Kafkas Univ Vet Fak Derg
22 (5): 671-678, 2016
DOI: 10.9775/kvfd.2016.15101

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

Research Article

Nitric Oxide and Glial Fibrillary Acidic Protein (GFAP) Expression in the Liver Parenchyma in Carbon Tetrachloride-Induced Hepatotoxicity

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Article Code: KVFD-2016-15101 Received: 02.03.2016 Accepted: 16.06.2016 Published Online: 16.06.2016

Abstract

Carbon tetrachloride (CCl_4)-induced liver injury causes necrosis and fibrosis, which may lead to cirrhosis and liver failure. Although a lot of study to understand the mechanism of liver fibrosis, our knowledge of the molecular level of this disease is still incomplete. The aim of this study was to investigate the effect and regulation of nitric oxide (NO) and glial fibrillary acidic protein (GFAP) on the severity of hepatic injury in male Wistar albino rats with CCl_4-induced hepatotoxicity and elucidate the underlying mechanism. A total of 20 male rats were randomly divided into healthy control group (A) and CCl_4-induced hepatic fibrosis model group (B), with 10 rats in each group. The results of this study suggest CCl_4-induced hepatic injury is mediated by excessive NO production and up-regulated by inducible and endothelial NO synthase ($P<0.05$), which may plays a role in increasing hepatic injury. Additionally, liver injury induced by CCl_4 demonstrated significant increases in GFAP ($P<0.05$). Histological and immunohistochemical analyses of the CCl_4-treated group exhibited increased inflammatory process and liver necrosis/fibrosis. Inflammatory response especially NO and GFAP expression is identified to play an important role in the development of liver injury and fibrosis induced by CCl_4.

Keywords: *Carbon tetrachloride, Nitric oxide, Glial fibrillary acidic protein, Fibrosis*

Karbon Tetraklorür İle Oluşturulan Hepatotoksisite Karaciğer Paranşimindeki Nitrik Oksit ve Gliyal Fibriller Asidik Protein (GFAP) Sunumları

Özet

Karbon tetraklorür (CCl_4) karaciğerde nekroz ve fibrozis meydana getirerek siroza ve karaciğer yetmezliğine neden olabilmektedir. Günümüzde karaciğer fibrozis mekanizmasının anlaşılmasına yönelik çok fazla çalışma olmasına rağmen, oluşum mekanizması ile ilgili moleküler düzeydeki bilgiler hala eksiktir. Bu çalışmada CCl_4 ile hepatotoksisite oluşturulan erkek Wistar albino sıçanlarda fibrozisin oluşum aşamasında nitric oksit (NO) ve glial fibriller asidik protein (GFAP) etkilerinin araştırılması ve altında yatan mekanizmanın aydınlatılması amaçlanmıştır. Çalışmada 20 adet erkek sıçan, her grupta toplam 10 sıçan olmak üzere sağlıklı kontrol grubu (A) ve CCl_4 ile oluşturulan karaciğer fibrozu grubu (B) olarak rastgele ikiye ayrıldı. Histolojik ve immunohistokimyasal analizlerle CCl_4 grubunda yangısal değişiklikler ile karaciğer nekroz/fibrozis tespit edildi. On iki hafta süreyle CCl_4 verilerek oluşturulan karaciğer hasarının oluşumunda, indüklenebilen ve endotelyal NO sentazın ($P<0.05$) yüksek sunumlarına bağlı olarak artan NO üretiminin önemli bir rol oynadığı belirlendi. Ayrıca, CCl_4 grubunda karaciğerde GFAP ($P<0.05$) sunumlarında önemli artış da gösterildi. Sonuç olarak, CCl_4 kaynaklı karaciğer hasarı ve fibrozisin gelişiminde NO ve GFAP'ın önemli bir rol oynadığı tespit edildi.

Anahtar sözcükler: *Karbon tetraklorür, Nitrik oksit, Gliyal fibriller asidik protein, Fibrozis*

INTRODUCTION

Carbon tetrachloride (CCl_4) is a strong hepatotoxin that causes centrilobular necrosis in experimental animals and is currently used to induce a model of chemical liver injury in experimental studies [1,2]. The hepatotoxicity and carcinogenic properties of CCl_4 have been well defined in humans [3]. Animal models of CCl_4 demonstrate a significant similarity to the symptoms of toxicity in humans including the morphological, biochemical, and pathological

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manifestations. Because of the superficial similarity of cirrhotic responses induced experimentally by CCl_4 in animals, as compared to humans, CCl_4 has been commonly used as a hepatotoxin in experimental studies [2,4-6].

