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ARAŞTIRMA / RESEARCH

L-carnitine effects in CCl4-nephrotoxicity: Immunohistochemical evaluation of glomerular nephrin and HIF-1alpha expressions

CCl4-nefrotoksisitesinde L-karnitin'in etkileri: Glomeruler nefrin ve HIF-1alfa ekspresyonlarının immunohistokimyasal değerlendirilmesi

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Abstract

Purpose: In this study, we aimed to demonstrate the effects of L-carnitine after carbontetrachloride (CCl_4) toxicity through nephrin and Hypoxia inducible factor-1 alpha (HIF-1α) expressions in the glomerular structure.
Materials and Methods: Forty male Sprague dawley rats were divided into 5 groups with animals in each group. Group I: Control group; 0.2 ml olive oil intraperitoneal (ip) twice weekly, Group II: L-carnitine group; 200 mg/kg L-carnitine (ip) twice a week, Group III: CCl_4 group; 0.2 ml CCl_4 (ip) twice a week for 6 weeks, Group IV: L-carnitine + CCl_4 group, 200 mg/kg ip L-carnitine 24 hours before CCl_4 twice a week, Group V: CCl_4 + L-carnitine group; 200 mg/kg L-carnitine half an hour after CCl_4 twice a week. Immunohistochemical staining was performed on kidney tissue sections to show nephrin and HIF-1α expression. Expression densities of the proteins were measured by ImageJ program.
Results: Nephrin expression was significantly increased in Group III compared to other groups. There was a significant increase in HIF-1α expression only between Group I and Group III. Expression densities of proteins in L-carnitine-treated groups were similar to control.
Conclusion: L-carnitine has both protective and therapeutic effects against CCl_4 toxicity in renal glomeruli.

Keywords: Carbontetrachloride, L-carnitine, kidney, nephrin, hypoxia inducible factor.

Öz

Amaç: Bu çalışmada glomerül yapısındaki nefrin ve hipoksi indüklenebilir faktör-1alfa ekspresyonları aracılığıyla karbontetraklorid (CCl4) toksisitesi sonrası L-karnitin'in etkilerinin gösterilmesini amaçladık.
Gereç ve Yöntem: 40 adet Sprague dawley erkek sıçan 5 gruba (n=8) ayrıldı. Grup I: Kontrol grubu; 0.2 ml zeytinyağı intraperitoneal (ip) haftada 2 kez, Grup II: L-karnitin grubu; 200 mg/kg L-karnitin (ip) haftada 2 kez, Grup III: CCl_4 group; 0.2 ml CCl_4 (ip) haftada 2 kez 6 hafta boyunca, Grup IV: L-karnitin + CCl_4 grubu, haftada 2 kez CCl_4 uygulamasından önce 200 mg/kg ip L-karnitin, Grup V: CCl_4 + L-karnitin grubu, haftada 2 kez CCl_4 uygulamasından 1 saat sonra 200 mg/kg ip L-karnitin. Böbrek doku kesitlerine nefrin ve HIF-1α ekspresyonunu göstermek için immunohistokimya boyama uygulandı. Proteinlerin ekspresyon yoğunlukları ImageJ programında ölçüldü.
Bulgular: Nefrin ekspresyonu diğer gruplar ile kıyaslandığında Grup III'de anlamlı olarak arttı. HIF-1α ekspresyonu yalnızca Grup I ve Grup III arasında anlamlı şekilde arttı. Proteinlerin ekspresyon yoğunlukları L-karnitin-tedavili gruplarda kontrol grubuna benzerdi.
Sonuç: L-karnitin böbrek glomerulusunda CCl_4 toksisitesine karşı hem koruyucu hem tedavi edici etkilere sahiptir.

Anahtar kelimeler: Karbontetraklorid, L-karinitin, böbrek, nefrin, hipoksi indüklenebilir faktör.

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INTRODUCTION

Carbontetrachloride (CCl_4) is a colorless, volatile, toxic substance which is rapidly miscible in air, water and soil[1]. CCl_4 causes the formation of harmful metabolites such as trichloromethyl in the cell, blocking the oxidative phosphorylation of free radicals containing an unpaired electron and directly affecting the mitochondrial membrane[2,3]. Because it induces oxidative stress, it causes damage to organs such as kidney, testis and heart, primarily to cause liver damage[4-6]. L-carnitine is essential for the transport of long chain fatty acids into the mitochondrial matrix and plays a role in β-oxidation[7,8]. Furthermore, lipid peroxidation is an antioxidant substance that prevents the accumulation of end products [9]. L-carnitine is mainly synthesized in the kidney and liver, but exogenously can also be taken into the body. Under normal physiological conditions, L-carnitine is highly conserved because a large amount is absorbed back into the proximal tubules of the kidneys.

