Sorafenib Alleviates Inflammatory Signaling of Tumor Microenvironment in Lung Cancer

Betul Cicek
Erzincan Binali Yildirim University: Erzincan Binali Yildirim Universitesi
Serhat Hayme
Erzincan Binali Yildirim University: Erzincan Binali Yildirim Universitesi
Mehmet Kuzucu
Erzincan Binali Yildirim University: Erzincan Binali Yildirim Universitesi
Ahmet Cetin
Erzincan Binali Yildirim University: Erzincan Binali Yildirim Universitesi
Yesim Yeni
Ataturk University: Ataturk Universitesi
Sidika Genc
Bilecik Sheikh Edebali University: Bilecik Seyh Edebali Universitesi
Serkan Yildirim
Ataturk University: Ataturk Universitesi
Ismail Bolat
Ataturk University: Ataturk Universitesi
Mecit Kantarci
Ataturk University: Ataturk Universitesi
Mustafa Gul (mgul@atauni.edu.tr)
Atatürk Üniversitesi Tıp Fakültesi: Ataturk Universitesi Tip Fakultesi https://orcid.org/0000-0002-0042-890X
Ahmet Hacimuftuoglu
Ataturk University: Ataturk Universitesi

Research Article

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Abstract

Background: This study was designed to assess the possible beneficial effects of sorafenib (SOR) in diethylnitrosamine (DEN) induced lung carcinogenesis in male rats and also to examine its probable mechanisms of action.

Methods and results: A total of 30 adult male rats were divided into three groups as (1) control, (2) DEN, and (3) DEN+SOR. The chemical induction of lung carcinogenesis was performed by injection of DEN intraperitoneally at 150 mg/kg once a week for two weeks. The DEN-administered rats were co-treated with SOR of 10 mg/kg by oral gavage for 42 alternate days. Serum samples were analyzed to determine SOX2 levels. Levels of TNF-α and IL-1β were measured in lung tissue supernatants. Lung sections were evaluated histopathologically. Also, COX-2 and JNK were analyzed by immunohistochemistry and immunofluorescence methods respectively. SOR reduced the level of SOX2 that maintenance of cancer stemness and tumorigenicity, and TNF-α and IL-1β levels. Furthermore, SOR reduced lung histopathological structure and suppressed COX-2 and JNK expression

Conclusions: These results suggest that SOR reduces inflammation in the tumor microenvironment and decreases the level of SOX2 which has an important role in maintaining cancer stem cell properties.

Introduction

Lung cancer remains the largest cause of cancer-related deaths worldwide with about 1.8 million new cases each year [1]. Chemotherapy is the standard therapeutic approach for most lung cancer patients; however, it does not effectively improve life expectancy due to drug resistance [2]. These agents destroy tumor cells by inducing apoptotic cell death, but over time the cancer cells get marked resistance to apoptosis [3]. Despite billions of dollars spent on research in hopes of finding a cure for cancer, the death rates remained virtually unchanged for major cancers. For this reason, the relationship between the mechanism of carcinogenesis, cancer origins and targeted therapies has been questioned [2, 4].

Considering oncogenic signaling pathways, the relationship between inflammatory response and cancer development is particularly noteworthy. The common factor among numerous carcinogenic agents is the activation of genes that control inflammation cell signaling pathways and that these signals control all aspects of the cancer process [5, 6]. Of these pathways, c-Jun N-terminal kinase (JNK) is considered to be a potentially suitable target for the treatment of inflammatory conditions [6]. On the other hand, JNK signaling has two faces in cancer. JNK is associated with cancer cell apoptosis; however many emerging evidence demonstrated that the JNK pathway promotes cancer cell survival [7]. Another inflammatory parameter is the inducible cyclooxygenase (COX)-2 enzyme that catalyzes prostaglandin synthesis, which is the main mediator of inflammation and angiogenesis [8]. Overexpression of COX-2 has been detected in lung cancer and found to relation with progressive tumor growth, as well as the resistance of cancer cells to conventional chemotherapy [9].

