# Amelioration of the Protein Expression of Cox2, NFκB, and STAT-3 by Some Antioxidants in the Liver of Sodium Fluoride–Intoxicated Rats

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## Abstract

The present study aimed to explore the efficiency of N-acetyl cysteine (NACC) or thymoquinone (TMQ) alone or in combination in the downregulation of inflammatory molecule expression and decreasing hepatic injury in response to sodium fluoride (SF). Sodium fluoride upregulated serum alanine and aspartate transferases activities, tumor necrosis factor α and hepatic malondialdehyde and nitric oxide levels, and the expression of cyclooxygenase 2, nuclear factor κB cell, and signal transducer and activator of transcription 3. In contrast, hepatic glutathione level, superoxide dismutase activity, and nuclear factor erythroid 2-related factor 2 expression were decreased. However, the concurrent treatment with antioxidants, alone or in combination, modulated the levels of these parameters. Histopathological examination revealed that SF treatment resulted in focal areas of massive hepatic degeneration and many degenerated hepatocytes, whereas the treatment with TMQ or NACC exhibited moderate improvement in cellular degeneration of the liver with many abnormal cells. Rats receiving a combination of TMQ and NACC showed marked improvement in cellular degeneration of liver with apparently normal hepatic architecture with very few degenerated hepatocytes. The results also revealed that the combination of TMQ and NACC is the most effective regimen in ameliorating SF toxicity, suggesting their efficacy against the toxicity of fluoride compounds. Their activities might be mediated via multiple molecular pathways.

## Introduction

Sodium fluoride (SF), a pollutant, is used as an insecticide and antihelminthic agent.[1,2] Fluoride can be present in the soil, water, and vegetation. It has been reported that chronic fluoride toxicity causes joint stiffness.[3-6] Its deleterious effects on the reproductive system and kidney tubules are well-documented.[7-9] It is known as a double-edged sword, and hence, it is an essential trace element in small doses to prevent dental caries and osteoporosis. Conversely, high doses cause harmful effects. It interferes with the metabolism of macromolecules,[10,11] inhibits the activity of enzymes involved in glycolysis and Krebs cycle and fatty acid oxidation, and suppresses the formation of polypeptides that block DNA synthesis.[12] The mechanism of fluoride-induced pathogenesis is accompanied by reactive oxygen species (ROS) and peroxyl and hydroxyl radical formation, resulting in oxidative stress (OS), apoptosis, and DNA damage.[13,14]

N-acetylcysteine (NACC) is a small molecule containing a thiol group and has an antioxidant effect. It can access intracellular compartments.[15] Its therapeutic efficacy is contributed to the existent of the cysteinyl thiol group; the ability of thiol

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group to scavenge oxygen-free radicals is well established.[15] N-acetylcysteine raises the intracellular concentrations of cysteine and in sequence decreases reduced glutathione (GSH) level.

Numerous active constituents from herbal and medical plants are widely documented for their favorable effects. Thymoquinone (TMQ) is known as an antioxidant and free radical scavenger.[16] Thymoquinone exhibits various pharmacological and immunomodulatory actions[17] and also has anti-inflammation, antioxidant,[18] and antitumorigenic activities against different cancer diseases.[19,20] Its protective role against oxidative damage of many organs induced by free radical-producing agents such as doxorubicin-induced cardiotoxicity and carbon tetrachloride-induced hepatotoxicity was well studied.[21-23]

The aim of this study is to explore the efficiency of NACC and/or TMQ alone or in combination in the downregulation of inflammatory molecule expression and hepatic injury in response to SF toxicity. Another aim is to study the influence of cyclooxygenase 2 (Cox2), nuclear factor κB (NFκB), signal transducer activator of transcription 3 (STAT-3), and nuclear factor erythroid 2-related factor 2 (Nrf2) protein expressions in SF toxicity and treatments.

