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Protection by *Nigella sativa* against carbon tetrachloride-induced downregulation of hepatic cytochrome P450 isozymes in rats

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Abstract

Nigella sativa (family *Ranunculaceae*) is an annual plant that has been traditionally used on the Indian subcontinent and in Middle Eastern countries. In this study, we investigated the effect of *N. sativa* oil on the drug-metabolizing cytochrome P450 (CYP) enzymes and whether it has a protective effect against the acute hepatotoxicity of CCl₄. Intraperitoneal injection of rats with CCl₄ drastically decreased CYP2E1, CYP2B, CYP3A2, CYP2C11, and CYP1A2 mRNA and protein expressions. Oral administration of 1 ml/kg *N. sativa* oil every day for one week prior to CCl₄ injection alleviated CCl₄-induced suppression of CYP2B, CYP3A2, CYP2C11, and CYP1A2. Moreover, CCl₄ increased iNOS and TNF α mRNA, while *N. sativa* oil administration for one week prior to CCl₄ injection downregulated the CCl₄-induced iNOS mRNA and up-regulated IL-10 mRNA. These results indicate that *N. sativa* oil administration has a protective effect against the CCl₄-mediated suppression of hepatic CYPs and that this protective effect is partly due to the downregulation of NO production and up-regulation of the anti-inflammatory IL-10.

Key words: carbon tetrachloride, cytochrome P450, *Nigella sativa* oil

Introduction

Nigella sativa (family *Ranunculaceae*), commonly known as black seed or black cumin, is an

annual plant that has been traditionally used in Arab countries. *N. sativa* seeds were prescribed by ancient Egyptian and Greek physicians to treat headache, nasal congestion, toothache, and intesti-

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nal worms, as well as a diuretic to promote menstruation and increase milk production⁴). Pharmacological study of the plant has shown its broad therapeutic value. *N. sativa* seeds contain more than 30% fixed oil and 0.40-0.45% w/w volatile oil. The volatile oil has been shown to contain 18-24% thymoquinone, and 46% comprises many monoterpenes such as *p*-cymene and α -pinene³). *N. sativa* oil (NSO) was recently subjected to extensive pharmacological investigations that revealed its antioxidant activity in different organs^{7,16}). Importantly, NSO is traditionally used as an anticancer drug alone or in combination with other anticancer drugs^{23,26}). However, the effects of this oil on drug metabolism remain unknown.

Cytochrome P450 (CYP) is a phase I enzyme that is involved in the oxidative activation or deactivation of both endogenous and exogenous compounds such as drugs, environmental toxins, and dietary constituents. Many factors modify CYP levels and activities, the most important of which is the inflammatory responses that interact with and alter the levels and activities of drug-metabolizing enzymes. It is well known that infectious or inflammatory stimuli induce the down-regulation of a number of different hepatic P450 mRNAs and proteins¹⁹).

There are several lines of evidence suggesting that nitric oxide (NO) is the mediator of the endotoxemia-induced suppression of CYP²⁴) and treatment of rats with an inhibitor of inducible nitric oxide synthase (iNOS), L-NAME, was found to prevent the endotoxemia-mediated suppression of CYPs⁹). Many food chemicals are known to modify CYPs and among them, herbal medicines are of great concern because many of them have been reported to alter drug metabolism and the inflammation that accompanies many pathological states. The alterations of drug metabolism due to herbal medicine administration and/or inflammatory process should be considered in clinical therapeutics. Carbon tetrachloride proves highly useful for experimental induction of endotoxemia in many species, and it is well known that administration of CCl₄ induces acute liver injury and endotoxemia in

rats.

In the present study, we investigated the effects of NSO on CYP activities and CCl₄-induced hepatotoxicity. We demonstrated that NSO has a protective effect against the CCl₄-mediated suppression of hepatic CYP isoforms, and our results suggest that NSO has a beneficial effect on CYPs and cytokines that counteract the effects of acute inflammation on drug metabolism.

