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Carbontetrachloride induced acute liver damage and protective effect of n-acetylcysteine on rats with regenerated and non-regenerated liver

Karaciğer rejenere olan ve olmayan sıçanlarda, karbontetraklorürle indüklenen akut karaciğer hasarı ve n-asetilsisteinin koruyucu etkisi

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Abstract: Objective: Our aim was to investigate 70% partial hepatectomy (PH) groups, compare with not subjected to PH groups after exposure to hepatotoxic agents for alterations in the protective effects of antioxidant agents and sensitivity of the liver. Accordingly, we aimed to investigate the toxicity of a hepatotoxic agent, carbon tetrachloride (CCl4), and protective effects of an antioxidant, N-acetylcysteine (NAC), in experimental animal model.

Methods: 67 male Wistar Albino rats were divided into 2 main groups to total 9 subgroups: group 1, underwent PH; group 2, not subjected to PH. 0.5 ml/kg CCl4 and 50 mg/kg NAC was given intraperitoneally (i.p.) to the groups. On postoperative day 9, 70% PH was performed according to the method of Higgins and Anderson. Finally, all rats were humanely killed.

Results: Catalase (CAT) and superoxide dismutase (SOD) activities were significantly lower in both groups when CCl4 was administered. NAC treatment was found to significantly increase these parameters (P<0.05). Malondialdehyde (MDA) and protein carbonyl (PC) levels were significantly greater in both groups when CCl4 was administered (P<0.05). NAC treatment was found to significantly reduce these parameters.

Conclusion: These results indicated that CCl4 increased oxidation products, reduced liver enzymatic activity and reduced proliferation activity in both hepatectomised and nonhepatectomised liver. The liver injury of CCl4, and the protective effect of NAC was similarly in both main groups. Consequently, making PH may not create a negative effect and an additional health problems in liver. Thus, these results can positively affect the decisions of the healthy liver donors.

Keywords: Partial hepatectomy, liver, NAC (N-acetylcysteine), CCl4 (carbon tetrachloride)

Özet: Amaç: Bu çalışmada amacımız %70 parsiyel hepatektomi (PH) yapılan grupların, PH yapılmayan gruplara göre karbontetraklorür (CCl4) gibi hepatotoksik ajanları maruziyetleri sonrasında antioksidan ajanların koruyucu etkilerindeki değişiklikleri ve karaciğer duyarlığını araştırmaktır. Buna uygun olarak, hepatotoksik birajan olan CCl4‘ün toksisitesinin ve bir antioksidan olan N-asetilsisteinin’in (NAC) koruyuculuğunun deneysel hayvan modeli ile araştırılması amaçlanmıştır.

Metod: Altmış yedi Wistar albino tipi erkek sıçan PH yapılan ve yapılmayan olmak üzere iki ana grup altında dokuz alt gruba ayrılırdı. Gruplara intraperitoneal (i.p.) olarak 0.5 ml/kg CCl4 ve 50 mg/kg NAC enjekte edildi.

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Postoperatif dokuzuncu günde, Higgins and Anderson metoduuna uygun olarak %70 PH gerçekleştirildi. Deneyin sonunda bütün sıçanlar usulünce kesildi.

Bulgular: Katalaz (CAT) ve süperoksid dismutaz (SOD) enzim aktiviteleri hem PH yapılan hem de yapılmayan gruplarda CCl₄ uygulandığında azaldığı görüldü. NAC uygulandığında ise bu parametrelerin anlamlı olarak arttığı görüldü (P<0.05). CCl₄ uygulandığında her iki grupta doku malondialdehit (MDA) ve protein karbonil (PC) düzeylerinin anlamlı olarak yükseldiği tespit edildi (P<0.05). NAC'ın bu parametreleri anlamlı olarak düşürdüğü görüldü.

