

Therapeutic effect of 6-shogaol on acetaminophen-induced hepatotoxicity in mice: an experimental study

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Abstract. – OBJECTIVE: Acetaminophen (APAP) is one of the most commonly used analgesics and antipyretics. It causes serious liver damage when taken in large quantities by adults or children. Also, 6-shogaol is an active compound obtained from ginger with anti-inflammatory and antioxidant properties. This study aimed at examining the therapeutic effect of 6-shogaol in APAP-induced hepatotoxicity.

MATERIALS AND METHODS: The mice were separated into five groups. After the mice were sacrificed, the levels of alanine aminotransferase (ALT), aspartate transaminase (AST), and alkaline phosphatase (ALP) in the blood, glutathione (GSH) level in the liver tissue homogenate, and levels of induced nitrite oxide synthetase (INOS) and total nitrite/nitrate were measured by spectrophotometric methods.

RESULTS: APAP administration significantly increased the serum levels of ALT, AST, and ALP, INOS activity in liver tissue, and total nitrite/nitrate levels compared with control and significantly decreased GSH levels. After APAP toxicity, 6-shogaol and N-acetylcysteine (NAC) administration significantly decreased the levels of ALT, AST, INOS, and total nitrite/nitrate levels and significantly increased GSH levels compared with control. Also, 6-shogaol was found to be better than NAC in increasing the GSH level.

CONCLUSIONS: The study showed that 6-shogaol might have an early therapeutic effect on APAP-induced liver damage.

Key Words:

Acetaminophen, Hepatotoxicity, Liver, 6-shogaol.

Introduction

Acetaminophen (APAP) is the most commonly used analgesic and antipyretic worldwide¹. It can

be sold without a prescription. Also, it is cost-effective and the most reported medication for its toxicity. It has an excellent safety profile when taken in adequate therapeutic doses. However, its overdose or misuse can lead to hepatotoxicity in adults or children. The most commonly used antidote is N-acetylcysteine (NAC), which protects intracellular glutathione (GSH) reserves to detoxify the N-acetyl-p-benzoquinone imine (NAPQI). Further, NAPQI is the electrophilic APAP metabolite in treating the acute phase of acetaminophen intoxication^{2,3}. However, the requirement for high doses in treatment and its side effects, such as anaphylactoid reaction, and alternate medications to NAC, have been investigated due to the preventive and therapeutic characteristics of NAC in APAP-induced liver damage; also, its ineffectiveness against high-dose poisoning has been investigated⁴. In our emergency clinic, the oral and parenteral forms of NAC have been used as an antidote for treating acetaminophen poisoning. The long duration of treatment with NAC and the unpleasant smell of parenteral forms used in emergency departments compared with their oral forms have led to the investigation of alternative medications with rapid action and more efficiency in acetaminophen toxicity. Many clinical or *in vivo* research have been undertaken to explore the effectiveness of some drugs considered to have a significant hepatoprotective capacity in treating APAP-induced liver poisoning, in addition to NAC⁴⁻⁶.

Ginger rhizomes are commonly used in Korean, Chinese, and Japanese traditional medicine due to their antiemetic, anti-inflammatory, anticarcinogen, antidiarrheic, antioxidant, and antiplatelet aggregation effects⁷. Also, 6-shogaol obtained from ginger is an active compound with

anti-inflammatory, anticarcinogen, and antioxidant properties. Hence, whether 6-shogaol could serve as an alternative to NAC needed exploration No animal and human studies conducted to date compared the antidotal efficacy of NAC and 6-shogaol in acetaminophen toxicity.

This study aimed at comparing the early therapeutic efficacy of previously known and currently used NAC and 6-shogaol in acetaminophen-induced hepatotoxicity in mice.

Materials and Methods

Materials

The study was carried out at Erciyes University, Faculty of Pharmacy, Department of Pharmacology. Also, multifunctional microplate reader (BioTek Synergy HT, Santa Clara, CA, USA), microbalances (Ohaus Adventurer, Parsippany, NJ, USA), vortex (UM10, Wisemix, Wertheim, GERMANY), centrifuge (NüveNF1200R, Nüve-Micro200R, Ankara, TURKEY), incubator (Biosan, Riga, LATVIA), heater and magnetic stirrer (Hei-Standart, Heidolph MR, Schwabach, GERMANY), micropipette and multipipette (Eppendorf, Jülich, GERMANY), blender (IKA T25 digital Ultra-turrax, Staufen, GERMANY), and -80°C Operon (Gimpo-si, Gyeonggi-do, South Korea) were used.