Materials and Methods

Materials: Bovine serum albumin (BSA), polyclonal goat-anti rat CYP1A2, CYP2B1/2, CYP2C11, and polyclonal rabbit-anti rat CYP3A2 antibodies were purchased from Daiichi Pure Chemical Co., Ltd. (Tokyo, Japan). Horseradish peroxidase-labeled rabbit anti-goat IgG was from Sigma Chemical Co. (St. Louis, MO, USA). Horseradish peroxidase-labeled goat anti-rabbit and polyclonal goat anti-rat β -actin antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Diaminobenzidine tetrahydrochloride was purchased from Kanto Chemical Co. (Tokyo, Japan). NSO was obtained by crude extraction of *N. sativa* seeds without any solvent. Other chemicals and solvents were of analytical grade.

Animals and treatments: All experiments using animals were performed under the supervision and approval of the Institutional Animal Care and Use Committee of Hokkaido University. Twelve-week-old male Wistar rats (SLC, Hamamatsu, Japan) were divided into four groups of four rats each. They were housed at 24 $\pm$ 1°C with a 12-hr light and 12-hr dark cycle, and given laboratory feed and water ad libitum. Rats were assigned to groups of CCl₄, CCl₄+NSO, NSO only, or distilled water as the control. The CCl₄+NSO and NSO groups were given *N. sativa* oil at a dose of 1 ml/kg by intragastric (i.g.) administration every day for 7 days, while the other two groups were given 1 mg/kg of water by the same method. On the 8th day, the CCl₄ and CCl₄+NSO groups were injected in-

traperitoneally (i.p.) with 0.4 ml/kg CCl₄ as a 20% solution in paraffin oil, while the other two groups were given an equal amount of paraffin oil. Twenty-four hours after CCl₄ administration, rats were sacrificed with carbon dioxide, and livers were removed.

Preparation of microsomal fraction: The microsomal fractions from livers were prepared according to the method of Omura and Sato²⁰⁾ with slight modifications. Livers were minced and homogenized in 3 volumes of ice-cold 1.15% potassium chloride solution with a Teflon homogenizer. Homogenized samples were centrifuged at 9,000 g at 4°C for 20 min. The supernatant fraction was centrifuged at 105,000 g at 4°C for 70 min to attain a mitochondria-free microsomal pellet. The washed microsomes were then suspended in 0.1 M potassium phosphate buffer, pH 7.4, divided into 1.5 ml tubes, snap-frozen in liquid nitrogen, and kept at -80°C until use. Microsomal protein concentrations were determined according to the method of Lowry *et al.*¹³⁾ using BSA as a standard.

CYP protein measurement by Western blotting: Aliquots of 12 µg of liver microsomal proteins from treated and control rats were applied to 10-12% sodium dodecylsulfate (SDS) polyacrylamide gels and separated by electrophoresis¹²⁾ using a Protean 2 mini 1-D cell (BioRad, Hercules, CA, USA). Western blot analysis was performed according to Towbin *et al.*²⁵⁾. The proteins were electrophoretically transferred to nitrocellulose membranes, blocked in 5% skim milk in phosphate buffer saline (PBS) containing 1% Tween 20 for 2 hr at room temperature, and probed with the polyclonal goat anti-rat CYP2B, CYP2E1, CYP1A2, β-actin, or CYP2C11, or polyclonal rabbit-anti rat CYP3A2 antibodies as solutions in PBS containing 1% Tween 20 on a shaker for 2 hr at room temperature. The secondary antibody was either horseradish peroxidase-labeled rabbit anti-goat IgG or horseradish peroxidase-labeled goat anti-rabbit IgG in PBS containing 1% Tween 20 on a shaker for 1 hr at room temperature.

Immunoreactive protein bands were colorimetrically revealed by oxidation of 0.025% 3,3-diaminobenzidine tetrahydrochloride with 0.0075% hydrogen peroxide and catalyzed by peroxidase in 50 mM Tris-HCl (pH 7.6). The intensities of the immunoreactive bands were densitometrically analyzed on a Macintosh computer using the public domain NIH image program (U.S. National Institutes of Health), available on the internet at <http://rbs.info.nih.gov/nih-image/>.