L-carnitine deficiency is a risk factor for nephrotoxicity and exogenous L-carnitine has been reported to improve glomerular and renal tubular structures in cisplatin-induced renal injury[10]. The kidneys are the main organs in which all toxic chemicals, metabolites, endogenous and exogenous substances are excreted in the urine [11]. The blood coming to the kidney is filtered through the renal corpuscle and water and valuable solutions are reabsorbed in the renal tubules. Thus, the resulting urine is concentrated and blood volume is regulated. Renal corpuscule is the functional unit of the kidney, consisting of glomerulus and bowman's capsules. The glomerular filtration barrier in its structure is responsible for the retention of water and soluble substances[12]. Podocytes responsible for the maintenance of the barrier structure are large cells. These cells form specialized cell-cell contact between their connections called slit diaphragm (SD)[13]. SD acts as a filtering barrier that prevents leakage of urine from the glomerular capillary to the urine between adjacent podocytes[14,15]. Nephrin is the transmembrane component of the immunoglobulin superfamily and constitutes the structural component of SD[13]. Nephrin is located on the lateral and apical faces of the podocytes, in the region between the podocytes. Therefore, preservation of nephrin expression is important in experimental models of glomerular diseases and other proteinuric syndromes[16,17].

Kidney damage caused by hypoxia contributes to the formation of chronic and acute kidney diseases. Hypoxia inducible factor (HIF) is a transcription factor that regulates responses to hypoxia, in particular regulating events such as angiogenesis, vasotone, glucose metabolism, and cell death/survival[18]. Recent insights into HIF regulation allow an understanding of the role of hypoxia in disease progression and the emergence of new options to induce nephroprotection[19].

In this study, we hypothesized that L-carnitine may be effective against the free radicals produced by the toxic effects of CCl_4. We aimed to demonstrate immunohistochemical changes in nephrin and HIF-1α expression in renal glomeruli for evaluation.

MATERIALS AND METHODS

Animals

Male Sprague dawley 40 rats adult, 2-3 months/ 8-12 weeks, weighing 200-300 g obtained from Erciyes University Experimental and Clinical Research Center (DEKAM), Kayseri, Turkey were used in this study. This study was conducted in strict accordance with the recommendations Guide for the Care and Use of Laboratory Animals. The research protocol with animal experimentation was approved by the Scientific Ethics Committee of Erciyes University (approval dated 14.02.2018, decision number 18/034). They were housed in plastic cages in a well-ventilated rat house and allowed ad libitum access to food and water and kept at a 12-h light: dark cycle. All surgery was performed under ketamine + xylazine anesthesia and every effort was made to minimize suffering.

Chemicals

Carbontetrachloride (Sigma-Aldrich, St. Gallen, Switzerland) was used as an inducer of kidney injury. L-carnitine (Sigma-Tau, Pomezia, Italy) was used as a antioxidant.

Experimental design

- **Group I:** Control group (n:8) received intraperitoneally (ip) 0.2 ml olive oil twice a week during 6 weeks.
- **Group II:** L-carnitine group (n:8), 200 mg / kg L-carnitine (ip) was administered twice a week during 6 weeks.

- **Group III:** CCl_4 group (n:8) was applied by dissolving twice a week in 0.2 ml of CCl_4 (ip) olive oil for 6 weeks.
- **Group IV:** L-carnitine + CCl_4 group (n:8) were given 200 mg / kg L-carnitine (ip) 24 hours before each CCl_4 administration during 6 weeks. 24 hours after L-carnitine administration, 0.2 ml / 100 g CCl_4 was applied.
- **Group 5:** CCl_4 + L-carnitine group (8), 200 mg / kg L-carnitine (ip) was administered half an hour after CCl_4 twice a week during 6 weeks.

At the end of the experiment, kidney tissues of the rats were removed under ketamine (75 mg/kg) + xylazine (10 mg/kg) anesthesia.

Histological examination

At the end of the experiment, kidney tissues were fixed in 10% formaldehyde solution. After one week in the fixing solution, it was dehydrated (50, 70, 80, 96%, 3 x absolute alcohol). Transparent with xylol and embedded in paraffin. Nephrin and HIF-1α immunoreactivity were applied to 5 μm thick sections from paraffin tissue blocks.

Immunohistochemistry

Immunohistochemistry was performed with avidin-biotin-peroxidase method to determine nephrin (PA5-20330, Invitrogen, USA) and HIF-1α (NB100-479, Novus Biologicals, USA), immunoreactivity in kidney tissue. Paraffin sections (5 μm) were deparaffinized in xylene. The sections were rehydrated, rinsed in deionized water and antigen retrieval was carried out by microwave treatment in 0.01 M sodium citrate buffer (pH 6.0) at 95°C for 5 min. The sections were washed with phosphate-buffered saline (PBS) and endogenous peroxidase activity was inhibited by 3% H_2O_2 in methanol for 10 min. The staining kit (Lab Vision, Ultra Vision Detection System Large Volume, Anti-Polyvalent Thermo Scientific HRP) was used for the next stages according to manufacturer instruction. The sections were visualized using 3,3P-diaminobenzidine tetrahydrochloride (DAB) and counterstained with hematoxylin [20]. Under the light microscope (Olympus BX51, Center Valley, PA, USA) and images were obtained. A total of 80 different glomeruli were evaluated each groups using the ImageJ program.