All glass materials used in the study were washed with deionized water. The solutions and buffers used in the experiments were prepared with deionized water.

Chemicals used in the study: 6-shogaol (39303-10 mg, Sigma-Aldrich, St. Louis, MO, USA), acetaminophen (Fluka, P0300000, Sigma-Aldrich, St. Louis, MO, USA), and NAC (A7250-5G, Sigma-Aldrich, St. Louis, MO, USA) were stored at the required environment temperatures.

Experimental Groups and Methods

The study was performed on 50 male BALB/c mice, weighing 30-46 g, which were obtained from Erciyes University Experimental and Clinical Research Center. Ethical approval was established with a 13/94 registration number on June 12, 2013. The experimental animals were housed under a 12-h light/dark cycle and fed a standard diet and water (*ad libitum*). They were randomly segregated into five groups.

Group 1 (control group): mice were administered only 0.9% NaCl intraperitoneally (i.p.)

Group 2: mice were administered 6-shogaol (20 mg/kg) i.p.

Group 3: mice were administered APAP (900 mg/kg) i.p.

Group 4: mice were administered APAP (900 mg/kg) + 6-shogaol (20 mg/kg) i.p.

Group 5: mice were administered APAP (900 mg/kg) + NAC (100 mg/kg) i.p.

In the treatment groups, 6-shogaol and NAC were administered i.p. 2 hours after acetaminophen administration. Referring to the study by James et al⁸ as an example, all animals were sacrificed under ketamine xylazine/anesthesia due to the effect of nitrotyrosine 4 hours later. Further, intracardiac blood samples (1-2 mL) were collected from the mice, following which they were euthanized by cervical dislocation.

The liver tissues were isolated from each mouse. A part of the liver tissue was stored for pathological imaging, and the remaining liver tissue was homogenized. The tissues were stored at -80°C until measurements. The blood samples were centrifuged at 5°C and 2,000 rpm for 10 min. The obtained plasma samples were stored at -20°C until further use.

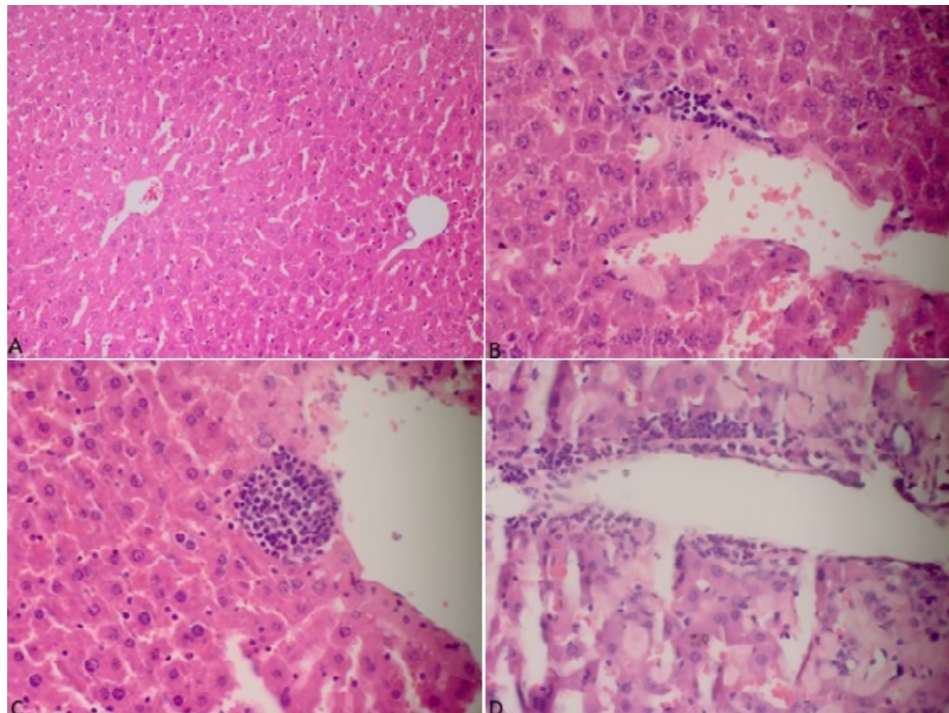
Histopathological Examination of Liver Tissues

The histopathological imaging of liver tissues was performed in the Kayseri Province General Secretariat of the Public Hospitals Association, Kayseri Training and Research Hospital's Pathology Laboratory. For this, the tissues were fixed in 10% formaldehyde solution for 24 hours and then embedded in paraffin blocks. Subsequently, 5-µm-thick sections were cut from the blocks and stained with hematoxylin-eosin (H&E) to examine the general structure and properties of the organs. Masson trichrome staining and reticulin staining were performed to examine the changes in the connective tissue and reticulin fibers. Several selected tissues were stained with Congo red stain for amyloid detection.