Sonuç: Bu sonuçlar, CCl₄'ün PH yapılan/yapılmayan karaçiğerde oksidan maddeleri artırdığını, karaciğer enzim aktivitelerini ve proliferasyonunu azaltığını göstermektedir. Ayrıca CCl₄'ün karaciğer hasarını ve NAC'ın koruyucu etkisini göstermektedir. Sonuç olarak PH yapılması karaciğer donörlerinde olumsuz yönde bir etki ve ek bir sağlık sorunu oluşturmayabilir.

Anahtar Kelimeler: (PH) parsiyel hepatektomi, karaciğer, NAC (N-Asetilsistein), CCl₄ (karbontetraklorür),

Introduction

The liver is the only organ that exhibits a tremendous potential to regenerate after injury or surgical resection in the mammalian body. The recovery of patients after resection of tumors or donation for a living-related liver transplantation depends on the regenerative capacity of the remnant liver [1]. Most commonly, regeneration of the liver is studied by performing a surgical procedure which removes 2/3 of the liver mass in rodents, a technique known as 2/3 PH [2].

Loss of liver mass can be induced by administering hepatotoxic chemicals [2]. CCl₄ is a lipid-soluble potent hepatotoxic that is widely used as an animal model of acute hepatocellular necrosis [3]. The administration of CCl₄ significantly increases the release of hepatic enzymes, increases destruction of cytochrome P-450, increases lipid peroxidation products, and elicits an inflammatory response [4]. While, after PH, the liver restores its mass and function within days, CCl₄ toxicity leads to an irreversible impairment of liver function [5]. Treatment with antioxidants may modulate the toxic effects of CCl₄ on the liver. NAC has antioxidant, anticyto- toxic and antiapoptotic properties and may therefore be useful in counteracting damaging events of CCl₄-induced hepatitis [6]. This drug has a diversity of applications, largely because of the chemical properties of the thiol moiety present in its structure. The ability of the reduced thiol moiety to sweep reactive oxygen species is well-established by NAC [7].

Partial liver grafts from living donors are usually transplanted to patients in liver transplantation. Therefore, in the present study our aim was to investigate alterations and sensitivities of the regenerated liver of healthy liver donors. Therefore regenerated liver, underwent 70% PH, compare with not regenerated liver, not subjected to PH, after exposure to hepatotoxic agents for alterations in the protective effects of antioxidant agents and sensitivity of the liver. Accordingly, we aimed to investigate the toxicity of a hepatotoxic agent, CCl₄, and protective effects of an antioxidant, NAC, in the regenerated liver and not regenerated liver in experimental animal model.

Materials and Methods

Chemicals

Carbon tetrachloride (CCl₄), 5,5'-dithiobis (2-nitrobenzoic acid; Ellman’s reagent), thiobarbituric acid (TBA), trichloroacetic acid (TCA), hypochlorous acid (HOCl), and 2,4-dinitrophenylhydrazine (DNPH) were from Sigma Aldich GmbH, Germany. NAC (Fluimucil, 10% solution) was purchased from Zambon Group (Italy). Ki-67 mouse monoclonal antibody (MMI) was purchased from Leica (United Kingdom). All other chemicals and solvents were of the highest grade commercially available.

Experimental animals

The animals involved in this study were produced, maintained and used in accordance with the Animal Welfare Act and the Guide for the Care and Use of Laboratory Animals prepared by the Inonu University, Animal Ethical Committee. All experiments were performed on male Wistar rats (each weighing 200–250 g at the time of the experiment). Rats were housed in polycarbonate cages with wire lids and given the standard laboratory chow and water. The housing room was maintained at 24°C with 42±5% relative humidity and had a 12-12-h light-dark cycle (lights on 06:00–18:00 h). Body temperature was maintained around 37±5°C throughout the surgical procedure.
Induction of hepatotoxicity by CCl₄

Oral LD₅₀ values for the CCl₄ ranging from 1000 to 12800 mg/kg of body weight were reported in mice and rats [8]. For CCl₄, the dose was administered on the basis of the LD₅₀ dose as determined by Lewis [9]. Previous studies indicated that animals were given a single dose of 0.5 ml/kg, i.p. CCl₄ [10]. The i.p. LD₅₀ of NAC was reported in the literature as 1205 mg/kg body weight in rats. [11]. In our study, animals were administered a single dose of 0.5 ml/kg, i.p. CCl₄ and 6 doses of 50 mg/kg, i.p. NAC as reported in the literature.