Statistical analysis

For statistical analysis, Graphpad Software, La Jolla California USA, Prism 7 version was used. D'Agostino & Pearson normality test was used to determine the normality analysis of the data. Oneway ANOVA and post hoc Tukey kidney were used to compare multiple variables. p value <0.05 was considered significant. Data were evaluated as ± standard deviation.

RESULTS

Expression densities of nephrin and HIF-1α proteins were measured in glomeruli. Compared to Group I, nephrin expression was statistically significantly increased in the Group III. A statistically significant difference was observed between Group III and other groups.

Nephrin expression was decreased in the groups treated with L-carnitine, this decline was close to Group I and II. HIF-1α expression increased in Group III compared to control. This increase was statistically significant. But there was no statistically significant difference among the other groups. Nephrin and HIF-1α immunohistochemistry stained sections are shown in Figure 1 and Table 1. Graphic of nephrin and HIF-1α expressions are given in Figure 2.

DISCUSSION

In the study, we evaluated the glomerular damage in the kidney after toxicity immunohistochemically based on the relationship between nephrin and HIF-1α. The disruption in the glomerular structure, which is the functional unit of the kidney, paves the way for various diseases. It is inevitable that the glomerular structure is not affected after CCL4 toxicity. Nephrin is one of the main proteins of podocytes. HIF-1α is a protein that occurs in the cell against adverse conditions. Therefore, it is important to evaluate the relationship between these two proteins in the glomerulus. The present study demonstrated that nephrin expression level was significantly increased in CCl_4-induced group. L-carnitine treatment (both Group IV and Group V) recovered the nephrin expression. HIF-1α expression increased after CCL_4-induced. HIF-1α has been reported to increase permanently after glomerular injury[21].

Table 1. Nephrin and HIF-1α immunohistochemistry measurement results.

Groups	Group I	Group II	Group III	Group IV	Group V
Nephrin	91.05±5.40[b]	92.56±6,52[b]	99.34±7.52[a]	93.39±7.68[b]	93.14±6.68[b]
HIF-1α	95.92±4.69	98.22±5.94	98.54±7.19[a]	97.64±6.09	97.9±04.39

[a] Significantly different from group I. [b] Significantly different from group III.; Data are expressed as mean±SD. Significance among groups were considered $p < 0.05$.

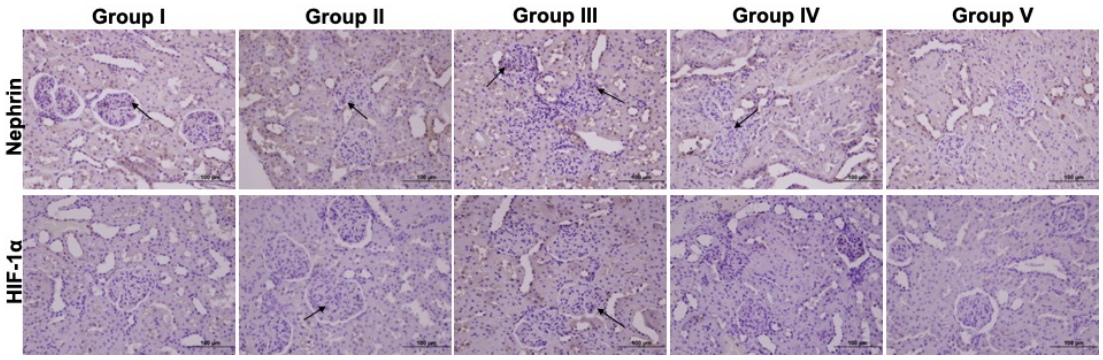

Figure 1. Immunohistochemistry staining of Nephrin and HIF-1α.

Group I: Control group, Group II: L-carnitine group, Group III: CCl_4 group, Group IV: L-carnitine + CCl_4 group, Group V: CCl_4 + L-carnitine group. The arrow indicates the expression densities in the glomerulus. Scala bar:100 μm.