The second critically important condition responsible for the cancer process is the presence of cancer stem cells (CSCs) [10]. CSCs are a small population of cells in tumors that have self-renewal, infinite proliferation capability, and lead resistance to conventional anticancer agents. SOX2, also known as sex-determining region Y (SRY)-box 2, is a transcription factor that is involved in maintenance of CSC characteristics [11]. SOX2 positively affects stem-like cells responsible for the initiation, maintenance, metastasis, and relapse of lung tumors. Also, inflammatory markers such as COX-2 are coexpressed with CSC markers including SOX2 in cancer [9]. As a result of an in-depth examination of the spectrum of genomic alterations in lung cancer, SOX2 is considered as a potential target for therapeutic intervention [9, 11].

Inflammation is essential not only for cancer induction through its mutagenic effects on stem cell DNA but also because the subsequent long-term behavior of these tumors is largely determined by the tumor microenvironment [5]. Exposure of stem cells' DNA to or prolonged, unrepaired assaults by pro-inflammatory cytokines and chemokines can lead to genetic mutations that over time can convert a somatic stem cell into a cancer stem cell [12].

Chemotherapy does not prolong patients' lifespan, but rather provides short-term recovery of survival (2). Therefore, directing the therapy to inflammation and stem cells, which are the main causes of malignancy, may make the treatment more efficient [5]. The most important development in cancer treatment is the effectiveness of targeted therapies in various types of cancer such as breast and liver [13].

Sorafenib (NEXAVAR; SOR) is a multi-kinase inhibitor approved for the treatment of hepatocellular carcinoma and advanced renal carcinoma and has also been the subject of extensive clinical trials in advanced lung cancer [14, 15]. It was declared that SOR significantly prolongs the recurrence-free survival of cancer patients. The efficacy of SOR is quite high even in patients who had no previous effective treatment and who had only minimal response to chemotherapy [16]. Studies concerning the relation between SOR and lung cancer are available [14, 15]; however, to the best of our knowledge, there is no study evaluating the relationship between JNK and COX-2 signaling and SOX-2 in the tumor microenvironment in lung cancer, given the above-mentioned information. Therefore, we aimed to determine the effects of mechanisms of action of SOR in diethylnitrosamine (DEN)-induced lung carcinogenesis in rats.

Materials And Methods

Animals, Grouping, and Experimental Design

In total, 30 male albino male Sprague Dawley rats (250-300 g) were obtained from Experimental Animal Laboratory of the Medicine and Experimental Application and Research Center of Ataturk University (Erzurum, Turkey). The rats were housed in plastic cages in well-ventilated room at 24 ± 1°C, with a normal 12-hr light/dark cycle. Commercially available pelleted rat chow and tap water were given ad

libitum. The use and care of laboratory animals were accepted by the Atatürk University Institutional Animal Care and Use Committee, and the experiments were performed according to international guidelines (E-42190979-000-2100281403).

Animals were assigned randomly to three groups (n = 10). Group I (control): 0.5 mL serum physiological (SF) was administered orally for 42 alternate days; Group II (DEN): 150 mg/kg DEN (Sigma Chemical Co., St. Louis, MO, USA) for was administered intraperitoneally (i.p.) in two doses every 7 days for 15 days [17]; Group III (DEN+SOR): 150 mg/kg DEN for was administered intraperitoneally and 0.5 mL SF was applied orally for 42 alternate days (i.p.) in two doses every 7 days for 15 days and 10 mg/kg Sorafenib [18] (Nexavar, Bayer HealthCare; dissolved in SF) was applied orally in an volume 0.5 mL for 42 alternate days. In our study, we applied 10 mg/kg SOR daily to rats, since higher concentrations were found to be toxic in long-term treatments (11, pilot experiments, and personal communication with Bayer AG).

At the end of this period, all rats were anesthetized with 80 mg/kg ketamine + 8 mg/kg xylazine administered i.p. The lung tissues were homogenized with ice cold lysis buffer for biochemical investigation. For histopatological, immunohistochemical and immunofluorescence analysis, lung tissues were kept in 10% buffered formalin solution.

Biochemical Analysis

The lung tissue samples from each rat were grinded in liquid nitrogen in a Tissuelyser II device (Qiagen, Germany). 100 mg of ground tissue was homogenized in lysis buffer with 1ml phosphate-buffered saline, and the sample lysates were centrifuged at 14,000 g for 15 min at 4°C and the collected supernatant were used for analysis. Concentrations of SOX2, TNF-α and IL-1β were determined using ELISA kits (Elabscience, United States) (using the Competitive-ELISA principle) according to the manufacturer's instructions.