Nitric oxide (NO) is a free radical synthesized from L-arginine by NO synthases activation in parenchymal and non-parenchymal cells [7-9]. In mammals, NO can be generated by three different isoforms of the enzyme NO synthase (NOS). Neuronal NOS (nNOS), which belongs to the constitutive class and is usually localized in neurons and endothelial NOS (eNOS), which is localized in the endothelial cells and is responsible for the regulation of vascular tone, shear stress, and receptor-dependent substances such as bradykinin and acetylcholine. Inducible NOS (iNOS) is expressed in various cells, especially macrophages and plays a part in the inflammatory response [9,10].

Nitric oxide, potentially has both cytotoxic and cyto-protective roles in the liver, which are critical. The amount, location, and duration of NO generated determines the predominance of its protective or harmful effects [11]. Previous studies demonstrated degenerations resulting from severe NO accumulation in the tissues, which triggered apoptosis [11]. In addition, NO creates severe oxidative stress by inducing high level of reactive oxidants from the parenchymal and non-parenchymal cells in the liver [8,12,13]. Furthermore, there are studies suggesting that NO protects the liver against oxidative damage and peroxidation in experimental CCl_4 intoxication [14]. It also contributes to the removal of CCl_4 from the liver by developing the microcirculation in the liver [15,16].

There are studies examining CCl_4 intoxication under conditions where the generation and accumulation of NO were simultaneously investigated [14,15,17,18]. However, the pathophysiology of severe liver injury has not been clarified yet. This is because of the complex role of NO in liver diseases and the conflicting effects that it demonstrates. It is believed that elucidation of the potential role of NO in liver injury, as well as determination of the isoforms of NOS responsible for its generation, would contribute to the development of alternative treatments.

Glial fibrillary acidic protein (GFAP) is an intermediate filament protein that is expressed by cells of the central nervous system and generally considered to be specific for astrocytes [19,20]. GFAP has been described in non neural tissues including liver [21-23]. Hepatic stellate cell (HSC), which can express GFAP, proliferation plays an important role in fibrosis as a result of chronic inflammatory liver diseases [24-26].

In this study, the interactions between NO and GFAP in CCl_4-induced hepatotoxicity as well as the possible roles of NO in the pathogenesis of the associated liver injury have been investigated. In addition, the relationship between the severity of the resulting liver injury and the source of NO generated have been determined.

MATERIAL and METHODS

Ethics Statement

This study was performed in strict accordance with the recommendations in the Guide of The National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs). The protocol was approved by the Committee on the Ethics of Animal Experiments of the Erciyes University (Permit Number: 05-11/59).

Animal Model

Wistar albino male rats weighing 200-250 g were used for all. Rats were randomly divided into two groups of ten animals each (n = 10). To induce chronic liver injury, a 1:1 (v/v) mixture of the CCl_4 (Merck, 1.02222) and corn oil was injected intraperitoneally (i.p., 0.25 mL/kg) twice a week for twelve weeks. Control animals received corn oil at a dose of 0.25 mL/kg in the same manner. Rats were fasted and sacrificed while under sodium pentobarbital anesthesia (65 mg/mL) 48 h after administration of the last dose of CCl_4 and corn oil.

Necropsy and Histopathologic Examination

The livers were collected for histopathology and immunohistochemistry. After harvesting, the livers were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) at Ph 7.4 for 48 h and then were thoroughly rinsed overnight, under tap water. After performing the routine tissue preparation procedures of dehydration using graded alcohol and xylene, the tissue samples were embedded in paraffin blocks. Paraffin serial sections were cut at a thickness of 4-5 µm and placed onto poly-L-lysine-coated glass slides. Hematoyxlin-Eosin (H&E) and immunohistochemical staining were performed, and the sections were histo-pathologically and immunohistochemically analyzed using a binocular light microscope.

Antibodies

Commercial anti-rat antibodies against eNOS (Thermo-scientific, USA, PA1-037), iNOS (Thermo Scientific, USA, PA1-036), nNOS (Santa Cruz Biotechnology, USA sc-5302), and GFAP (Thermo Scientific, USA, MS-1376) were used in the present study. GFAP antibody was diluted to 1:100, ready to use eNOS antibody was used, nNOS antibody was diluted to 1:100 and ready to use iNOS antibody was used.