## Materials and Methods

### Chemicals

All chemicals (high analytical grade) were purchased from Sigma and Merck (St. Louis, Missouri, United States); SF, NACC, and TMQ were purchased from Sigma Chemical Co (St Louis, Missouri). Commercial kit for the assay of alanine aminotransferase (ALT) was purchased from Randox (United Kingdom). Primary and secondary antibodies for COX-2, NF-κB, STAT-3, and Nrf2 were purchased from Santa Cruz Biotechnology (California, USA).

### Experimental Animals

Fifty Wistar adult male albino rats weighing 170 to 210 g were kept at a temperature of 20°C to 22°C. The rats were procured from the Experimental Animal House, Faculty of Pharmacy, King Saud University, Saudi Arabia. They were fed with standard rat pellet chow with free access to tap water ad libitum. The experimental protocol was approved by the Experimental Animal Ethics Committee at the same University. After 1 week of acclimation, rats were divided into 5 groups, each of 10 rats.

### Experimental Design

Fifty rats were allocated into 5 groups, each of 10 rats; the first group was the normal control and was given distilled water, the second group was intoxicated with 10 mg/kg SF[24] once daily, the third group was treated with TMQ at a dose of 10 mg/kg/d intraperitoneally,[25] the fourth group was treated with 20 mg/kg/d of NACC,[26] and the fifth group was treated with TMQ and NACC. All treatments were given daily along with SF for 1 month.

Rats were killed; serum was separated by blood centrifugation at 3000 rpm for 20 minutes. Some livers were homogenized in phosphate buffer to yield 20% homogenates. The homogenates were centrifuged for 20 minutes at 3000 rpm at 5°C and were kept at −80°C. Other parts of the livers were rapidly frozen under liquid nitrogen for Western blotting. Three livers from each group were kept in 4% formalin for histopathological examination.

### Biochemical Serum Analysis

*Determination of serum content of ALT and aspartate aminotransferase.* The content of ALT and aspartate aminotransferase (AST) was determined using the kits obtained from Randox.

*Determination of lipid peroxidation (malondialdehyde) in the hepatic tissues.* The degree of lipid peroxidation in the liver tissue was determined according to the method of Uchiyama and Mihara.[27]

*Determination of hepatic GSH content.* The content of GSH was determined by the method of Ellman.[28]

*Determination of hepatic total nitrite content (nitric oxide).* The total nitrite content was measured according to the method of Moshage et al.[29]

*Determination of superoxide dismutase activity.* The activity of superoxide dismutase (SOD) was measured according to the method of Marklund and Marklund.[30]

*Determination of tumor necrosis factor level.* The level of tumor necrosis factor α (TNF-α) in the serum was measured using a high-sensitive rat enzyme-linked immunosorbent assay kit (Immuno-Biological Laboratories Co, Ltd, Takasaki-Shi, Gunma, Japan).

### Histological Analysis

The liver samples were stored in 4% paraformaldehyde embedded in paraffin wax. Thinly sliced sections were used for the histopathological examination using hematoxylin and eosin (H&E) stain.