Histopathological Analysis

Lung tissues were fixed in 10% buffered formalin for 48 h, dehydrated in a graded ethanol series, and embedded in paraffin. Sections (3-4 μm thick) were placed on slides, stained with hematoxylin-eosin (H&E) for histopathological analysis, and examined under a light microscope (Leica DM 1000, Germany).

Immunohistochemical And Immunofluorescence Examination

All sections taken on the adherent (poly-L-Lysin) slides were passed through the xylol and alcohol series. The sections were dipped in 3% H_2O_2 after washing in distilled water. Protein blocks were dripped onto the tissues that were boiled with antigen retrieval. For immunohistochemical analysis, tissues were

incubated at 37°C for 30 minutes with the primary antibody (COX2, Catalog no: sc-293182, Santa Cruz, USA). 3-3' Diaminobenzidine (DAB) was used as chromogen. Sections were examined under a light microscope. For immunofluorescence examination, the primary antibody (JNK, Catalog no: sc-514539, santa cruz, USA) was kept at the appropriate temperature and time for the conditions of use. FITC (Cat No: ab6785 Dilüent Oranı: 1/1000) was used as a secondary marker. Then, DAPI with DNA (Catalog no, D-1306 dilution ratio:1/200) marker mounting medium was used. Sections were examined under laser scanning confocal microscope (Zeiss LSM 710). The processed tissues were examined under a fluorescent light microscope (Zeıss Leica DM 1000). The sections were evaluated as no (-), mild (+), moderate (++), severe (+++) according to their immunopositivity.

Statistical Analaysis

All statistical analyses were performed using SPSS for Windows 20 (SPSS Inc, Chicago, IL, USA). Shapiro Wilks test was used to assess the normality assumption and Levene Test was used to assess the assumption of homogeneity of variances. Descriptive statistics for continuous variables were expressed as mean ± standard deviation. The significance of the mean difference between the three groups was investigated by the Welch One Way Analysis of Variance (ANOVA) Test. Tukey HSD multiple comparison tests were used for pairwise comparisons. Data were illustrated by the graphical representation. A two-sided p-value <0.05 was considered statistically significant.

Results

Biochemical results

Effects of SOR on lung SOX2 levels in DEN-induced lung carcinogenesis in rats

As shown in Fig. 1 serum SOX2 protein levels were significantly increased in the DEN group when compared to the control group (p< 0.001). The SOX2 decreased significantly in DEN+SOR group when compared with the DEN group (p < 0.001).

Effect of SOR treatment on lung TNF-α and IL-1β levels in DEN-induced lung carcinogenesis in rats

As shown in Fig. 2 lung tissue TNF-α and IL-1β levels were significantly increased in the DEN group when compared to the control group (p< 0.001). TNF-α and IL-1β levels decreased significantly in DEN+SOR group when compared with the DEN group (p < 0.001).

Histopathological Examination Of Lung

Control group: The lung tissue of healthy rats was observed to have normal histological structure. **DEN group**: General appearance of the lung tissue was compatible with tumorogenesis findings. Severe peribronchiolar cell infiltrations, severe exudation and alveolar macrophages in the alveolar lumens, proliferation and degeneration in the bronchiole epithelium, severe thickening due to cell proliferation in the interstitial spaces, and severe hyperemia in the vessels were observed. **DEN+SOR group**: Compared with the DEN group, the treatment of SOR had partial signs of improvements. Mild peribronchiolar cell infiltration, mild thickening of the interstitial spaces, and moderate hyperemia in the vessels were detected (Fig. 3 and Tab.1).

Table 1
Summary of the histopathological scores based on H&E staining, immunohistochemical and immunofluorescence results for lung tissues.

	Control group	DEN group	DEN+SOR group
Peribronchiolar cell infiltrations	-	+++	+
Macrophage in alveolar lumens	-	+++	-
Proliferation in bronchial-bronchiole epithelium	-	+++	+
Hyperemia	-	+++	++
COX-2	-	+++	+
JNK	-	+++	+

Control: healthy group (no treatment), DEN: diethylnitrosamine group, DEN+SOR: DEN+ 10 mg/kg sorafenib. COX-2: Cyclooxygenase-2; JNK: c-Jun N-terminal Kinase. None (-), mild (+), moderate (++), and severe (+++).