RNA extraction: Total RNA was isolated from 50 mg liver using TRIzol reagent (Life Technologies, Inc., Grand Island, NY, USA). Briefly, the liver tissue sample was homogenized in 1 ml TRIzol, then 0.3 ml chloroform was added. The mixture was then shaken for 30 sec followed by centrifugation at 4°C and 15,000 g for 20 min. The supernatant layer was transferred to a new tube, and an equal volume of isopropanol was added to the sample, shaken for 15 sec, and centrifuged at 4°C and 15,000 g for 15 min. The RNA pellet was washed with 70% ethanol. RNA was dissolved in diethylpyrocarbonate-treated water. The prepared RNA was checked by electrophoresis, which demonstrated the RNA integrity; then, the optical density was measured. The optical density of all RNA samples was 1.7-1.9 based on the 260/280 nm ratio.

RT-PCR: A mixture of 5 µg total RNA and 0.5 ng oligo dT primer was incubated at 70°C for 10 min in a total volume of 24 µl sterilized ultra-pure water, then removed from the thermal cycler and made up to 40 µl with a mixture of 8 µl 5x RT-buffer, 2 µl 10 mM dNTP, 2 µl diethylpyrocarbonate-treated water, and 2 µl reverse transcriptase (Toyobo Co., Ltd, Osaka, Japan). The mixture was then re-incubated in the thermal cycler at 30°C for 10 min, at 42°C for 1 hr, and at 90°C for 10 min to prepare the cDNA. Aliquots (1 µl) of the synthesized cDNA were added to 19 µl of a mixture containing sterilized ultra-pure water, 2 µl 10x PCR buffer, 2 µl dNTP (2.5 mM), 0.3 µl sense and anti-sense primers (10 mM), and 0.1 µl Taq polymerase (Takara, Kyoto, Japan). Specific

primer sets for iNOS (sense 5'-TTCTTTGCTTCTG TGCTTAATGCG-3'; antisense 5'-GTTGTTGCTGA ACTTCCAATCGT-3'), TNF α (sense 5'-GTAGCCCA CGTCGTAGCAAA-3'; antisense 5'-CCCTTCTCCA GCTGGAAGAC-3'), IL-10 (sense 5'-GGAGTGAAG ACCAAAGG-3'; antisense 5'-TCTCCCAGGGAATT CAAATG-3'), G3PDH (sense 5'-TGAAGGTCGGTG TGAACGGATTGTC-3'; antisense 5'-CATGTAGG CCATGATGAGGTCCACC-3') were used. Amplification was initiated by denaturation by one cycle at 95°C for 1 min, followed by, in each cycle, denaturation at 94°C for 1 min, and annealing at the proper temperature for 1 min, and then extension at 72°C for 1 min for the proper number of cycles for each gene mRNA using a thermal cycler (Bio-Rad). Finally, the samples were incubated for 7 min at 72°C after the last cycle of amplification. Amplified PCR products were separated by electrophoresis through 1.0-1.5% agarose gels. Bands

of cDNA were stained with ethidium bromide and visualized by ultraviolet illumination. Intensities of the bands were densitometrically analyzed on a Macintosh computer using the public domain NIH image program.

Statistical analysis: All data are expressed as means $\pm$ SE. Statistical significances were evaluated using ANOVA and the Tukey-Kramer test. The differences at $p < 0.05$ were considered to be statistically significant.

Results and Discussion

N. sativa is an important medicinal herb that has been used since ancient times as a natural remedy for a wide range of diseases, but the mechanism of its action is still unclear¹. CYP su-