In the histopathological examination of the liver tissues, normal liver tissues and the liver tissues of mice in the study groups were compared in terms of inflammation, cell damage, increased connective tissue, and other pathologies. The inflammation was evaluated semi-quantitatively as follows: A/0: none; B/1: mild; C/2: moderate; and D/3: severe scores (Figure 1).

The material in the perisinusoidal and perivenous areas showed staining compatible with the collagen in Masson trichrome stain. The accu-

Figure 1. Degrees of inflammation in liver tissues (H&E, $\times 200$). A/0: none; B/1: mild; C/2: moderate; and D/3: severe scores.



mulation of collagenous material was scored as follows: A/0: none; B/1: accumulation of focal perisinusoidal, perivascular collagenous material (mild); C/2: collagenous material accumulation,

covering half of the liver in the same regions and periportal areas (middle); and D/3: collagenous material accumulation, covering more than 75% of the liver (severe) (Figure 2).

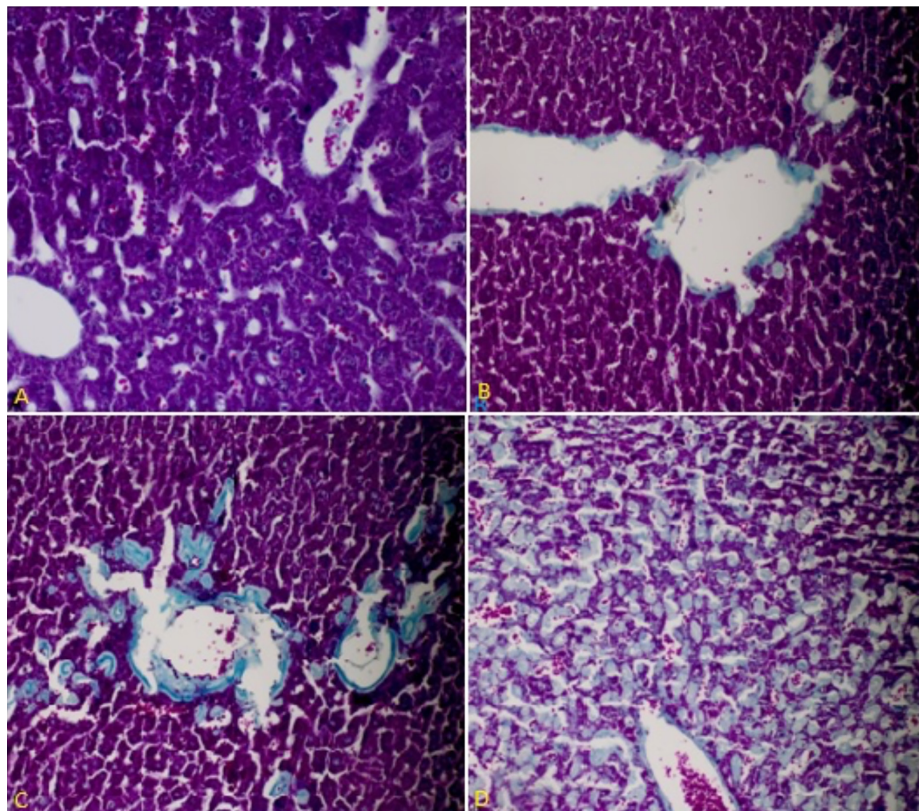


Figure 2. Degrees of accumulation of collagenous matter in liver tissues (Masson trichrome, $\times 400$). A/0: none; B/1: accumulation of focal perisinusoidal, perivascular collagenous material (mild); C/2: collagenous material accumulation, covering half of the liver in the same regions and periportal areas (middle); and D/3: collagenous material accumulation, covering more than 75% of the liver (severe).

Table I. Blood ALT, AST and ALP levels and glutathione level in liver tissue.