Immunoperoxidase Examination

Immunohistochemistry was performed to investigate eNOS, iNOS, nNOS, and GFAP expressions. Commercial antibodies were visualized on 4- to 5-µm-thick paraffin sections using an indirect streptavidin/biotin immuno-peroxidase kit (HRP; Thermo Scientific, USA, PHL100413). All steps were carried out following the procedure described by Dincel and Yıldırım [27]. Tissue sections were incubated with the primary antibody (eNOS, iNOS, nNOS, and GFAP)

for 60 min in a humidified chamber at room temperature. Finally, sections were incubated in aminoethylcarbazole chromogen (Thermo Scientific, USA, HA24938) for 5-10 min to induce the color reaction. Mayer's hematoxylin was applied as a counterstain for 30 sec. As a control for non-specific endogenous peroxidase and biotin activities in each test, the primary antibody step was omitted.

Masson Trichrome with Anilin Blue Staining

The extent of liver fibrosis was evaluated by Masson trichrome staining from the CCl_4-treated and control groups rats. After deparaffinization and rehydration, liver sections were stained with Masson's trichrome. Masson trichrome staining was performed following the manufacturer's instructions (Bio Optica, CND Code: W01030799, Milano).

Histomorphometric Analysis and Statistics

The density of immunpositive staining was measured using a computerized image system composed of a Leica CCD camera DFC420 (Leica Microsystems Imaging Solutions, Ltd., Cambridge, UK), connected to a Lecia DM4000 B microscope (Leica Microsystems Imaging Solutions, Ltd.) was used according to the procedure described by Dincel and Atmaca [28]. The pictures of five random fields selected and consecutive 20x objective microscopic fields were captured by the Leica QWin Plus v3 software (Leica Microsystems Imaging Solutions) at a setting identical to the image system. For examining the staining for each antibody, we used the same setting for all slides. Integrated optical density of all the positive staining of eNOS, iNOS, nNOS and GFAP in each photograph was measured. For the quantification of mean was quantified as the eNOS-, iNOS-, nNOS- and GFAP-positive area/total area were measured and calculated by Leica Qwin Plus on the pictures. Data were statistically described in terms of mean and standard deviation (mean±SD) for area %. For evaluating the non-parametric data, Mann-Whitney U-test was performed to compare eNOS, iNOS, nNOS and GFAP immunoreactive cells and immunopositively stained areas in the CCl_4-treated animals versus the healthy controls. A P value of < 0.05 was considered significant. The data were presented as means ± SD. All statistical analyses and graphs were prepared using GraphPad Prism version 6.0 (GraphPad Software, La Jolla California, USA).

RESULTS

Histopathologic Findings

Histopathological examination of rat livers from healthy control group showed normal hepatic lobular architecture *(Fig. 1a)*. The most prominent histopathological findings in the livers of CCl_4-treated rats were characterized diffuse vacuolar fatty changes, large areas of extensive pericentral/centrilobular necrosis/fibrosis, loss of hepatic architecture and hemorrhage *(Fig. 1b)*. Fatty degeneration and coagulative necrosis, especiallyamong hepatocytes settled in the central zone and midzone, were clearly identified in the CCl_4-treated group *(Fig. 1b)*. In addition, necrotic cells were evident in centrilobular areas, and these were surrounded by inflammatory cells.

Masson Trichrome with Anilin Blue Staining Findings

The degree of liver fibrosis in CCl_4-treated group was more severe than that in the control group. The collagen fibers were heavily deposited around portal tracts and central veins in CCl_4-treated group *(Fig. 1c)*. In addition, pseudolobules formations were showed in CCl_4-treated rats.

Nitric Oxide Synthase (NOS) and Glial Fibrillary Acidic Protein (GFAP) Expression

In this study, immunohistochemical staining score that iNOS, eNOS and GFAP immunoreactivity was dramatically enhanced in activated rat parenchymal and nonparenchymal liver cells. iNOS, eNOS and GFAP expressions in the livers of CCl_4-treated rats were higher than healthy control group rats (P<0.05) *(Table 1)*. Increased expression of GFAP expression was observed in the more extensively injured liver samples *(Fig. 2 a,b,c)*. High GFAP expression was observed in limited to the fibrosis area in HSC *(Fig. 2 a,b)*. In addition to GFAP expression increased significantly in the vascular endothelial cells *(Fig. 2b)*, which was also significantly higher than the levels in the healthy control group. The liver sections from CCl_4-treated rats showed strong GFAP immunopositive bands lining the hepatic sinusoids and pericentral hepatocyte cytoplasm *(Fig. 2c)*. GFAP-positive cells exhibited diffuse distribution in the livers of CCl_4-treated rats.