Experimental protocol

67 male Wistar Albino rats (12 weeks old) weighing around 200–250 g were used. Rats were divided into 2 main groups to total 9 subgroups: group 1, underwent PH; group 2, not subjected to PH. Both main groups were assigned into four subgroups respectively: subgroup 1, control; subgroup 2, CCl₄ (0.5 ml/kg, i.p.); subgroup 3, NAC+CCl₄ (50 mg/kg, i.p. NAC and 0.5 ml/kg, i.p. CCl₄); subgroup 4, NAC (50 mg/kg, i.p.). Besides to the groups that underwent a 70% PH, a sham control group was added. In subgroups, which underwent PH were designated 8 tried numbers, in sham control and subgroups which were not subjected to PH were designated 7 tried numbers. 0.5 ml/kg CCl₄ and 50 mg/kg NAC was given i.p. to the groups which underwent PH in the 8th day of operation and to the groups which were not subjected to PH 24 hours before the tissues were taken off. Treated subgroups were administered a total of 6 doses of NAC (50 mg/kg, i.p.), 3 hrs before CCl₄ and 1, 3, 6, 12, 18 hrs after CCl₄ administration. On the other hand, the same volume of serum physiologic was given to the control groups. Treatment to the whole groups was carried out synchronously. As for PH, approximately 70% of the rat liver was surgically removed under general anesthesia. On postoperative day 9, all rats were killed ethical and then serum and liver tissues were removed.

Partial hepatectomy

70% PH was performed according to the method described by Higgins and Anderson [2]. On postoperative day 9, all rats were killed and then serum and liver tissues were removed. A sample of removed liver tissue was kept in 10% formalin for histopathological analysis. Livers were rapidly frozen and stored at -70°C.

Histopathological examination

For all histopathological examinations, liver tissues were fixed overnight in 10% formaldehyde solution (dissolved in phosphate buffer, pH 7.4), and then the incubation solution was altered successively from 70% alcohol to 100% alcohol. The incubating solution was further changed from 100% alcohol to xylene, and the tissues were then embedded in paraffin wax, sectioned in 5 µm slices and stained with H&E.

Ki-67 immunostaining

Immunostaining for Ki-67, a marker for cell proliferation, was performed to evaluate the proliferation of hepatocytes. It was determined as previously described [12]. Slides were examined by a single pathologist unaware of the group identity. The mean of hepatocytes labeled with the anti-Ki67 mouse monoclonal antibody (MMI) over 400 high-power fields was measured in the nine experimental groups.

Biochemical determination

Liver was washed, finely minced, and submerged in ice-cold 0.2 mmol/L Tris-HCl (pH 7.4) and mechanically homogenized (Ultra Turrax T 25 basic; IKA, Wilmington, NC, United States) at 16000 g for 2 min at 4–8°C. In order to evaluate the prooxidant-antioxidant balance, we determined the free radicals production by measuring of lipid peroxidation, PC, glutathione (GSH) levels, and activity of SOD, CAT, and glutathione peroxidase (GSH-Px).

Determination of CAT, SOD, and GSH-Px activity

CAT activity was determined according to Aebi’s method [13]. The principle of the assay is based on the determination of the rate constant (s⁻¹, k) or the H₂O₂ decomposition rate at 240 nm.