### Western Blot Analysis

Western blotting was performed to determine the expression of COX-2, NF-κB light-chain enhancer of activated B cells, STAT-3, and Nrf2. The proteins bands were visualized using the ECL-Plus detection system (Amersham Life Sciences, Little Chalfont, Buckinghamshire, United Kingdom), according to the manufacturer's instruction. Positive immunoreactive bands were quantified densitometrically and compared with that of the control.[31] Liver sections were homogenated in lysis buffer (20 mM) 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid, N-(2-Hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid), (HEPES), 2 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol
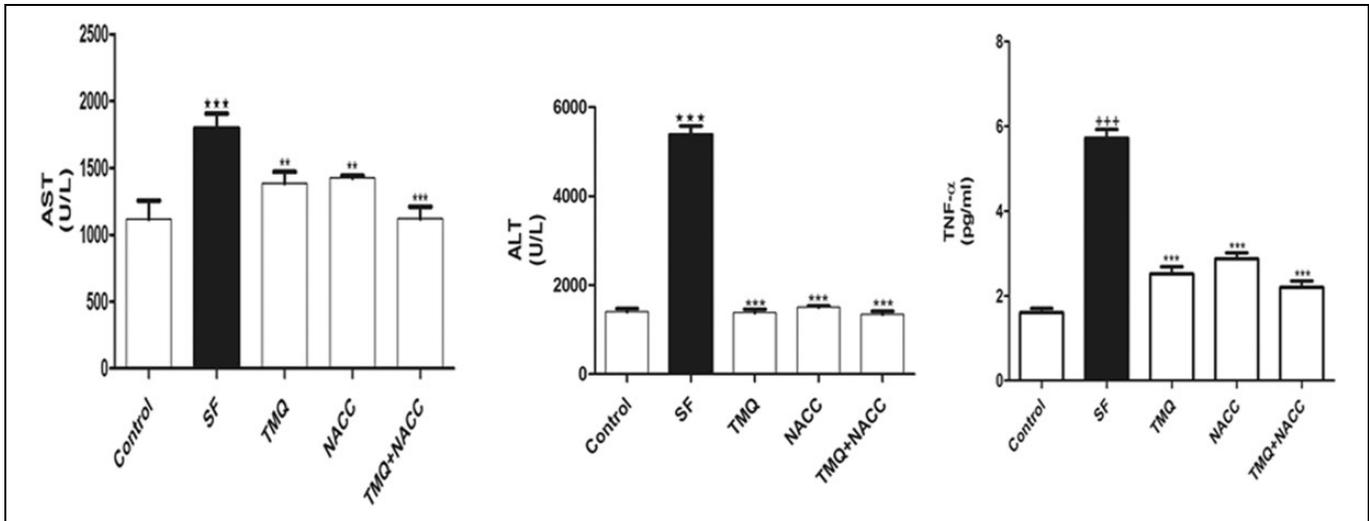
**Figure 1.** Liver function enzymes (ALT and AST) and inflammatory marker (TNF-α) in hepatic tissues of rats in control, SF-intoxicated, and all treated groups. Data are presented as mean ± SEM (N = 6). $^{+++}P \leq .001$ versus control and $^{***}P \leq .001$ versus SF-intoxicated group. ALT indicates alanine aspartate; AST, aspartate transferase; TNF-α, tumor necrosis factor α; SEM, standard error of the mean.

(DTT), 0.1% sodium dodecyl sulfate, 1 mM phenylmethane sulfonyl fluoride, pH 7.4 on ice. The supernatants were harvested by centrifugation at 12 000g at 4°C for 10 minutes. Protein concentrations were determined by a Bradford assay. Protein (20 mg) was separated on a 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes.

### Statistical Analysis

The data are expressed as mean ± standard error of the mean. Comparisons between different groups were performed by the 1-way analysis of variance, followed by Tukey-Kramer multiple comparisons test. The level of significance was set at $P < .05$, $P < .01$, and $P < .001$. The statistical analyses were conducted using the software GraphPad Prism version 5 (Graph-Pad Prism, San Diego, California) and SPSS version 21 (IBM).

## Results

Effect of SF and TMQ or/and NACC on the levels of serum ALT and AST (Figure 1) revealed the effect of SF, TMQ, or NACC and their combination on serum liver function. Sodium fluoride exposure resulted in a significant increase in the activity of ALT and AST. Coadministration of TMQ or/and NACC resulted in a significant decrease in these activities compared to the control group (Figure 1). Also, serum TNF-α was markedly increased upon SF intoxication and ameliorated by the antioxidants in question.

Effect of SF and TMQ or/and NACC treatments on hepatic tissue administration of SF evoked significant increment in hepatic malondialdehyde (MDA; $P < 0.001$) and nitric oxide (NO) levels compared to the control group. The TMQ and/or NACC administration to SF-intoxicated rats alleviated the enhanced MDA and NO levels in the liver tissue (Figure 2).