Kupffer and endothelial cells iNOS and eNOS immunoreactivity were evaluated as a remarkable finding in the

Table 1. *Immunoperoxidase test results and statistical data*
Tablo 1. *İmmünoperoksidaz test sonuçları ve istatistiksel verileri*

Animals	N	eNOS		P <	iNOS		P <	nNOS		P <	GFAP		P <
		Mean	Sd		Mean	Sd		Mean	Sd		Mean	Sd	
Control animals	10	5.379	0.732	0.014	5.117	0.453	0.001	1.800	0.509	0.821	2.937	0.423	0.009
CCl_4-treated animals	10	6.379	0.735		6.935	0.670		1.880	0.382		3.548	0.525	

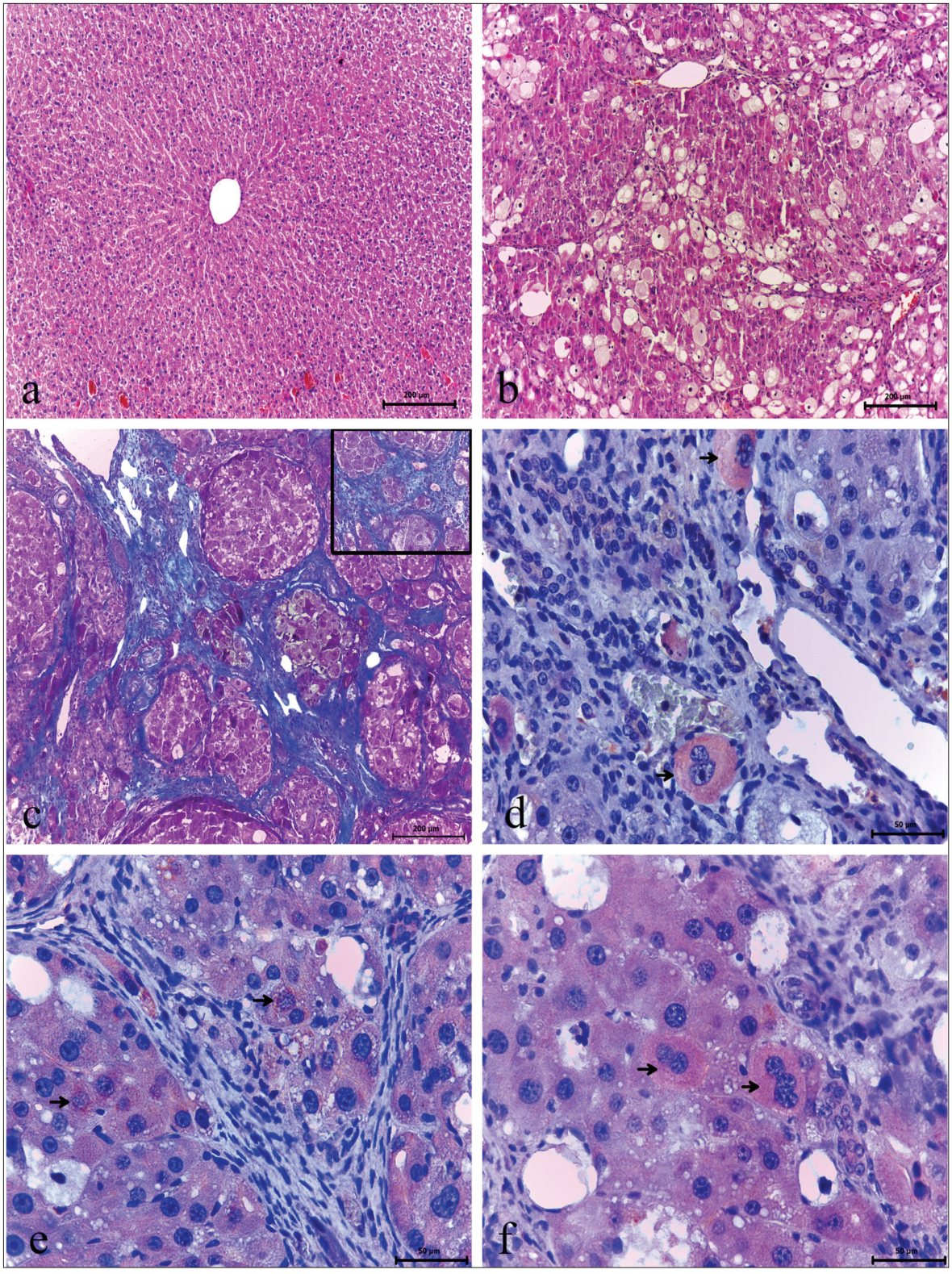

Fig 1. (a) Healthy liver tissue, H&E; **(b)** In the CCl_4 group, observed more severe degeneration and balooned/necrotic hepatocytes, H&E; **(c)** Excessive collagen fibers deposition. Collagen fibers of the connective tissues are identified by their blue color. Masson's trichrome stain and upper small photo large magnification, 40 µm; **(d,e,f)** Strong expression of iNOS in degenerated hepatocytes *(arrows)*. ABC technique (anti-iNOS), Mayer's hematoxylin counterstain

Şekil 1. (a) Sağlıklı karaciğer dokusu, H&E; **(b)** CCl_4 group, şiddetli dejeneratif ve balonumsu/nekrotik hepatositler, H&E; **(c)** Yoğun kolajen fiber depolanması. Konnektif dokudaki kolajen fiberler mavi görülüyor. Masson's trichrome boyaması ve küçük fotoğraf büyük büyütme, 40 µm; **(d,e,f)** Dejeneratif hepatositlerde *(oklar)* şiddetli iNOS sunumları. ABC teknik (anti-iNOS), Mayer›s hematoksilin arka plan boyaması