Effects of SOR on the COX-2 levels in lung tissue

Control group: The lung tissue of healthy rats was observed immunohistochemically and found negative COX-2 expression. **DEN group**: As a result of DEN application, a severe level of COX-2 expression in peribronchiolar cells, alveolar lumens, and perivascular areas were determined. **DEN+SOR group**: When SOR treatment was applied to DEN groups, mild COX-2 expression was detected in peribronchiolar cells, around bronchial bronchioles and alveoli (Fig. 3 and Tab.1).

Effects of SOR on the JNK levels in lung tissue

Control group: The lung tissue of healthy rats was examined by immunofluorescence and negative JNK expression was observed. **DEN group**: As a result of DEN application, severe JNK expression was observed in the bronchi, bronchioles and alveolar epithelium. **DEN+SOR group**: When SOR treatment was applied to DEN groups, mild JNK expression was present at bronchi, brochioles and alveolar epithelium (Fig. 3 and Tab.1).

Discussion

In order to control the emergence and progression of cancer, survival mechanisms, especially cancer stem cell factors, and inflammation, become very important [5, 8, 10, 12]. In contrast to traditional cytotoxic chemotherapies, targeted cancer therapy involves treating cancer cells with greater sensitivity and potentially fewer side effects and mechanisms of carcinogenesis [13]. In this study, we analyzed the effects of low doses of sorafenib treatment on DEN-induced lung carcinogenesis in rats and determined that DEN effectively induced lung carcinogenesis when applied alone and sorafenib was significantly effective in decreasing the inflammatory parameters in the tumor microenvironment and level of SOX2 protein, which mediates the self-renewal of cancer stem cells.

Diethylnitrosamine (DEN) is a well-known and commonly used chemical compound to induce cancers of the esophagus, liver, and lung in experimental animals [17]. Some literature data demonstrated that DEN may be a potent lung carcinogen in strains with a higher incidence of lung tumors than other cancers [19, 20]. We selected a dose of 150 mg/kg DEN based on previous studies, which demonstrated that the application of DEN at this dose remarkably reflects the pathological and biochemical findings in lung cancer in humans [17, 21]. In parallel with the previous literature, we determined that DEN administration significantly increased histopathological scores and inflammation in the present study. Lung tissues displayed extensive pathological changes in the DEN group. Widespread inflammatory cell infiltration, thickened alveolar wall, proliferation, degeneration in the bronchiole epithelium, hyperemia in the vessels were observed in the DEN group. In contrast to the DEN group, the histopathological scores decreased and partial histopathological improvement was observed in the group treated with SOR.

Chronic inflammation is often a hallmark of tumor initiation and progression [5]. JNK takes an important role in these inflammation-related events. Many studies demonstrated that JNK is a positive regulator of tumor growth in the lung tissue of experimental models [6, 22]. In line with such roles of JNK, it has been documented that JNK maintain the tumor-initiating capacity of lung stem cells and another role has been added to it as "one of the pre-tumor roles of JNK" [10]. Besides this, JNK has activated the inflammatory lung environment to support tumor development by regulating cytokines including IL-1β and TNF-α [23]. IL-1β, a proinflammatory cytokine, is associated with tumor progression in lung cancer patients in multiple studies [24, 25]. An examination of the Cancer Genome Atlas database shows that for lung cancer, IL-1 and TNF reveals a tendency as an oncogene, as patients with higher IL-1β and TNF-α mRNA levels are linked to shorter overall survival, although this correlation does not reach statistical significance [26]. In another study, it is well documented that TNF-α and IL-1β signaling support COX-2 promoter activity as well as COX-2 and mRNA expressions. Moreover, recent studies have demonstrated that COX-2 expression also requires the activity of JNK intracellular signaling [28, 29].