The activity of SOD and the level of GSH in the liver homogenates were reduced significantly ($P < .001$) in response to SF treatment. Ingestion of TMQ and/or NACC to SF-intoxicated rats successfully restored their values matched to the control group (Figure 2).

### Western Blot

Western blot analysis results indicated that SF administration induced a significant elevation in COX-2, NF-κB, and STAT-3 with concomitant significant depletion in Nrf2 protein expressions in hepatic tissue compared with control ($P < .001$), while TMQ and/or NACC administration alleviated the activation of COX-2, NF-κB, and STAT-3 expressions and increased the expression of Nrf2 compared with an SF-treated group ($P \leq .001$; Figure 3). Of the aforementioned measured parameters, the treatment with the combination of TMQ and NACC was the most effective regimen in ameliorating SF toxicity.

### Histological Examination

Figure 4 presents light photomicrographs of H&E-stained sections of the liver. Liver of control rat revealed normal hepatic architecture, normal hepatocytes, and blood sinusoids. Sodium fluoride treatment caused focal areas of massive hepatic degeneration and many degenerated hepatocytes, while TMQ administration caused mild improvement in the hepatic changes with many degenerated cells. Liver from rat receiving NACC also showed moderate improvement in hepatic cellular degeneration with many abnormal cells, whereas liver from rat receiving TMQ and NACC showed the marked improvement in the hepatic cellular degeneration with apparently normal hepatic architecture with very few degenerated hepatocytes.
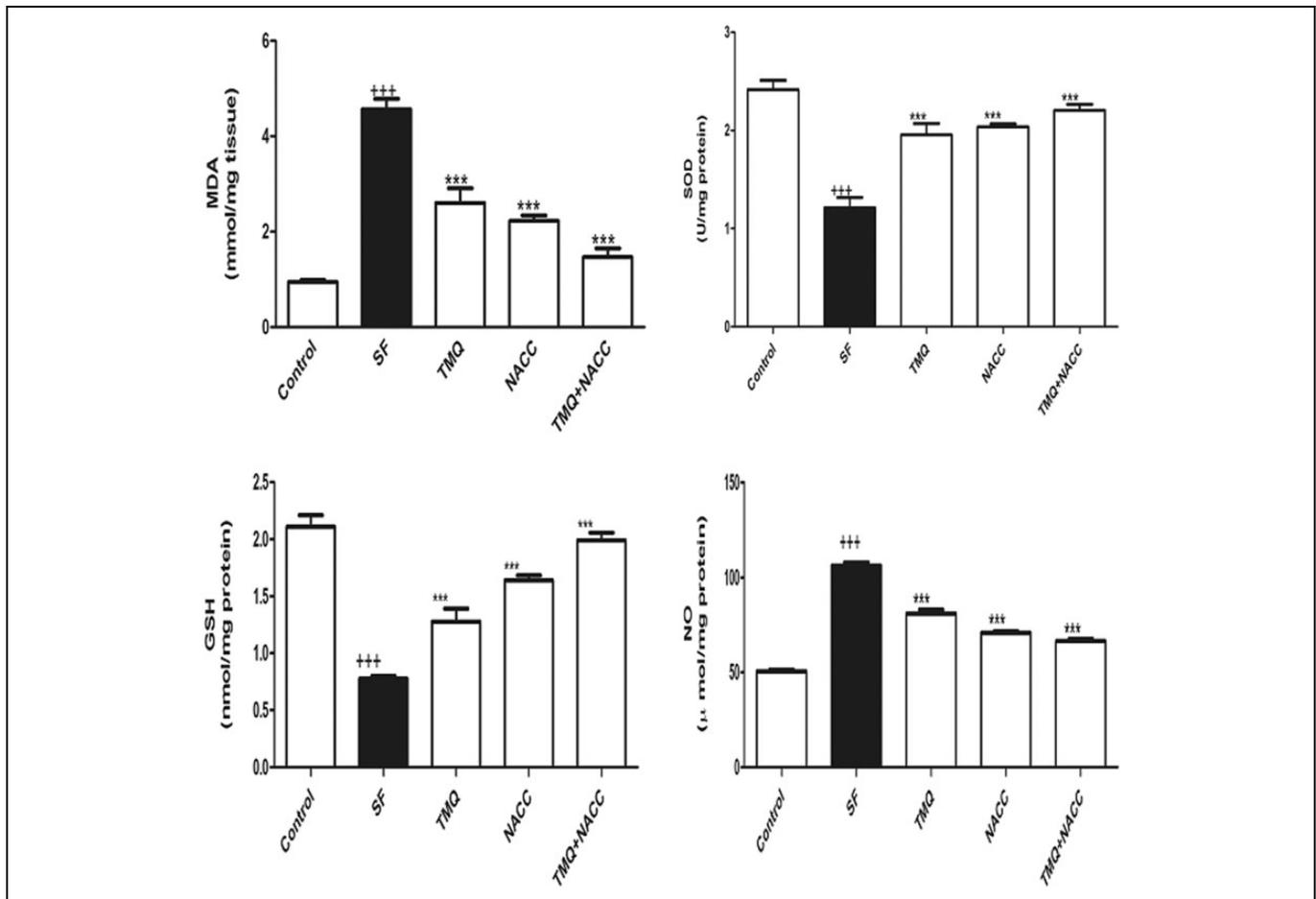
**Figure 2.** Oxidative stress and antioxidant biomarkers (MDA, SOD, GSH, and NO) in hepatic tissues of rats in control, SF-intoxicated, and all treated groups. Data are presented as mean ± SEM (N = 6). [+++]$P \leq$ .001 versus control and [***]$P \leq$ .001 versus SF-intoxicated group. GSH indicates glutathione; MDA, malondialdehyde; NO, nitric oxide; SEM, standard error of the mean; SOD, superoxide dismutase.