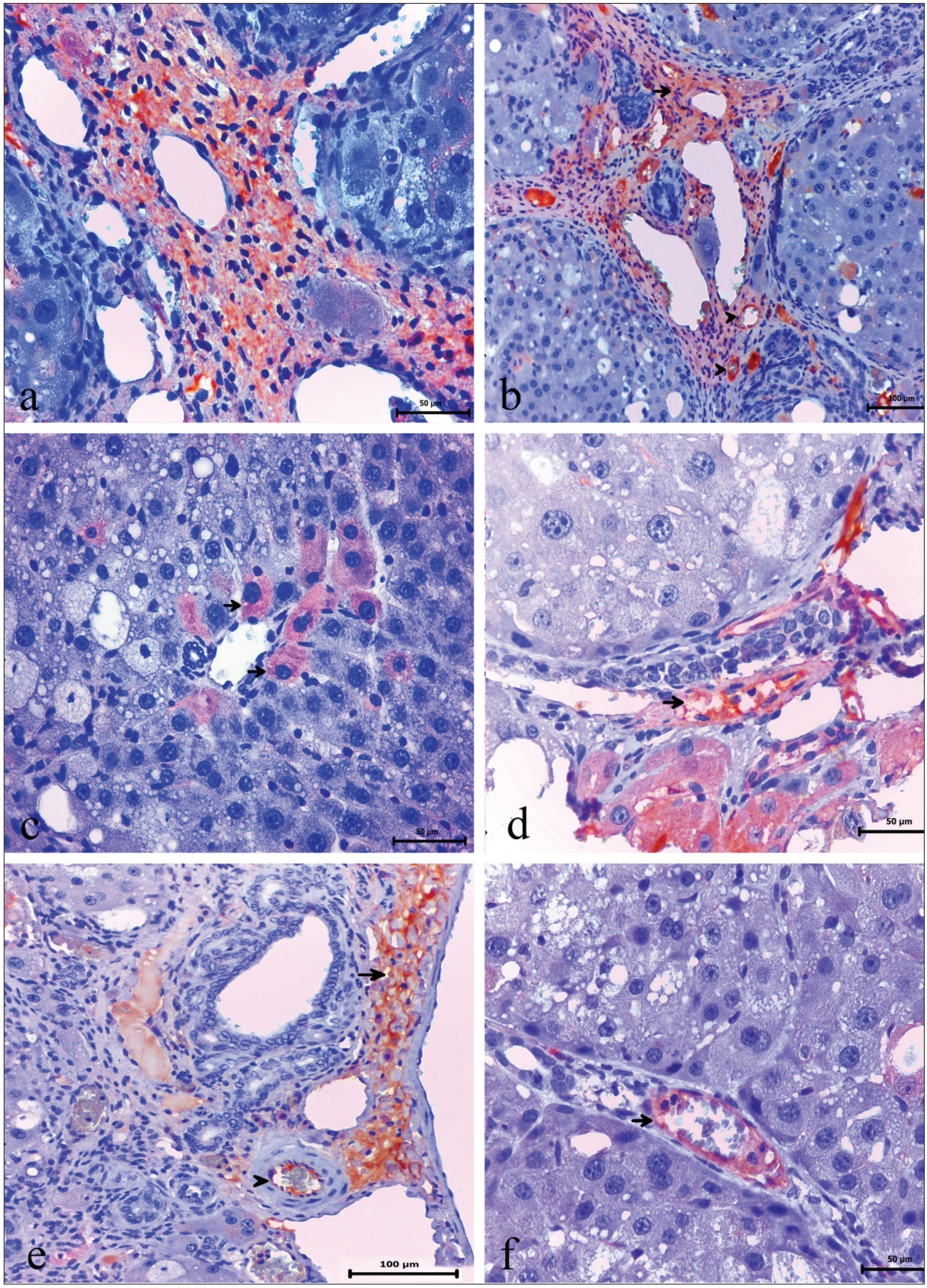

Fig 2. (a,b,c) In the CCl_4 group, strong expression of GFAP in scar and degenerated area *(arrow)*, endothelial cells (arrowhead), pericentral hepatocytes. ABC technique (anti-GFAP), Mayer's hematoxylin counterstain; **(d,e,f)** Modarete expression of eNOS in the endothelial cells *(arrow)*, strong expression of eNOS in some scar area *(arrow)* and endothelial cells *(arrowhead)*. ABC technique (anti-eNOS), Mayer's hematoxylin counterstain.

Şekil 2. (a,b,c) CCl_4 group, skar ve dejenere olmuş alanlarda *(ok)*, endotelyal hücrelerde (okbaşları) ve perisentral hepatositlerde