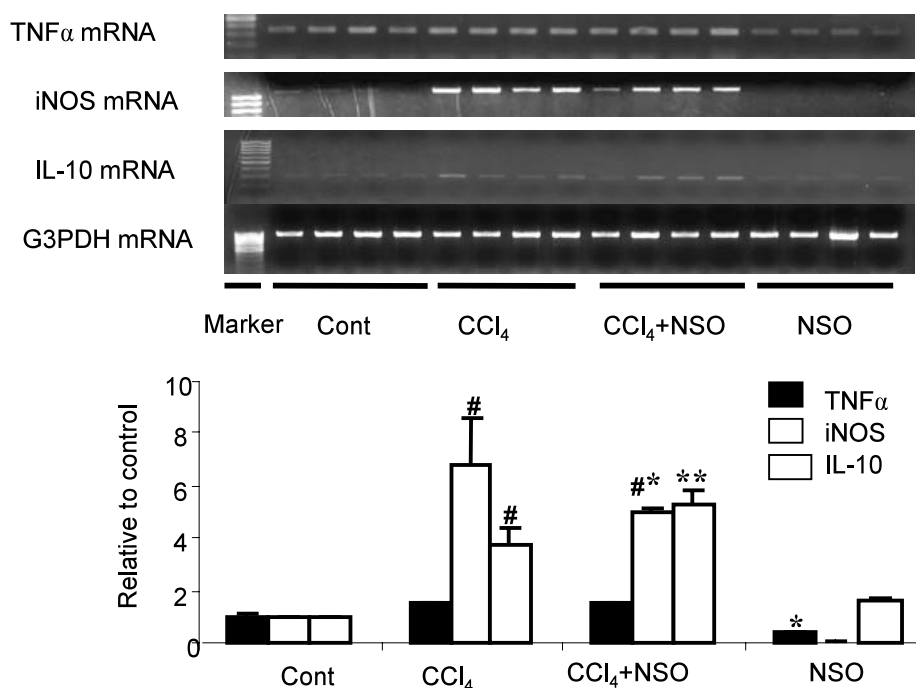


Fig. 1. Effects of NSO on liver iNOS and cytokines mRNA.

Male Wistar rats were orally administered either 1 ml/kg NSO (2 groups) or an equal volume of distilled water (2 groups) for one week; then rats of the CCl₄ and CCl₄+NSO groups were injected i.p. with 0.4 ml/kg CCl₄. Total RNA was isolated, and RT-PCR was performed as described in Materials and Methods. Data are presented in columns representing mean $\pm$ SE (n=4). *Significantly lower than control $p < 0.05$. #Significantly higher than control $p < 0.05$. #*Significantly lower than CCl₄-treated group, $p < 0.05$. **Significantly higher than CCl₄-treated group, $p < 0.05$.

perfamily monooxygenases are important for the metabolism of variety of endogenous and exogenous compounds. During infection and inflammation, the hepatic drug-metabolizing capacity is reduced in experimental animals and humans^{8,18,19}. Our study investigated the effect of NSO as a supplement on hepatic drug-metabolizing CYP enzymes and whether it has a protective effect against some environmental hazards. The present

studies demonstrated that NSO pretreatment protected against CCl₄-induced CYP suppression.

Interleukin (IL)-10 is a major anti-inflammatory cytokine that potently inhibits production of proinflammatory mediators such as TNF α and IL-1²⁾. Treatment of rats with NSO for one week prior to CCl₄ administration increased the mRNA expression of IL-10, as shown in Fig.1, which adds more evidence of the anti-