## Discussion

Fluoride, one of the most dangerous environmental pollutants, disrupts metabolic pathways at toxic doses.[32] Its effect on many organs, such as liver, pancreas, lungs, heart, skeletal muscles, and kidney, is well established.[33-36] In the present study, SF increased the activity of serum ALT and AST and the level of TNF-α, hepatic MDA, and NO with contaminant decrease in GSH level and SOD activity. Liver dysfunction after SF administration is due to increased OS, leading to liver injury. Abnormal levels of some serum biomarkers, such as ALT and AST, are attributed to the destruction of the membrane structure of the hepatic cells, leading to a noticeable increase in ALT activity.[37] Lipid peroxidation is the essential index of OS, and MDA is the end product of lipid peroxidation and reflects the degree of lipid peroxidation.[11]

Glutathione is an antioxidant toward ROS and is considered as a primary indicator of the OS.[35] Chen et al[38] and Liu et al[39] reported that in broiler chickens, high dietary fluoride increases the MDA content in their serum. The present data showed that the activity of SOD and GSH level were significantly decreased in the liver after SF treatment. This finding designated that SF

can induce the high generation of ROS and reactive nitrogen species. Herein, NO level was significantly increased in the liver after treatment with SF compared with the control group. The analogous observation was gained by Zhou et al[40] and Liu et al[39] who reported that fluoride-induced OS is involved in the morphological damage and dysfunction of the liver in female mice and elevated serum NO levels in chicks, respectively.