SOD activity was determined according to the method of Sun et al. [14] with a slight modification [15]. The principle of the method is based on the inhibition of NBT reduction by the xanthine-xanthine oxidase system as a superoxide generator. Activity was assessed in the ethanol phase of the sample after 1.0 ml ethanol/chloroform mixture (5/3, v/v) was added to the same volume of sample and centrifuged. One unit of SOD was defined as
the enzyme amount causing 50% inhibition in the NBT reduction rate.

GSH-Px activity was measured by the method of Paglia and Valentine [16]. The enzymatic reaction in the tube, which is containing following items: NADPH, reduced glutathione (GSH), sodium azide, and glutathione reductase, which was initiated by addition of H₂O₂ and the change in absorbance at 340 nm was monitored by a spectrophotometer.

### Detection of liver MDA, and GSH levels

The formation of thiobarbituric acid reactive substances (TBARS) during an acid-heating reaction was measured as an index of oxidative stress as previously described [17]. Briefly, the samples were mixed with 1 ml of trichloroacetic acid 10% and 1 ml of thiobarbituric acid 0.67% (Sigma Chemical) and then heated in a boiling water bath for 15 mins. Malondialdehyde equivalents were determined by the absorbance at 535 nm using 1,1,3,3-tetramethoxypropane (Sigma Chemical) as an external standard. Results were expressed as malondialdehyde equivalents per milligram of protein (Lowry assay).

GSH content was determined as described by Ellman [18]. Briefly, liver homogenates (500 µl) were precipitated by adding 500 µl 4% sulfosalicylic acid and centrifugation at 2,858 x g for 10 min. The supernatant (900 µl) was mixed with 100 µl 0.004% 5,5’dithiobis-2-nitrobenzoic acid (DTNB). After standing for 10 min at room temperature, the absorbance of chromophoric products (2-nitro-5-thiobenzoic acid) was determined at 412 nm using a spectrophotometry. GSH content was calculated using a standard curve.

### Methods for detection of PC groups

The oxidative damage to proteins was assessed by the determination of carbonyl groups based on the reaction with dinitrophenylhydrazine as previously described [19]. Briefly, proteins were precipitated by the addition of 20% trichloroacetic acid and redissolved in dinitrophenylhydrazine, and the absorbance was read at 370 nm. Protein was determined by the method of Lowry et al [20].

### Statistical analysis

The distribution of the groups was analyzed by the Shapiro Wilk test. Except GSH, all groups showed a normal distribution, so parametric statistical methods were used to analyze the data. Non-parametric tests were used for GSH. One Way ANOVA, LSD and Kruskal-Wallis were used for the differences between groups. Mann-Whitney U test was employed in comparison of data between groups. All values are given as mean and standard error of mean (SEM). Significance was determined at P<0.05.

### Results

#### Hepatic antioxidant enzyme activities

We measured activities of CAT, SOD, GSH-Px in the liver and results are shown in Table 1.

SOD activity decreased significantly in the PH (-) CCl₄ group (0.115±0.006, p=0.001) when compared with its control group. The decrease in SOD activity was signifi-
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SOD activity increased significantly in the PH (+) CCl₄+NAC group (0.199±0.012, p=0.001) and PH (-) CCl₄+NAC group (0.185±0.015, p=0.001).

CAT activity decreased significantly in the PH (+) CCl₄ (1.030±0.164, p=0.015) and PH (-) CCl₄ (1.756±0.164, p=0.034) groups when compared with their control groups. The decrease in CAT activity was significantly suppressed by NAC treatment. CAT activity increased significantly in the PH (+) CCl₄+NAC group (2.516±0.276, p=0.001) and PH (-) CCl₄+NAC groups (2.377±0.210, p=0.035).

A significant statistical difference of GSH-Px level was not found when the subgroups of PH (+) group compared with the subgroups of PH (-) group (P>0.05, Table 1).