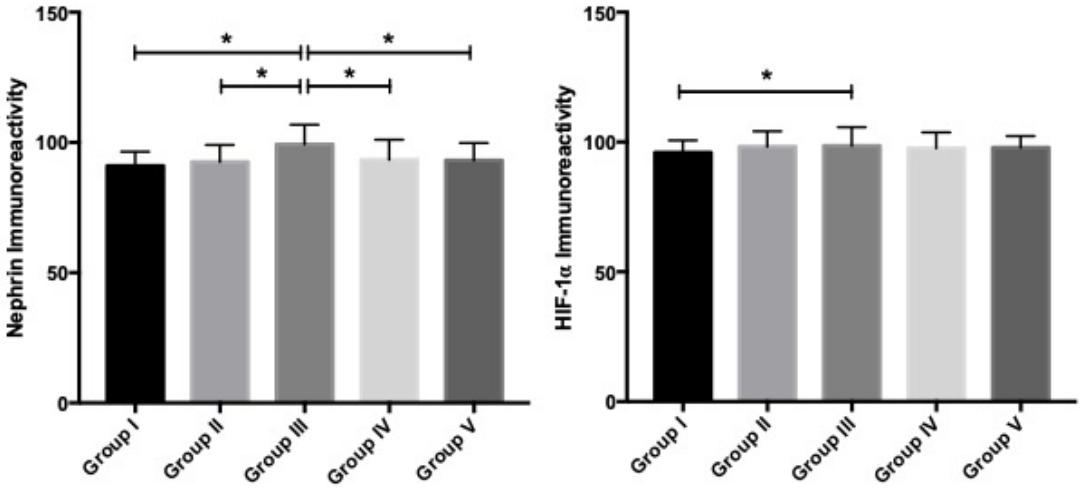

Figure 2. Nephrin and HIF-1α immunohistochemistry results shows the immunoreactivity of proteins among the groups.

* shows the significant difference among groups.

No change was observed in both Group IV and Group V after L-carnitine administration. The nephrin is essential for normal glomerular function. Preservation and permeability of the glomerular filtration barrier integrity is associated with the presence of proteins such as nephrin and podocin[22]. Podocytes are interlocking cells with filtration processes between them. In these cells, proteinuria

occurs as a result of injury, mutations or decrease in protein structure. Nephrin expression has been reported to start at the first stages of development in mice podocytes and from the moment the slit diaphragm first appeared[23]. Therefore, preservation of nephrin expression is important for maintaining glomerular function. Nephrin expression decreased in patients with nephropathy[24], in contrast, nephrin mRNA expression was reported to be increased in nonobese diabetic mouse and rat models[25]. Decrease in nephrine expression has always been associated with proteinuria, but limited information is available, except for signaling pathways associated with its increase. We found that nephrin expression increased after CCl_4 toxicity. This increase suggests that it increases nephrin expression as a precaution against deterioration of structure by CCl_4 toxicity. We know that L-carnitine is a powerful antioxidant that removes harmful metabolic residues and products. Reduction of nephrin expression in groups IV and V compared to Group III indicates that the metabolic products formed after CCl_4 toxicity are removed by L-carnitine, resulting in nephrin expression approaching normal levels in tissue.

HIF-1α is known to be a transcription factor induced under hypoxic conditions. The kidney is highly susceptible to hypoxic injury because hypoxia leads to a change in the gene expression of the glomeruli as it causes energy deprivation[26]. Hypoxia has been reported to induce apoptosis resulting in glycosuria and proteinuria by inhibiting the electron transport chain in the mitochondria[27]. Increased HIF-1α expression is concomitant with proteinuria in podocyte feet degeneration resulting from decreased nephrin and podocin expression[28]. In one study, hypoxia has been shown to have increased HIF-1α expression to adapt to hypoxic conditions and to prevent possible cell damage [29]. In our study, a significant increase in HIF-1α level was observed only in Group III. However, we found that nephrin expression increased in the same group. Increased nephrine expression suggests that the robustness of the glomerular structure may be enhanced, and that HIF-1α expression may be increased to adapt the glomerular structure to toxic conditions. Nephrin expression decreased in diseases and generally proteinuria was observed[30,31]. But in this study there was a significant increase in nephrin expression. Based on the above information, overexpression of nephrin suggests that it inhibits CCl_4-induced possible proteinuria and consequently increases HIF-1α expression in amounts. According to our immunohistochemistry results, it was effective on both nephrin and HIF-1α expressions in L-carnitine treated groups. According to these results, L-carnitine is a powerful antioxidant which has both protective and therapeutic effect on kidney.

Yazar Katkıları: Çalışma konsepti/Tasarımı: DK; Veri toplama: EÖ, ATA, AL, HMÖ, HMU, MS; Veri analizi ve yorumlama: DK, EÖ, ATA; Yazı taslağı:DK; İçeriğin eleştirel incelenmesi: DK, EÖ, ATA; Son onay ve sorumluluk: DK, EÖ, ATA, AL, HMU, TMÖ, MS; Teknik ve malzeme desteği: DK, EÖ, ATA, AL, HMU, TMÖ, MS; Süpervizyon: DK; Fon sağlama (mevcut ise): yok.
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