The elevated NO level induced by fluoride could disrupt protein functions, affect energy metabolism, reduce adenosine triphosphate and NADPH, and cause DNA damage and react with superoxide anions ($O_2^-$), to produce peroxynitrite (ONOO–), which is responsible for oxidation and destruction and hence cytotoxicity.[41,42] This anion is capable to diffuse across plasma cell. Based on the presence of iron, thiols, and SOD, peroxynitrite undergoes 3 types of reactions, leading to depletion of thiols radical, chain peroxidation, and nitrosylation of proteins.[43]

The bioactivities of TNF-α are mediated through the activation of the NF-κB pathway.[44] The crucial role of TNF-α in many diseases is that the mediation of organs injury is well-documented.[45,46]
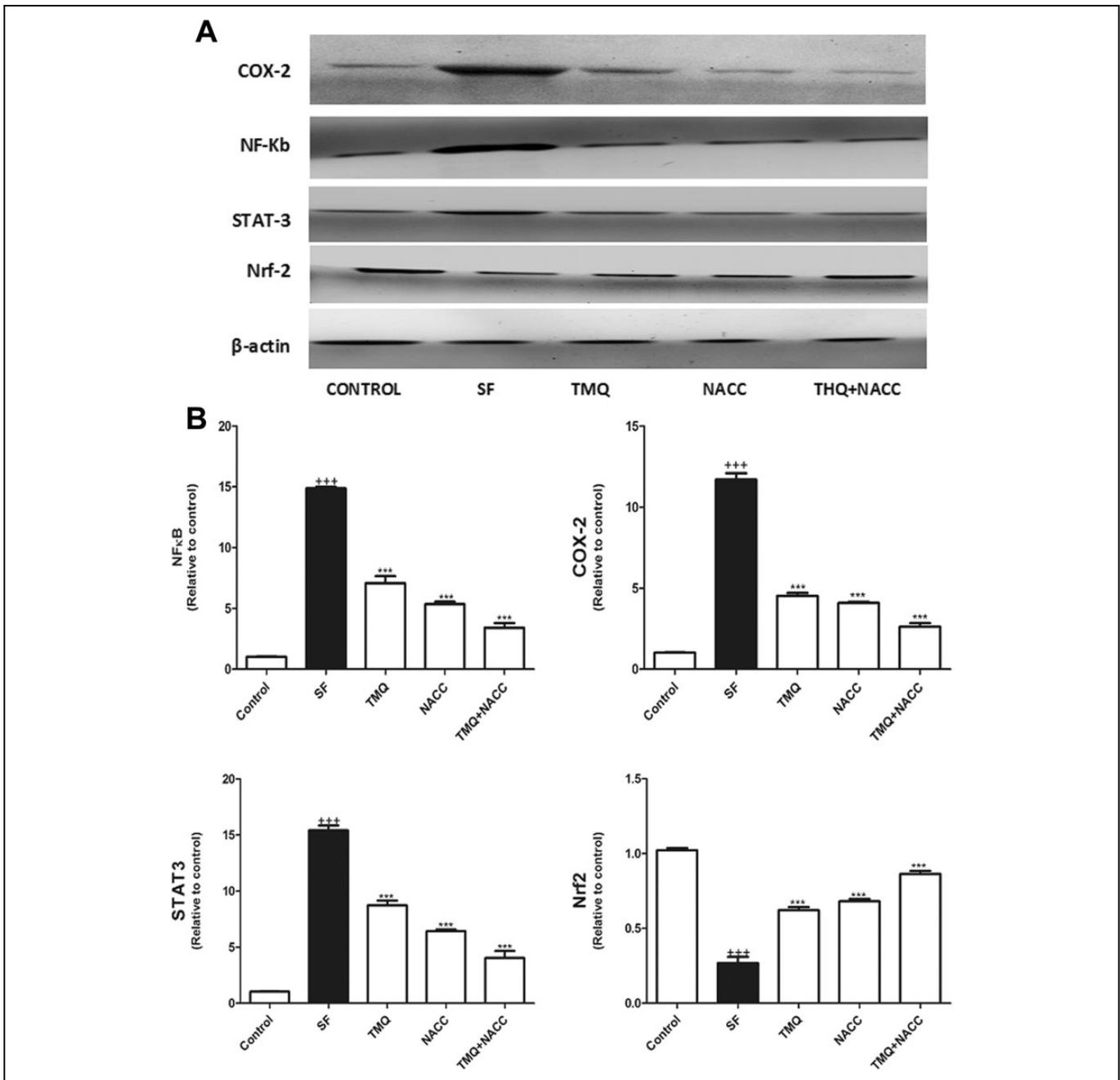
**Figure 3.** A, Western blot analysis of the expression of NF-κB, COX-2, STAT3, and NrF2 proteins in control, SF-intoxicated, and all treated groups. B, The densitometry analysis of the expression of NF-κB, COX-2, STAT3, and NrF2 proteins in control, SF-intoxicated, and all treated groups. (Data corrected by β-actin and expressed as protein/β-actin). Data are presented as mean $\pm$ SEM (N = 6). $^{+++}P \leq .001$ versus control and $^{***}P \leq .001$ versus SF-intoxicated group. COX-2 indicates cyclooxygenase-2; NAC, N-acetylcysteine; NF-κB, nuclear factor-κB; NrF2, nuclear factor erythroid 2-related factor 2; SEM, standard error of the mean; SF, sodium fluoride; STAT-3, signal transducer and activator of transcription 3; TMQ, thymoquinone.