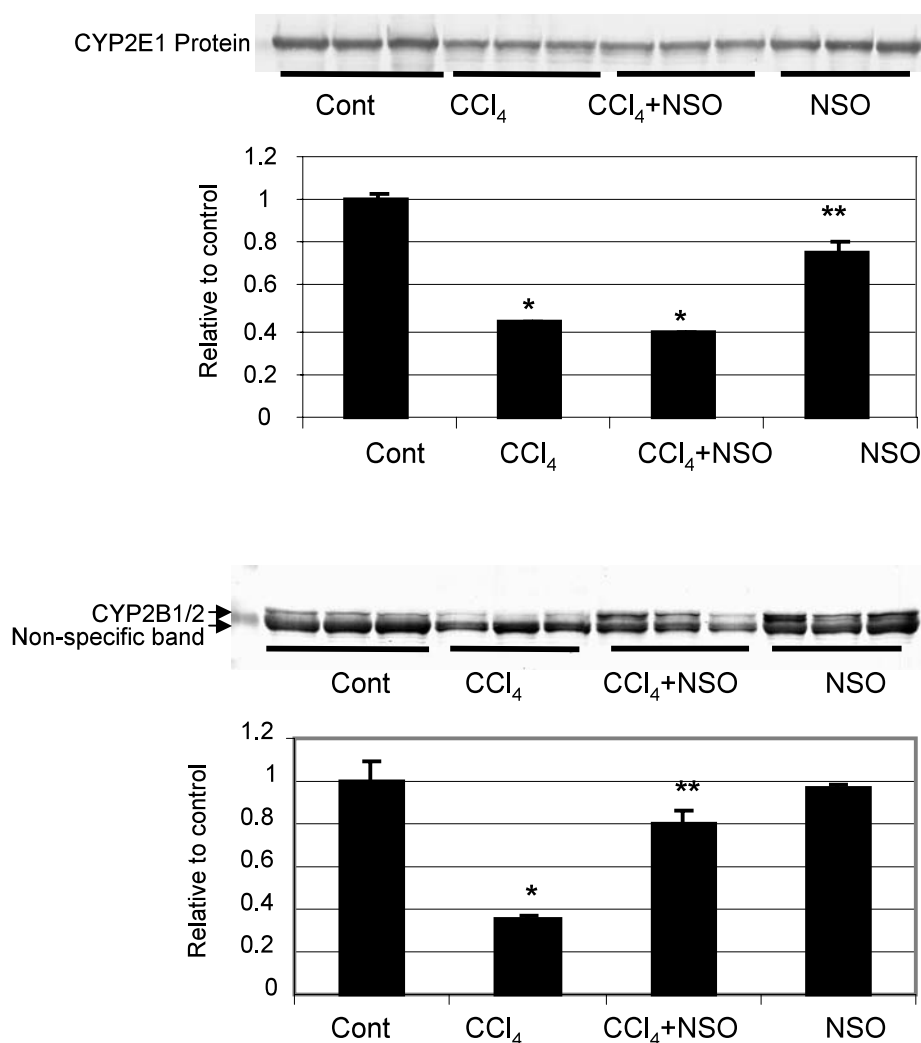


Fig. 2. Effects of NSO on CYP2E1 and CYP2B expression.

Male Wistar rats were grouped as CCl₄, CCl₄+NSO, NSO, or control (distilled water). Hepatic microsomal protein samples (12 μ g/lane) were applied to 10% SDS-PAGE, transblotted onto nitrocellulose membranes, and reacted with CYP2E1 or CYP2B antibodies as described in Materials and Methods. Protein signals were calculated in relation to control. Data are presented in columns representing mean $\pm$ SE (n=4). *Significantly lower than control, $p < 0.05$. **Significantly lower than control and higher than CCl₄-treated group, $p < 0.05$.

inflammatory effect of NSO in the acute phase response to CCl₄. The up-regulation of IL-10 by NSO indicates its ability to downregulate the inflammatory cytokine.

The protein level of CYP2E1 in CCl₄ group was significantly downregulated to approximately 40% of those in control group (Fig. 2). CCl₄ is metabolized by CYP2E1 and other cytochrome enzymes to CCl₃ radicals, which are reactive enough to bind covalently to either the active site of CYP2E1 or to the heme group, thereby causing

suicidal inactivation^{5,15}. In our study, the down-regulation of CYP2E1 expression in rats treated with CCl₄ could be due to the CCl₄-mediated inactivation, and thereby enhancement, of its degradation. Treatment with NSO alone for one week decreased CYP2E1 protein expression about 20% from the control level. The ability of NSO to decrease the expression level of CYP2E1 may show that its protective effects occur through its partial reduction of CCl₄ bio-activation.