şiddetli GFAP sunumları. ABC teknik (anti-GFAP), Mayer's hematoksilin arka plan boyaması; **(d,e,f)** Endotel hücrelerde orta şiddette eNOS sunumları *(ok)*, skar bölgesinde *(ok)* ve endotel hücrelerde *(okbaşı)* şiddetli eNOS sunumları. ABC teknik (anti-eNOS), Mayer›s hematoksilin arka plan boyaması

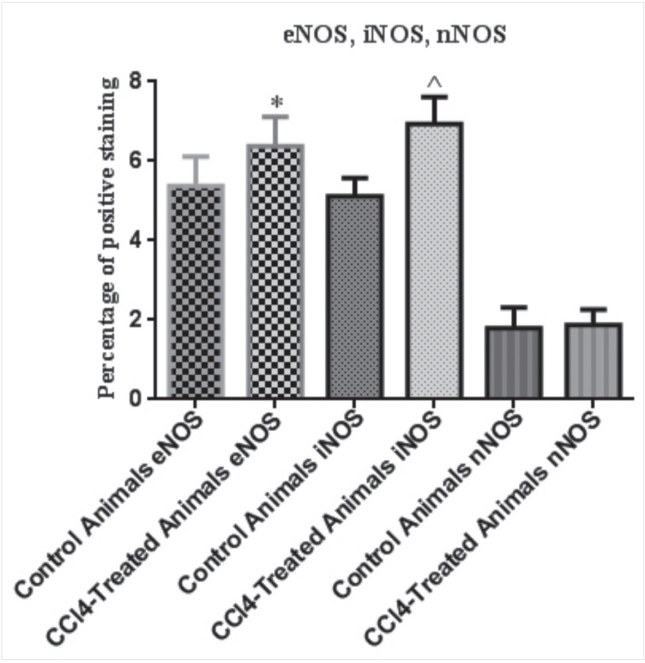

Fig 3. Comparison of eNOS, iNOS and nNOS immunopositivity. '*' indicates values that are significantly greater than those of the healthy control group for eNOS. '^' indicates values that are significantly greater than those of the healthy control group for iNOS.