Herein, SF induced a significant rise in hepatic NF-κB, COX-2, and STAT-3, while Nrf2expression was decreased; this is in agreement with the findings of Afolabi et al,[47] Gutowska et al,[48] Jain et al,[49] and Mukhopadhyay et al,[50] who declared that fluoride affected the previous parameters in the same manner.

Cyclooxygenase expression is linked with inflammation[51] and is released by a variety of pro-inflammatory stimulus.[52,53] It was documented that Nrf2 is the key manager of all cellular oxidation–antioxidation system at the transcriptional level.[54]

From the current investigation, it can be concluded that the consumption of TMQ or/and NACC to SF-intoxicated rats
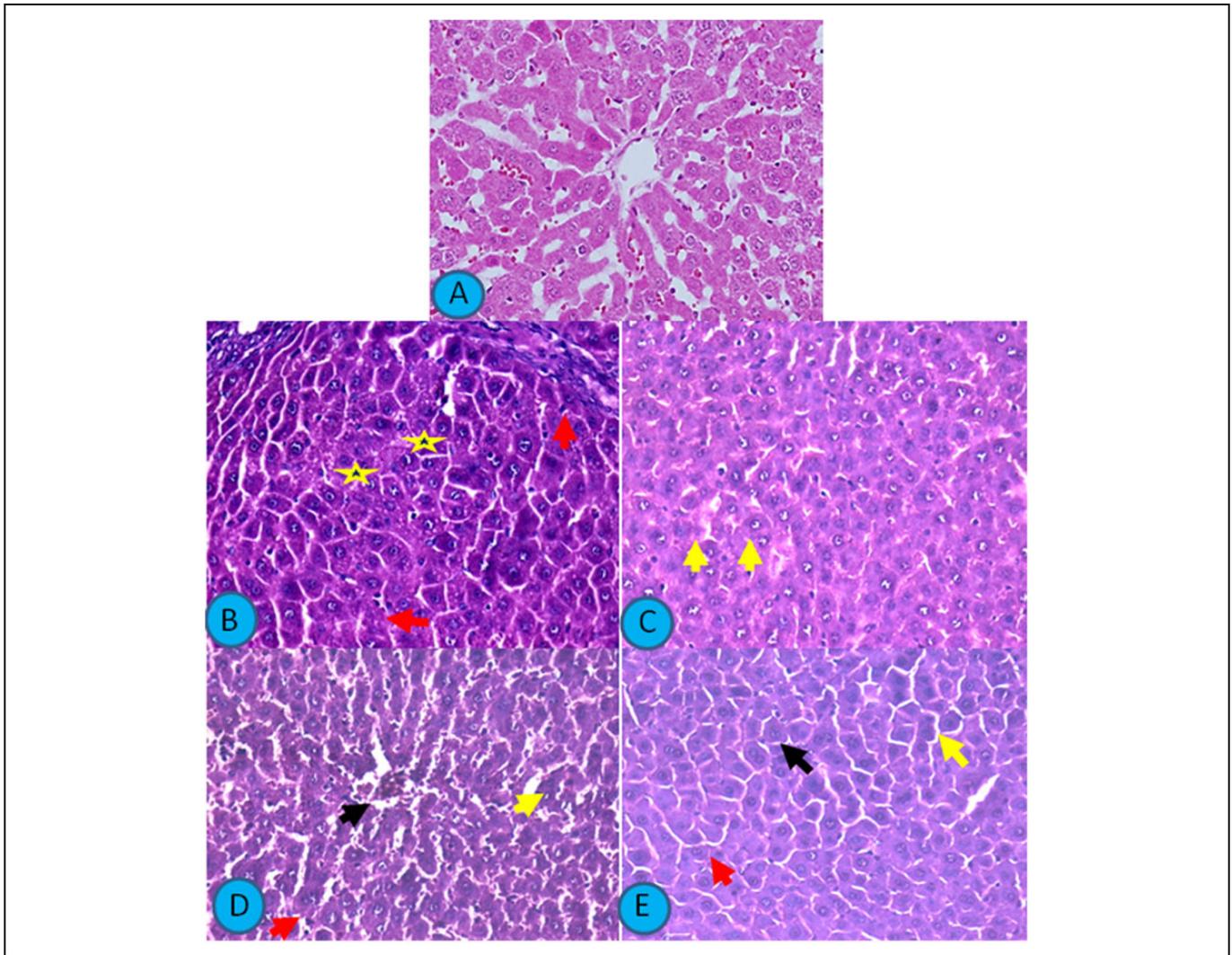
**Figure 4.** Light photomicrographs of H&E-stained sections of the liver. Scale bar 400 μm. A, Liver from control rat showing normal hepatic architecture with normal hepatocytes and blood sinusoids. B, Liver from rat receiving SF showing focal areas of massive hepatic degeneration (yellow star) and many degenerated hepatocytes (red arrows). C, Liver from rat receiving thymoquinone showing mild improvement in the hepatic changes with many degenerated cells (yellow arrow). D, Liver from rat receiving N-acetylcysteine showing moderate improvement in hepatic cellular degeneration with many abnormal cells (arrows). E, Liver from rat receiving thymoquinone and N-acetylcysteine showing marked improvement in the hepatic cellular degeneration with apparently normal hepatic architecture with very few degenerated hepatocytes (arrows). H&E indicates hematoxylin and eosin; SF, sodium fluoride.