In our study, JNK and COX-2 mRNA in lung tissue and TNF-α and IL-1β protein levels in serum increased in animals as a result of DEN administration. Studies on lung tissue and serum demonstrate that DEN treatment increased COX-2, TNF-α, and IL-1β levels [30, 31]. To the best of our knowledge, the results of the present study were first demonstrating that DEN leads to high level of JNK expression in lung tissue.

With the application of SOR, it was observed that JNK and COX-2 mRNA in lung tissue decrease with the amelioration of tissue damage and inflammatory response. In parallel to this, serum TNF-α and IL-1β levels were decreased with the administration of SOR. There were no studies evaluating the effect of SOR on lung JNK and serum TNF-α and IL-1β levels after DEN administration in rats previously. However, it has been reported that sorafenib reduces JNK expression in lung cancer cell line in vitro [32], and it has therapeutic properties by reducing TNF-α and IL-1β levels in liver cancer [33]. It has also been reported that, treatment of low concentrations of SOR significantly inhibits the proliferation of A549 tumor cells in vitro and suppressed tumor growth in vivo [34].

Inflammation also leads to cancer cells dedifferentiating into CSCs through several signaling pathways such as COX-2 and JNK signaling [6, 9]. SOX2 has demonstrated the potential to be a clinically useful biomarker to maintain lung CSCs [11]. There are also studies in the literature reporting that SOX2 is overexpressed and acts as an oncogene in lung cancer [35]. Although the role of SOX2 in lung cancer has been determined as a result of clinical and preclinical studies [11, 35] the effect of DEN administration on SOX2 expression in experimental animals remains unclear. In our study, we observed that DEN increased SOX2 levels as compared to the control group. In the literature, we could not find any study demonstrating SOX2 level in the DEN-induced lung cancer model. However, in line with our result, a recent study declared that stem cell gene SOX2 increased significantly in liver tissue with DEN application [36]. In this study, SOR treatment significantly reduced SOX2 level in comparison to the DEN group. Consistent with this result, SOR was reported to decrease SOX2 level in an in vitro study [37]. In the present study, we show for the first time that DEN exposure in rats promotes SOX2 levels through JNK/COX-2 signaling, endowing lung cells with malignant properties. These effects of DEN were reversed by the administration of sorafenib. It was also shown for the first time in this study that SOR reduces inflammation-associated JNK and COX-2 signaling and TNF and IL-1β protein levels in the tumor microenvironment and decreases the level of SOX2, which is a marker related to CSCs. These findings are noteworthy because of the close proximity to clinical trials investigating sorafenib for the treatment of lung cancer.

Declarations

Author contributions: BC: Conceptualization, Methodology, Formal analysis, Writing - original draft, Writing - review & editing, Investigation, Data curation. SH: Data curation. MK Investigation, Resources, Data curation. AC: Investigation, Data curation. SY: Data curation, Writing. IB: Data curation. MK: Writing - original draft, Visualization. MG: Writing - review & editing, Supervision. AH: Conceptualization, Visualization.

Author declaration: All authors declare that we have seen the final version of the manuscript being submitted. We certify that the manuscript represents the original work and has not been published or under consideration for publication elsewhere.

Compliance with Ethical Standards: The use and care of laboratory animals were accepted by the Atatürk University Institutional Animal Care and Use Committee, and the experiments were performed according

to international guidelines (E-42190979-000-2100281403).