	ALT (U/L)	AST (U/L)	ALP (U/L)	Glutathione (µmol/g)
Control (n = 10)	226 ± 35.29	632.02 ± 65.77	439.22 ± 106.27	5.089 ± 0.622
6-shogaol (n = 10)	214 ± 98.35	705.45 ± 108.88	545.02 ± 79.65	5.863 ± 1.281
APAP (n = 10)	1400 ± 294.54	1303.68 ± 130.78	619.87 ± 191.88	3.757 ± 1.042
APAP/6-shogaol (n = 10)	769 ± 108.22	903.24 ± 114.22	597.38 ± 135.33	5.987 ± 1.112
APAP/NAC (n = 10)	283 ± 85.99	651.68 ± 64.90	575.68 ± 114.06	5.414 ± 1.547

ALT, Alanine aminotransferase; AST, Aspartate transaminase; ALP, Alkaline phosphatase; APAP, Acetaminophen; NAC, N-acetylcysteine. Data are expressed as mean ± standard deviation.

Statistical Analysis

Before the study, a power analysis was performed using G-Power 3.1.9.7. The sample size ($n = 10$) was decided according to the test power of 0.80 and the significance level of 0.05. The parameters measured in the samples obtained from the study groups were analyzed using the SPSS 18.0 software (IBM Corp., Chicago, IL, USA). Data were expressed as mean±standard deviation. Kolmogorov-Smirnov and Shapiro-Wilk tests were used to determine whether the variables had normal distribution. The comparison between the groups was performed using one-way analysis of variance or Kruskal-Wallis tests. The Tukey test was used for post-hoc analysis. Paired comparisons between groups were made using the Mann-Whitney U test. The histopathological assessments of the groups were performed using the Chi-square test. The significance level was considered as $p < 0.001$ and $p < 0.05$.

Results

The acetaminophen administration increased ALT activity statistically significantly ($p < 0.001$), while this increase decreased statistically significantly ($p < 0.001$) with 6-shogaol and NAC administration. Also, the administration increased AST activity statistically significantly ($p < 0.001$), while this increase decreased statistically significantly with 6-shogaol and NAC administration ($p < 0.05$ and $p < 0.001$). AST activity (U/L) was significantly different in the treatment groups compared with the control ($p < 0.001$) and APAP groups ($p < 0.05$) (Table I).

ALP activity statistically significantly increased in the acetaminophen-administered group compared with the control group ($p < 0.05$), while it reduced with 6-shogaol and NAC administration. However, this decrease was not found to be statistically significant ($p < 0.01$) (Table I).

When the GSH levels in the liver tissue were evaluated, a statistically significant decrease was observed in the acetaminophen-administered group compared with the control group and other study groups ($p < 0.05$). An increase was observed in the 6-shogaol-administered groups compared with the other groups (Table I).

After acetaminophen administration, the GSH level increased in the NAC-administered group. The change in this level was not statistically significant. The increase in the 6-shogaol-administered groups after acetaminophen administration was found to be statistically significantly different from that in the acetaminophen-only group ($p < 0.05$) (Table I).

High-dose acetaminophen administration increased the iNOS activity compared with that in the control group. This increase was not statistically significant. The activity decreased statistically significantly in the treatment groups, but then increased with acetaminophen administration ($p < 0.05$) (Table II).

A statistically significant increase in the supernatant total nitrite/nitrate level was found in the liver tissue homogenate in the acetaminophen-administered group ($p < 0.05$) compared with the control and 6-shogaol-administered groups. Increased total nitrite/nitrate levels de-

Table II. iNOS, total nitrite and nitrate levels in liver tissue.

	iNOS	Total nitrite/nitrate (µM)
Control (n = 10)	100	3.83 ± 0.94
6-shogaol (n = 10)	104.37	9.23 ± 3.20
APAP (n = 10)	125.14	13.66 ± 6.09
APAP/6-shogaol (n = 10)	98.98	9.59 ± 2.26
APAP/NAC (n = 10)	84.69	8.81 ± 2.23

iNOS, Induced nitrite oxide synthetase; APAP, Acetaminophen; NAC, N-acetylcysteine. Data are expressed as mean ± standard deviation.

creased statistically significantly in the treatment groups after 6-shogaol and NAC administration ($p < 0.05$) (Table II).

Histopathological Evaluation Results

No significant results were found except the inflammation and perisinusoidal and perivenous material accumulation, which were localized in the portal area, central vein surrounding areas, and partly in the parenchyma in the liver tissue. The histopathological examination of the liver revealed no statistically significant difference between the groups.

