The effects of formaldehyde intoxication on the inducible nitric oxide synthase expression and nitric oxide level in the liver tissue of rats

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Aim: To investigate inductive activity of formaldehyde (FA) on the expression of inducible nitric oxide synthase (iNOS) and level of nitric oxide (NO) in the liver of rats.

Materials and methods: Sixteen male Wistar albino rats of the same age (3 months old) were divided into 2 experimental groups, with 8 rats as the control and 8 rats formaldehyde-treated (FAt). The FAt group received intraperitoneal injection of 10 mg/kg FA for 10 days. Activity of iNOS was shown with immunohistochemical stain kits, and the concentration of NO level was measured by spectrophotometric method in the liver tissue.

Results: The FAt group had increased iNOS expression and NO levels in liver tissue compared to controls.

Conclusion: FA increased iNOS expression and NO production in liver tissue of rats.

Key words: Formaldehyde, nitric oxide, iNOS, rat liver

1. Introduction
Formaldehyde (FA), which is a one-carbon very reactive compound, can react with different cellular components, such as lipids, proteins, and nucleic acids (1). Humans are exposed to FA every day because it is found in different kinds of medicine and industrial products such as building materials, cosmetics, cigarette smoke, photochemical smog, and even various fruits (2). It can also be physiologically formed by the metabolism of some amino acids or methylamine, and it can contribute to biological methylation by folic acid (3,4). After intraperitoneal, oral, or inhaler administration, FA rapidly diffuses into many tissues, including the brain, testis, and liver (5). Formaldehyde can cause gene mutations, carcinogenesis, neurodegenerative disorders, digestive symptoms, and spermatogenic inhibition (6–8). Many studies have already been done to learn the pathogenesis of FA toxicity, but answers are not clear yet. Nitric oxide (NO) is one of the crucial regulators of numerous biological processes, including vascular tone, transfer of nerve impulses, inflammation, thrombosis, motor activity, secretion, and macrophage-mediated immunity. NO is produced by nitric oxide synthase (NOS) activity from arginine and it has double-sided effects. It is synthesized from the terminal guanidine nitrogen of the semiessential amino acid L-arginine, which is converted to L-citrulline in a complex reaction involving molecular oxygen and NADPH as cosubstrates; and enzyme-bound heme and flavin nucleotides including FAD and FMN, and tetrahydrobiopterin, as cofactors; and it is catalyzed by nitric oxide synthases (9). Three major isoforms of NOS, which are each encoded by different genes, have so far been identified: endothelial NOS (eNOS) and neuronal NOS (nNOS), which are expressed constitutively and are collectively termed constitutive nitric oxide synthase (cNOS); and induced NOS (iNOS) by endotoxin and inflammatory mediators, such as cytokines (10–12). The aim of the present study was to investigate the inductive activity of FA on the expression of iNOS and levels of NO in the liver of rats.

2. Materials and methods
2.1. Animals and design
Three-month-old Wistar albino rats, weighing between 250 and 300 g, were obtained from the Fatih University Medical Faculty Experimental Research Center and housed...
in separate cages in standard conditions, with a 12/12-h light–dark cycle. The animals were given standard rat chow and water ad libitum. Twelve male rats were randomly allotted into 1 of 2 experimental groups: control and FA-treated (FAt) groups. Each group consisted of 6 animals. The FAt group received intraperitoneal injection of 10 mg/kg FA (37% formaldehyde, Merck, Darmstadt, Germany) for 10 days at the same time in the morning (13). After the treatment, the animals were sacrificed and liver tissues were removed for biochemical and immunohistochemical investigations.

2.2. Biochemical analysis

2.2.1. NO determination
As NO measurement is very difficult in biological specimens, tissue nitrite and nitrate were estimated as an index of NO production. The method for the nitrite and nitrate levels in the tissues was based on the Griess reaction (13). Samples were initially deproteinized with Somogyi reagent. Total nitrite (nitrite+nitrate) was measured after conversion of nitrate to nitrite by copperized cadmium granules with a spectrophotometer at 545 nm. A standard curve was established with a set of serial dilutions of sodium nitrite. Linear regression was done using the peak area from the nitrite standard. The resultant equation was then used to calculate the unknown sample concentrations. Results were expressed as micromoles per gram protein in the tissues. Protein assays in the samples were done by the method of Lowry et al. (14).

2.2.2. Immunohistochemical detection of iNOS in tissue samples
Sections of 5 µm in thickness were prepared from paraffin-embedded tissues. After deparaffinization, endogenous peroxidase was quenched with 3% H₂O₂ in deionized water for 5–10 min. Nonspecific binding sites were blocked by incubating the sections in 10% normal rabbit serum for 10–15 min. The sections were then incubated with polyclonal rabbit anti-iNOS (dilution 1:25) or anti-NF-κB p65 (dilution 1:100) overnight at 4 °C, followed by incubation with biotinylated goat-antirabbit IgG at room temperature for 10–15 min. After phosphate buffered saline (PBS) rinses of 3 × 3 min, the horseradish-peroxidase-conjugated streptavidin solution was added and incubated at room temperature for 10–15 min. The antibody binding sites were visualized by incubation with a diaminobenzidine–H₂O₂ solution. The sections incubated with PBS instead of the primary antibody were used as negative controls. Brown-yellow granules in cytoplasm or nuclei were recognized as positive staining for iNOS. Activity of iNOS tissues was shown by commercial iNOS immunohistochemical stain kits. Streptavidin–biotin complex was used. Cytoplasmic stainings with iNOS were revealed to be positive.