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Figures

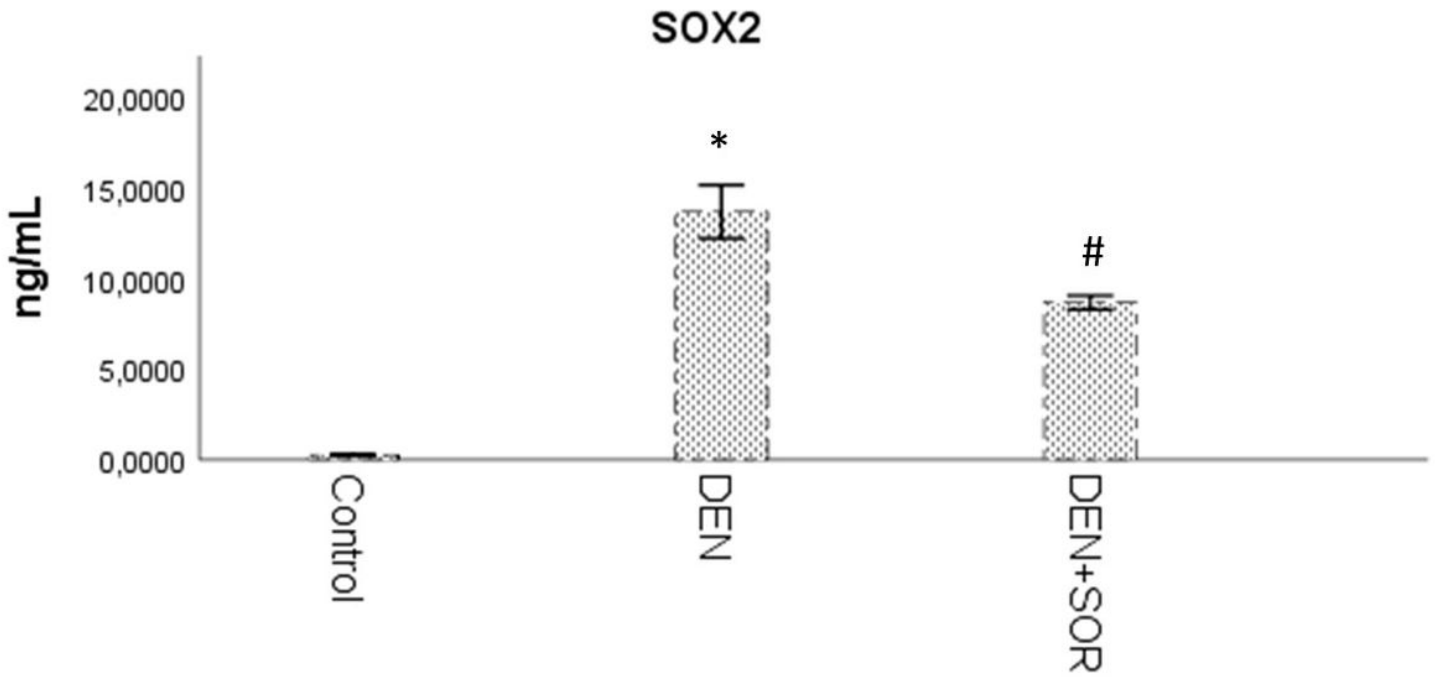

Figure 1

Effects of SOR on serum SOX2 level. (n=10) Data are expressed as the means ± SD *p < 0.001 vs. control group, #p < 0.001 vs. DEN group DEN: diethylnitrosamine; SOR: Sorafenib.

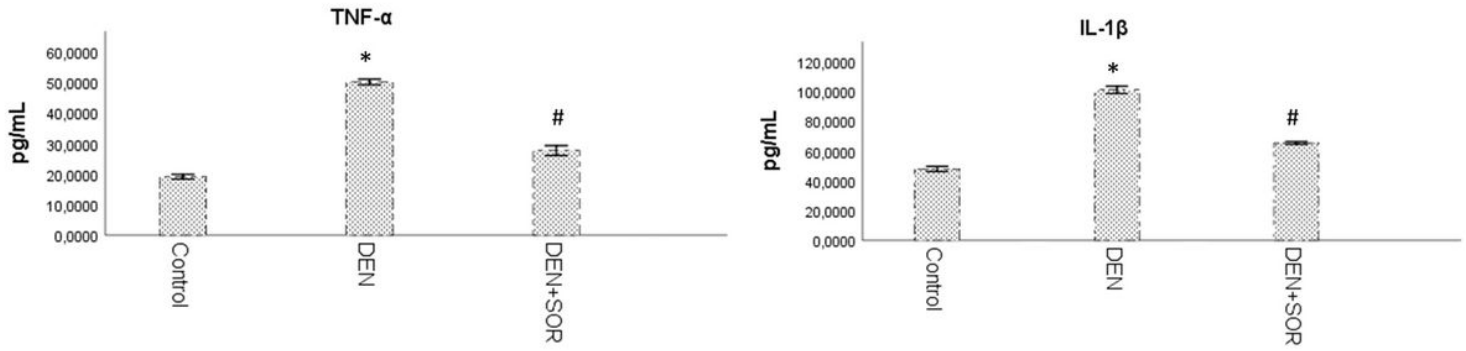

Figure 2

Effects of SOR on TNF-α and IL-1β in lung tissue. (n=10) Data are expressed as means ± SD. *p < 0.001 vs. control group, #p < 0.001 vs. DEN group DEN: diethylnitrosamine; SOR: Sorafenib; SOX2: Sex-