Carbon tetrachloride decreased CYP2B pro-

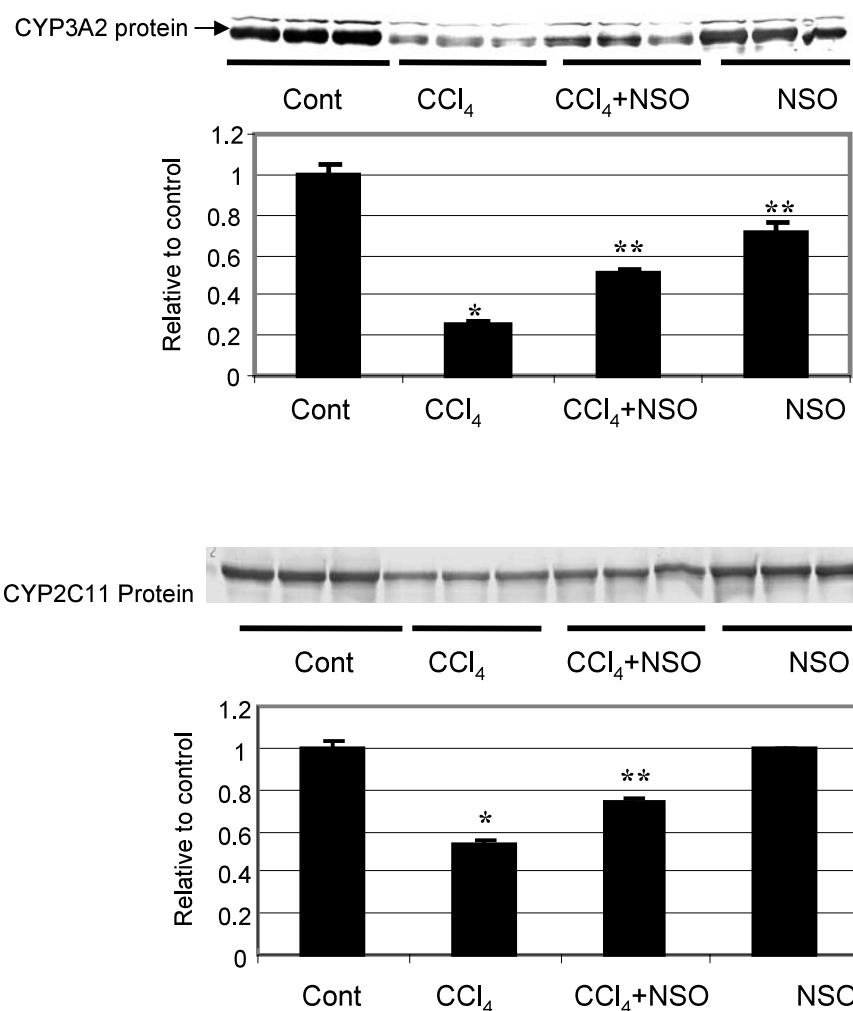


Fig. 3. Effects of NSO on CYP3A and CYP2C11.

Male Wistar rats were grouped as CCl₄, CCl₄+NSO, NSO, or control (distilled water). Western blotting analyses were performed using anti-rat CYP3A2 or CYP2C11 antibodies as described in Materials and Methods. Protein signals were calculated in relation to control. Data are presented in columns representing mean $\pm$ SE (n=4). *Significantly lower than control, $p < 0.05$. **Significantly lower than control and higher than CCl₄-treated group, $p < 0.05$.

tein expression to 40% of that of the control. In the rat groups treated with NSO for one week before CCl₄ administration, CYP2B protein expression returned to nearly normal levels (80%), while NSO alone did not affect CYP2B expression (Fig. 2). These results indicate the ability of NSO to protect CYP2B from the suppressive effect of hepatotoxicity produced by CCl₄. Phenobarbital-induced expressions of CYP2B1/2mRNA and protein were reported to be inhibited by lipopolysaccharide (LPS) in the liver, and this effect was demonstrated to be attenuated in rats treated with inhibitors of nitric oxide synthase⁹. Thus, the protection in rat CYP2B from the suppressive effect of CCl₄ could be partly due to the inhibitory effect of NSO on NO production through its downregulation of iNOS because the rat group treated with NSO one week before the CCl₄ injection had decreased CCl₄-mediated induction of iNOS mRNA (Fig. 1).

Treatment of rats with CCl₄ suppressed CYP3A2 protein expression to only 20% of control levels, while NSO treatment for one week before CCl₄ treatment returned CYP3A protein expression to 50% of the control expression (Fig. 3). The

activation of CYP3A has been shown to be controlled by the nuclear receptor PXR¹⁰. CCl₄ intoxication triggers a TNF α -mediated increase in IL-6 synthesis¹⁴. The CCl₄ suppression of CYP3A could be the result of IL-6 induction because negative regulation of PXR has been reported to occur via an IL-6-mediated mechanism in human hepatocytes²¹. Meanwhile, the downregulation of CYP3A may be due to the high level of NO produced by CCl₄ intoxication, since NO has been proved to have an inhibitory effect on the induction of CYP3A4 mRNA in Caco-2 cells⁶. Therefore, it is possible that the partial protection of CYP3A by NSO against the CCl₄-suppressive effect could be due to the reduction of NO either by decreasing the CCl₄-induced iNOS mRNA or through the scavenging of NO because NSO was shown to have radical scavenging activity²².