successfully restored ALT and AST activities; SOD, MDA, GSH, and NO levels; and COX-2, NF-κB, and STAT3 protein expressions back to control values. This positive effect of either TMQ or/and NACC was formally recognized in different models of hepatotoxicity.[16,55]

The STAT3 protein expression is one of the major members of the unit of signal transducers and activators of transcription that functions by adaptable cell growth, differentiation, and angiogenesis and participates in the pathogenesis of diabetic retinopathy.[56] It is the first time to evaluate the effect of SF on STAT3.

Histological examination reported that SF treatment caused focal areas of massive hepatic degeneration and many degenerated hepatocytes, while TMQ administration caused mild improvement in the hepatic changes with many degenerated cells. Liver from rat receiving NACC also showed moderate improvement in hepatic cellular degeneration with many abnormal cells. Liver from rat receiving the combination of TMQ and NACC showed marked improvement in the hepatic cellular degeneration with apparently normal hepatic architecture with very few degenerated hepatocytes.

The results of the present study revealed that the combination of TMQ and NACC is the most effective regimen in the amelioration of SF toxicity. The hepatoprotective effects of NACC and TMQ against the toxicity of fluoride compounds might be mediated via multiple molecular mechanisms and can be considered a promising candidate against hepatic damage induced by SF toxicity.

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