**Lipid peroxidation**

We measured MDA level in the liver and the results are shown in Table 2. Liver MDA level in the PH (-) CCl₄ group was significantly higher than PH (-) control group (94.531±3.752, p=0.001). The elevation of MDA induced by CCl₄ was significantly inhibited by the treatment with NAC in the PH (+) CCl₄+NAC group (53.018±4.547, p=0.001) and PH (-) CCl₄+NAC groups (53.018±4.547, p=0.001).

**GSH**

A significant statistical difference of GSH level was not found when the subgroups of PH (+) group compared with the subgroups of PH (-) group (P>0.05, Table 2).

**PC**

We measured PC level in the liver and the results are shown in Table 2. Liver PC level increased in the PH (+) CCl₄ (0.836±0.088, p=0.044) and PH (-) CCl₄ (0.846±0.165, p=0.016).
p=0.001) groups when compared with their control groups. The elevation of PC induced by CCl₄ was significantly inhibited by the treatment with NAC in the PH (+) CCl₄+NAC (0.398±0.060, p=0.001) and PH (-) CCl₄+NAC (0.480±0.062, p=0.003) groups when compared with their control groups.

### Table 3: Ki-67 levels in liver tissue of Carbontetrachloride (CCl₄) intoxicated rats. The reduction of proliferation activity induced by CCl₄ was inhibited by the treatment with N-Acetylcysteine (NAC) and improved higher than the values of control groups.

| Group          | Grades |
|----------------|--------|
| PH(+)control   | ++     |
| PH(+)CCl₄      | +      |
| PH(+)NAC+CCl₄  | +++    |
| PH(+)NAC       | ++     |
| Sham control   | ++     |
| PH(-)control   | ++     |
| PH(-)CCl₄      | +      |
| PH(-)NAC+CCl₄  | ++++   |
| PH(-)NAC       | +      |

Grades are follows: - = absent, + = 0.1–1.9%, ++ = 2.0–3.9%, +++ = 4.0–5.9%, ++++ = 6.0–7.9%.

### Discussion

The liver is an important organ which plays a central role in the metabolism, detoxification and excretion of various endogenous and exogenous substances such as xenobiotics. Liver injury can be caused by bacterial and viral infections, alcohol abuse, environmental pollutants, and several other factors [21].

Hepatic injury induced by CCl₄, a classic experimental model, has been extensively used for evaluation of hepatoprotective activity. CCl₄ has been used for years to induce acute liver damage in rats [22]. Liver injuries induced by CCl₄ are the best-characterized system of the xenobiotic-induced hepatotoxicity and is a commonly used model for the screening the anti-hepatotoxic/hepatoprotective activity of drugs [23]. The typical hepatotoxin CCl₄ is known to be rapidly transformed by cytochrome P4502E1 of the hepatocyte endoplasmic reticulum to a trichloromethyl radical (CCl₃*) which is converted into a peroxyl radical (CCl₃O₂*) in the presence of oxygen [24].

In recent decades, the pharmaceutical application potential of NAC supplement has attracted much interest from researchers in treating liver damage. NAC exerts its antioxidant action by facilitating glutathione biosynthesis and scavenging the reactive oxygen species (ROS) formed during oxidative stress [25]. The NAC, the N-ace-
tetyl derivative of the amino acid L-cysteine, is a sulphydryl group donor that crosses the cell membrane and restores glutathione levels by providing cysteine for its synthesis [26]. NAC has antioxidant, anticytotoxic and antiapoptotic properties and may therefore be useful in counteracting liver injuries induced by CCl₄ and treatment of liver. For comparison, we used NAC, which has been known for its therapeutic effects linked to the antioxidant and free radical scavenging action, and is commonly used as an antidote against drug-induced hepatopathies [27].

Liver regeneration is a physiological response to the loss of hepatic tissue as in the case of surgical resection of the liver [28]. This regeneration after injury with CCl₄ followed by PH is a complex model involving toxicological, inflammatory, and necrotic processes [29]. PH is generally regarded as the strongest reproducible stimulus to hepatocyte proliferation. It is well known that the remnant liver after 70% PH can regenerate and restore its original mass within 7–10 days in rats [30].