determining region Y (SRY)-box 2

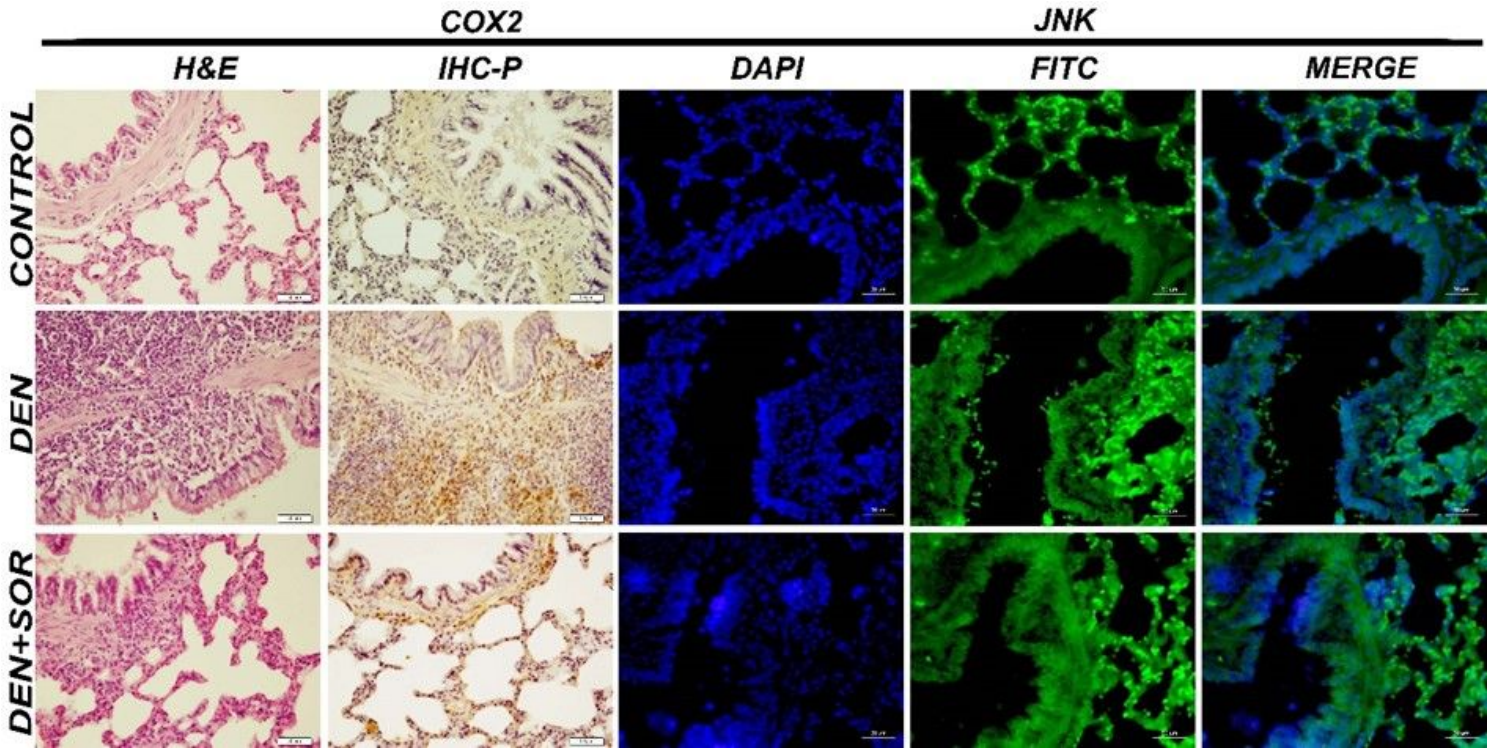

Figure 3

Histopathological (H&E), immunohistochemical (COX-2) and immunofluorescence (JNK) images of lung tissue samples, bar: 50 μm