Discussion

This study investigated the early effect of 6-shogaol on acetaminophen-induced hepatotoxicity in mice. Yemitan and Izegebu⁹ investigated the protective efficacy of ethanolic extract of ginger rhizome on CCl₄- and acetaminophen-induced liver damage in rats and showed that the extract might be useful against in chemical-induced acute liver damage. In a study similar to ours, Sabina et al¹⁰ used 6-gingerol (active metabolite of ginger) and standard medication silymarin after liver toxicity was induced by administering a single dose of acetaminophen to the mice. It was found that 6-gingerol decreased the activities of liver ALT, AST, and ALP after intoxication.

The antioxidant and anti-inflammatory effects of 6-shogaol obtained from ginger were proved by both *in vitro* and *in vivo* studies. Also, 6-shogaol has been used in alternative medicine in far Eastern countries and is thought to be useful against the toxicity of acetaminophen. No animal and human studies to date compared the antidotal efficacy of NAC and 6-shogaol in acetaminophen toxicity. In the present study, the therapeutic effects of previously known and currently used NAC and 6-shogaol in hepatotoxicity in the mouse model were compared.

In our study, a single dose of intraperitoneal (i.p.) acetaminophen was administered to mice so as to induce toxicity, and the serum levels of ALT, AST, and ALP were measured as an indicator of liver function. After APAP administration, the ALT level increased seven times and the levels of AST and ALP increased approximately two and 1.5 times compared with those in the control group. The highest increase after APAP administration was in the ALT level. The ad-

ministered dose of acetaminophen increased the serum levels of ALT, AST, and ALP statistically significantly compared with those in the control group and caused liver damage without mortality.

Choi et al¹¹ administered NAC in the treatment group after APAP administration, which was used as a reference control group against APAP toxicity in mice. They observed a statistically significant decrease in the increased levels of ALT and AST after NAC treatment. Similar to this study, NAC, which is the current antidote, was selected to compare the response of 6-shogaol to treatment in our treatment groups. The serum levels of ALT and AST statistically significantly decreased more in the group treated with 6-shogaol than in the APAP-administered group; however, they were lower than those in the NAC-treated group. Further, 6-shogaol given after APAP administration was not found to be effective in lowering the levels of ALT and AST compared with NAC. The effect of administering different doses of 6-shogaol for a long time should be investigated *in vivo*. Although NAC and 6-shogaol decreased the ALP levels, the decrease was lower in the NAC-treated group. Also, the decrease was not statistically significant in both groups. We believed that the ALP level decreased later compared with the levels of ALT and AST. Both 6-shogaol and NAC were found to decrease the ALP level statistically insignificantly. Ajith et al¹² investigated the protective effect of 3 g/kg acetaminophen and 200 and 400 mg/kg ginger extract given orally (p.o.) to rats 1 hour prior to the administration of acetaminophen. The rats were sacrificed after 24 h. The serum levels of ALT, AST, and ALP, especially in the group administered 400 mg/kg ginger extract, decreased significantly. In the present study, we wondered whether the serum ALP levels statistically significantly decreased in the treatment groups when the mice were sacrificed in the later hours or when they were administered a dose below and/or above the given 6-shogaol dose. This needs further investigation.

In the study by Göksel et al¹³ 900 mg/kg (i.p.) APAP application resulted in a significant decrease in the hepatic GSH level compared with that in the control group. Yapar et al¹⁴ induced hepatotoxicity with 500 mg/kg acetaminophen administration in mice and observed a significant decrease in serum GSH levels at the 4th, 8th, and 24th hour. James et al⁸ induced APAP toxicity in mice; the mice were sacrificed after 1, 2, 4, and 24 hours. They detected nitrotyrosine in the

liver at the 4th and 24th hour after sacrifice. In our study, the mice were sacrificed after 4 h, and inflammation was found in the liver tissue. The study by James et al⁸ and our study supported each other. Meotti et al¹⁵ observed a significant decrease in the total hepatic GSH level after 4 hours of APAP application, while no significant change was reported at the end of the 24 hours. In the same study reported that APAP administration significantly decreased the hepatic GSH level, whereas the GSH level returned to normal after 8 hours. In our study, the GSH levels in the liver were examined at the tissue level at the fourth hour after intoxication. Similar to literature, a statistically significant decrease in liver GSH levels was observed in the APAP group compared with the control group. The decrease in GSH levels with the given APAP dose in our study was an important indicator of liver damage, and it supported our hypothesis. NAC administration increased the GSH level statistically significantly; however, the increase in the GSH level was found to be higher in 6-shogaol-administered groups compared with the NAC-administered group. Our findings indicated that 6-shogaol was a better provider of GSH than NAC, which required no further confirmation.