The enzymatic antioxidant defense system is the nature protector against excessive free radicals. Several studies have demonstrated that antioxidant enzymes such as SOD, and CAT represent one protection against oxidative tissue-damage and contribute to oxygen radicals scavenging activity [31]. Previous studies have indicated that CCl₄ decreased activities of antioxidant enzymes [32,33]. In our study, NAC markedly elevated the levels of SOD and CAT, and protected the liver from CCl₄ intoxication, indicating that inhibition of the oxidative cascading stress was one of the main mechanisms in CCl₄-induced liver injury in the rats. Our results show that NAC treatment may contribute to the antioxidant system in the liver following CCl₄ administration. Therefore, the regeneration may be assumed to be related with increasing CAT and SOD enzymatic activities.

TBARS are indicators of the oxidative stress, since they are generated from the breakdown of lipid peroxyl radicals [34]. Earlier it was shown that NAC treatment during CCl₄ administration was able to prevent liver necrosis, to decrease partially the covalent binding of CCl₄ reactive metabolites and to diminish an increase in lipid peroxidation [35]. Similarly, in an experimental model of 10-week long-term rat CCl₄ intoxication, NAC (150 mg/kg, starting 7 days before CCl₄ administration) inhibited liver malondialdehyde formation [36]. Elevated levels of MDA reflect an enhanced lipid peroxidation leading to liver tissue damage and failure of antioxidant defense mechanisms. In our study, the elevation of MDA induced by CCl₄ was significantly inhibited by the treatment with NAC. Therefore, NAC treatment may decrease the MDA content, may diminish an increase in lipid peroxidation and may contribute to the antioxidant system in the liver following CCl₄ administration.

Another indicator of oxidative stress is the PC content which is the oxidation products of specific amino acid residues [37]. Previous studies have indicated that NAC treatment in rats with CCl₄-induced liver injury decreased the PC accumulation and partially attenuated mixed protein-glutathione disulphide levels due to thiol-disulphide exchange between the incoming thiol and oxidized glutathione [38]. Our findings indicated that the increase of PC formation might be a result of oxidative stress and decrease of detoxification process. Therefore, NAC treatment may increase the generation of sulphydryl groups and may contribute to the antioxidant system in the liver. Thus, the regeneration may be assumed to be related with decreasing PC level by NAC.

Ki-67 immunostaining, a marker for hepatic cell proliferation, was performed to evaluate the proliferation of hepatocytes. Previous studies indicated that hepatocyte proliferation inhibited by CCl₄ [39]. In our study, treatment with NAC increased the amount of Ki-67 positive hepatocytes. Accordingly the reduction of proliferation activity induced by CCl₄ was inhibited by the treatment with NAC. This positive effect of NAC may contribute to the liver regeneration. Thus, the regeneration may be assumed to be related with increasing Ki-67 level.

In conclusion, these results indicate that CCl₄ increased oxidation products, reduced liver enzymatic activity and reduced proliferation activity in both hepatectomised and non-hepatectomised liver. In addition, these findings indicate that the liver injury of CCl₄ and the protective effect of NAC were similarly in above both groups. Consequently, making PH not created a negative effect and additional health problems in the liver donors. Thus, these results can positively affect the decision of the donors. On the other hand, no difference was observed in the sensitivity of regenerated liver tissue to CCl₄ toxic and in response to the protective effect of NAC. The absence of an increase of hepatic sensitivity in the regenerated liver against hepatotoxic agents can be positively affect volunteers decisions for donation.

In addition, the present study can contribute to the formation of data about the sensitivity of the donors with regenerated liver against hepatotoxic agents. Thus, both health professionals and community can raise awareness of such an issue, but knowledge about this is not sufficient. Nonetheless, more studies are needed to fully comprehend the issue.

Conflict of Interest: The authors have no conflict of interest.
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