Şekil 3. eNOS, iNOS ve nNOS immünopozitifliklerin karşılaştırılması. eNOS için '*' ve iNOS için '^' istatistiksel olarak sağlıklı kontrol grubundan yüksek olduğunu vurgulamaktadır

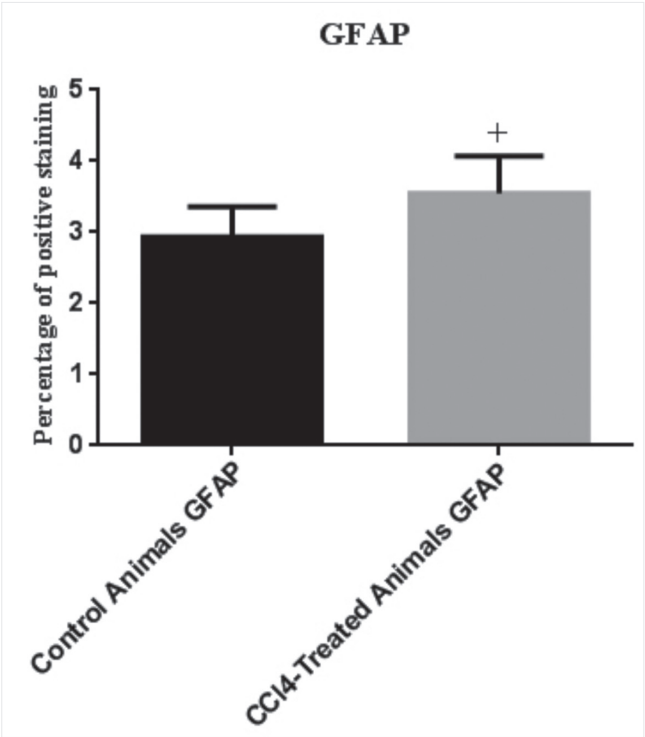

Fig 4. Comparison of GFAP immunopositivity. '+' indicates values that are significantly greater than those of the healthy control group for GFAP

Şekil 4. GFAP immünopozitifliklerin karşılaştırılması. '+' istatistiksel olarak sağlıklı kontrol grubundan yüksek olduğunu vurgulamaktadır

fibrosis area. The more severe iNOS immunoreactivity was determined in parenchymal cells and infiltrating inflammatory cell (lymphocytes) area *(Fig. 1 d,e,f)*. Exclusively in the expression of eNOS a certain amount of increase was observed in endothelial cells *(Fig. 2 d,e,f)*.

nNOS immunopositive stainings was observed in vascular endothelia, hepatocytes and Kupffer cells in livers of CCl_4-treated rats and healthy control group animals. However, the differences was not statistically important between in CCl_4-treated and healthy control group ($P>0.05$) *(Fig. 3)*.

iNOS and eNOS expressions in CCl_4-treated group were higher when healthy control group difference was statistically important *(Fig. 3)*. In addition to GFAP expressions in CCl_4-treated group were higher when healthy control group Difference was statistically important *(Fig. 4)*.

DISCUSSION

In this study, HSC numbers activated with GFAP staining and NO generation in the liver, have been studied immunohistochemically. It has been determined that the NO generated was from the non-parenchymal cells (endothelial, Kupffer, and natural killer cells related to the liver hepatic stellate cells) and from parenchymal cells. It was noteworthy that compared to the healthy control, stellate cell numbers in rats with CCl_4 toxicity, demonstrated a significant increase in iNOS, eNOS and GFAP ($P<0.05$). There was no change in nNOS generation ($P>0.05$).

Liver fibrosis is the pathologic result of chronic liver diseases, and it is characterized by HSC proliferation [24]. HSCs, which express GFAP in normal livers, play important roles in hepatic fibrogenesis [25,26]. This study demonstrated that a high level of GFAP immunopositivity in the HCS was associated with CCl_4 toxicity and activated the HCS considerably. Additionally, the presence of intense GFAP immunopositivity, particularly in the perisinusoidal regions suggests that the fibrogenesis commences from these regions. Furthermore, increasing GFAP immunoreactivity suggests that active fibrogenesis may still be on going. Immunohistochemical demonstration of GFAP expressing HCS count was strongly predictive of the degree of liver fibrosis in CCl_4-induced hepatotoxicity.