In the acetaminophen-induced acute liver damage, excess NO is synthesized by means of INOS from various liver cells¹⁶. Levy et al¹⁷ investigated the anti-inflammatory effect of 6-shogaol (6.2 mg/kg) *in vivo* and showed that 6-shogaol decreased the chronic inflammation induced by Freund's adjuvant in the knees of rats. Pan et al¹⁸ showed that 6-shogaol was effective in inhibiting the expression of inflammatory mediators such as INOS and cyclooxygenase-2. Wang et al¹⁹ administered 200 mg/kg ginger extract against aspirin-induced gastric ulcers in rats and observed a decrease in INOS activity, which increased with the administration of aspirin in the gastric mucosa. We investigated INOS and total nitrite/nitrate levels in liver tissue to show the inflammation caused by acetaminophen at the tissue level and the therapeutic effect of 6-shogaol. After acetaminophen administration, the INOS activity and related NO levels increased in liver tissues; however, this increase was not found to be statistically significant. Post-APAP total nitrite/nitrate levels in liver tissues statistically significantly increased compared with those in the control group. The more important part was that 6-shogaol and NAC statistically significantly decreased INOS and total nitrite/nitrate levels

compared with those in the APAP-only group. Although the decrease by NAC was slightly higher than that by 6-shogaol, no statistically significant difference was observed while comparing the treatment groups. The inflammation at the tissue level occurred with APAP administration, and this inflammation was reduced by 6-shogaol and NAC. We concluded that 6-shogaol protected the cells against the damaging effect of peroxynitrite in acute liver damage.

Qiu et al²⁰ stated that 6-Shogaol exerts an anti-inflammatory effect by suppressing the NF-κB pathway in acute liver injury caused by the administration of carbon tetrachloride (CCl₄). Guo et al²¹ showed that the anti-inflammatory effect of 6-shogaol on hepatocytes exposed to lipopolysaccharide (LPS) was related to suppression of the MAPK/NF-κB pathway. In our study, we think that 6-Shogaol inhibits the MAPK/NF-κB pathway and reduces inflammation secondary to paracetamol intoxication.

In the histopathological evaluation, the inflammation and collagenous material accumulation in the liver were re-observed in all groups (Groups 2, 3, 4, and 5), except the control group. No statistically significant difference was found between groups regarding inflammation and collagenous material accumulation in liver tissues. The material accumulation in the collagenous or amyloid structure was the most remarkable finding in liver tissues in our study. This material displayed similar staining as amyloid and collagen. However, it could not be directly evaluated as amyloid and was defined as a collagenous-fibrinoid substance. This finding can be explored in more detail with new experimental studies involving acute and chronic administrations of 6-shogaol, NAC, and APAP.

Limitations

This study had certain limitations. A pilot study was not conducted to determine the 6-shogaol dose to be administered to mice because of the limited budget received from the hospital before the study. The literature review suggested the administration of 20 mg/kg 6-shogaol, and significant results were obtained at this dose.

Conclusions

The early therapeutic effects of 6-shogaol in APAP-induced liver damage were demonstrated in this study at the enzymatic and tissue lev-

els. NAC showed better effect than 6-shogaol in all parameters except GSH levels. However, 6-shogaol was superior to NAC in increasing the GSH level in tissues and reducing inflammation. Although the findings validated the use of 6-shogaol as an alternative to NAC, new studies are needed for further confirmation. The therapeutic effects of different doses of 6-shogaol, for longer periods and using different ways of administration, should be examined *in vivo*.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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Ethics Approval

Ethical approval for this study was obtained from Erciyes University Faculty of Medicine Animal Experiments Local Ethics Committee (Approval date and number: 12.06.2013 and 13/94).

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None.

Authors' Contribution

Mustafa Oğuz Cumaoglu designed the research, analyzed and interpreted the data, and prepared the article. Bilge Cumaoglu analyzed and interpreted the data and evaluated laboratory blood and tissue parameters. Yücel Tekin evaluated tissue pathology. Nurullah Günay designed the study and provided the coordination between the authors. All authors participated in the intellectual content of the manuscript.

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