Measurement of CYP2C11 protein expression revealed that CCl₄ suppressed CYP2C11 protein to only 50% of the control while NSO pretreatment of rats retained CYP2C11 protein expression at 75% of control levels (Fig. 3). CYP2C11 downregulation by CCl₄ is consistent with the report by Wright

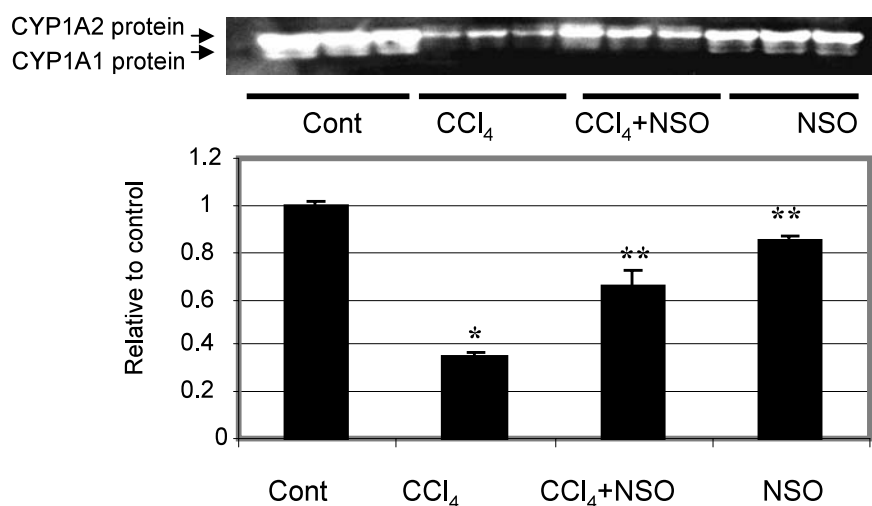


Fig.4. Effect of NSO on CYP1A2.

Male Wistar rats were grouped as CCl₄, CCl₄+NSO, NSO, or control (distilled water). Western blotting analyses were performed using anti-rat CYP1A2 antibody as described in Materials and Methods. Protein signals were calculated in relation to control. Data are presented in columns representing mean $\pm$ SE (n=4). *Significantly lower than control, $p < 0.05$. **Significantly lower than control and higher than CCl₄-treated group, $p < 0.05$.

and Morgan²⁷⁾, who showed that induction of systemic inflammation by an endotoxin or turpentine depressed the hepatic mRNAs for CYP2C11 and CYP2C12, presumably via cytokine production. However, in our study, the alteration pattern of the CYP2C11 level differed from that of TNF α (Fig. 1).

Measuring CYP1A2 enzyme protein expression revealed that treatment of rats with CCl₄ decreased CYP1A2 to less than 40% of the control, while NSO pretreatment of rats reduced the CCl₄-mediated suppression of CYP1A2 and maintained it at about 70% of the control levels (Fig. 4). These results provide more evidence of the ability of NSO to ameliorate the CCl₄-mediated inflammatory response, and thereby protect CYPs from the suppressive effect of CCl₄. In our study, a single injection of CCl₄ suppressed CYP1A2 to an undetectable level after 24 hours, and this is in line with a previous report stating that CCl₄ was able to suppress CYP1A2¹¹⁾. TNF α may take part in the acute phase response-mediated CYP1A2 downregulation, based on a study that indicated that TNF α is directly involved in the regulation of CYP1A and CYP2B. The inhibition of TNF α production by pentoxifylline after LPS administration partially prevented the inhibitory effects of LPS on CYP1A and CYP2B¹⁷⁾. Therefore, protection of CYP1A2 from the severe suppressive effect of CCl₄ could be due to the anti-inflammatory effect of NSO through which IL-6 and TNF α are decreased.

In conclusion, we demonstrated that administration of *N. sativa* oil slightly decreased CYP2E and CYP3A protein expressions. NSO also has a protective effect against the CCl₄-mediated suppression of hepatic CYPs, and this protective effect seems to be due partly to reduction of NO through downregulation of iNOS, in addition to the reduction of TNF α and the up-regulation of the anti-inflammatory IL-10.

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