Kupffer cell infiltration and activation lead to significantly increases the level of activation of stellate cells. In addition, Kupffer cells create reactive oxygen species (ROS) in the liver. In this case, there is an apparent increase in the stellate cell activation and collagen synthesis [29]. However, the increase in the stellate cell activation is thought to be linked to CCl_4 hepatotoxicity because there are studies that suggest that NO could modulate the activation of stellate cells and thereby, decrease fibrosis [11]. In this study, we speculate that Kupffer and stellate cells serve as critical effector cells in the liver damage linked fibrosis induced CCl_4 toxicity. Furthermore, it is obvious that these

cells have complicated the underlying functions in the molecular mechanism of CCl_4 toxicity.

It has been observed that inhibition of iNOS activity enhances the sensitivity of the liver of rats to hepatotoxic agents. Furthermore, in sensitized rats, the severity of CCl_4-induced liver necrosis increased [18]. In previous studies, it was demonstrated that the increase in hepatic iNOS in rats with liver injury [17]. This finding is consistent with other studies demonstrating that NO increases in CCl_4-induced hepatotoxicity [15,17,30]. In this study, NO is thought to be one of the most significant mediators in activated macrophages and contribute greatly to the pathogenesis of CCl_4 hepatotoxicity. Collectively, these findings support the idea that the NO generated in macrophages and hepatocytes has both protective and pathological roles in the development of CCl_4-induced liver damage.

Cytokine production in healthy liver is very low [31]. Additionally, the balance between the types and in the amounts of cytokines produced is an important factor in the recovery of the liver [32]. However, the roles of cytokines in the regenerative process are still not clear. In pathological conditions, a significant increase has been observed in the release of effector molecules. In this situation, cytokines have a serious role in the pathogenesis of liver diseases. It has been shown that Kupffer cells are involved in the chemical liver damage induced by CCl_4 and alcohols [33-35]. It has also been shown that Kupffer cells enhance tissue damage in the liver through mediators such as biologically active ROS and cytokines [36]. In this study, it is thought that Kupffer cells contribute to the pathogenesis of chemical-mediated liver damages, by releasing biologically active mediators.

In this study, we determined that hepatic NO generation increases significantly, and the major enzyme involved in its production is iNOS. Furthermore, it has been shown that there is a significant increase in eNOS, which also contributes to the existing NO. However, it is not clear which of the NOS isoforms contribute to the change in the hepatic arterial blood flow in acute liver damage. In addition, we speculated that the high level of iNOS expression suggests that this isoform maybe responsible for generating the NO that regulates the hepatic circulation during inflammation and reduces the effect of degeneration. Additionally, endothelial cells express both constitutive NOS and iNOS [37]. Another effect that was observed with chronic CCl_4-induced hepatotoxicity was the iNOS-mediated vasodilation, which appeared to reduce the liver damage. However, more studies need to be conducted to clarify the mechanism involved.

Our findings suggest that NO plays an important role in the advanced phases of the pathogenesis of liver degeneration and fibrosis. In summary, the enhanced NO expression persists over a long period, and contributes to the liver fibrosis. We speculate that the persistent generation of NO observed in chronic CCl_4 toxicity plays an important role in the continuous degeneration and fibrosis, rather than in hepatoprotection.

In liver diseases, different chemical toxins show variations in the rate and time of toxicity. We believe that the increased activation and numbers of HSCs in the livers of CCl_4-treated rats depending on degeneration of liver tissue occurring as a result of the pathological production of NO. The obvious relationship between NO and GFAP in the liver should be further evaluated using different immunohistochemical markers and molecular studies. The results of such in-depth studies would be expected to contribute to providing further explanations for the possible liver damage and repair mechanisms. In addition, these studies may even be beneficial to the process of developing new therapeutic interventions.

In conclusion, the differences in the NO levels observed in CCl_4 hepatotoxicity may be critical to the pathophysiology of NO-induced liver damage. This suggests that the existing major source of NO is also mainly iNOS-generated. The increase in the NO level may be responsible for the hemo-dynamic changes occurring in patients experiencing CCl_4 toxicity. Maintaining the stability of NO levels or an increase under certain conditions may imply that a protective mechanism connected to NO levels can be activated. Furthermore, we saw that there is a definite relationship between stellate cell counts and CCl_4 toxicity. GFAP expressing stellate cells may play an active role in the chemical-induced liver pathology and hepatic fibrogenesis and it count had predictive values for perisinusoidal fibrosis in CCl_4-induced hepatotoxicity.

Conflict of Interests

The authors declare no conflict of interest.

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