2.3. Statistical analysis
All statistical analyses were carried out using SPSS for Windows, version 11.0. All data were presented as mean ± standard deviation (SD). Dual comparisons between groups that presented significant values were evaluated with the Mann–Whitney U test. The differences were considered to be significant at P < 0.05.

3. Results
In the examination of hematoxylin and eosin (H&E)-stained sections, hepatocytes in control group rats were observed to be normal; no sign of Kupffer cell hyperplasia and inflammation was encountered and portal areas appeared normal (Figure 1a). In the examination of H&E-stained preparations of FAt rat liver tissue, mild edema, mild degeneration in hepatocytes, and Kupffer cell hyperplasia were observed (Figure 1b). In the immunohistochemical examination (Figures 2a, 2b, and 3), cytoplasmic and perinuclear localization of brown-colored iNOS expressions were observed in...
FAt rat liver tissues (Figures 2b and 3). However, in liver tissues of control group rats, no sign of any expressions were observed (Figure 2a). The semiquantitative results of tissue iNOS immunohistochemical staining are given in the Table. Tissue iNOS expression in the FAt group was found to be higher compared to the control group. The Table indicates the mean values and standard deviations of the concentrations of tissue NO in the FAt and control groups. The level of NO in the tissue was significantly increased in the FAt group as compared to the control group.

4. Discussion
Inducible nitric oxide synthase, which is an isoform of the NOS enzyme, is found in a wide variety of tissues, including the liver (15). In this study, compared to the control group in iNOS expression, an increase in the amount of liver cells in the FAt group was observed with immunohistochemical study. While there was no expression monitored in hepatocytes of the control group, iNOS expression was monitored in intracytoplasmic and perinuclear areas of hepatocytes in the FAt group. This also shows that FA has an effect on iNOS. Furthermore, the amount of NO in the liver tissues of the FAt group was detected to be significantly higher than in the control group. Increased activity of iNOS is assumed to be the reason for this. Therefore, an increment in the amount of NO is consistent with immunohistochemical findings.

FA can cause severe tissue injury by producing reactive oxygen species (16). In a literature review, no solid understanding of the impact of FA on the tissue expression of iNOS was encountered. In several different experimental studies on animal models, it was reported that FA caused an increase in the amount of NO in tissue and serum. Gulec et al. claimed that FA caused the amount of NO in and liver tissues to increase (17). In addition, the results of the present study are supported by the articles recently written by Franklin et al. They demonstrated an association between inhaled FA concentrations and exhaled NO levels in their study performed in children with no previous airway damage and atopy (18). The mechanism of the effect of FA on the expression of iNOS is not exactly clear. We assume that this effect occurs in 1

**Table.** The liver NO levels (mean ± standard deviation) and semiquantitative iNOS expression in rat tissues.

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<th>Control (n = 6)</th>
<th>FAt (n = 6)</th>
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<td>NO (µmol/g protein)</td>
<td>0.126 ± 0.016</td>
<td>0.198 ± 0.038*</td>
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<tr>
<td>iNOS expression</td>
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*P < 0.005 compared to control group.
of 2 ways. The first is that FA or its metabolites act like a stimulator/activator that induces iNOS protein synthesis directly. The fact that FA is a water-soluble molecule, which easily diffuses into membranes and directly cross-reacts with DNA-protein chains, supports this approach (19). Nevertheless, this approach should be supported with cell cultures and/or advanced analysis methods. The second way may be through cytokines (20).

Several studies showed that cytokines such as TNF-α and IL-1β are effective on iNOS mRNA synthesis and iNOS activity or expression (20,21). The researchers reported that FA also has a stimulating impact on cytokines, which affects iNOS metabolism (22,23). Therefore, the increase in liver cell expression iNOS is thought to be mediated by cytokines. The liver is an organ that metabolizes many toxic/nontoxic compounds, including FA. In this study, edema and degeneration findings were observed in hepatocytes by examining the liver tissues of the FAT group with H&E staining.

Indeed, in other studies of light and electron microscopy, the following observations were reported in the liver cells of experimental animals exposed to FA: flat endoplasmic reticulum hypertrophy and hyperchromatic nucleus (24), rough endoplasmic reticulum and mitochondrial damage (25), and impairment of membrane integrity (26). It was also reported that FA caused damage to intrahepatic and extrahepatic bile ducts in addition to hepatocytes (27). Moreover, it was asserted that FA led to similar cellular changes in other organs (28,29). Along with all these histopathologic findings, it was also reported that FA caused increase in the activities of enzymes that were used as indicators of liver tissue damage, such as serum aspartate transaminase, alanine transaminase, and alkaline phosphatase, and decrease in the amount of albumin and total protein (30).

In conclusion, this study showed that FA caused an increase in iNOS expression in hepatocytes and an increase in the amount of NO in liver tissues.

References


