* and *Akt2*). Replicating the study with an enhanced sample pool, as well as incorporating different organs such as kidneys may paint a broader and more accurate picture of the relationship between cadmium and L-carnitine with elements of the *mTOR* pathway.

Statements and Declarations

Funding

Authors declare that no funding was received for conducting this study.

Competing interests

The authors have no competing interests to state.

Author Contributions

Duaa Abdulmohsin: RNA extraction, qRT-PCR, statistical analysis and manuscript writing.

Hakan Aytacoglu: RNA extraction, qRT-PCR, statistical analysis and manuscript writing.

Gamze Kocamaz: Maintenance of the experimental rat groups, liver harvest from the rats.

Aysel Kukner: Idea owner of the study, study design, editing of the final draft.

Pınar Tulay: Idea owner of the study, study design, editing of the final draft.

All authors have approved of the final draft of the manuscript prior to its submission.

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