Comparison of Anti-Inflammatory Effects of Theopylline and Resveratrolin in Chronic Bronchitic Rats

Kronik Bronşitli Farelerde Teofilin ve Resveratrolün Anti-İnflamatuar Etkinliklerinin Karşılaştırılması

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ABSTRACT Objective: The aim of this study was to compare the anti-inflammatory effects of resveratrol and theophylline in rats with chronic bronchitis exposed to passive cigarette smoke. **Material and Methods:** Thirty-five Wistar-Albino rats were enrolled into five groups. After exposure to passive cigarette smoke for 20 weeks, the rats in the first four groups were treated with intraperitonaeal administration of resveratrol solved in dimethylsulfoxide (DMSO), DMSO, theophylline solved in physiologic saline (PS) or PS for 21 days. The rats in the fifth group were healthy controls. At the end of the experiment, blood samples were obtained to measure serum tumor necrosis factor alpha (TNF-α) levels and lung samples were obtained for light and electron microscopic examination. **Results:** Serum TNF-α level was lower in rats treated with resveratrol and theophylline compared to control rats ($p< 0.05$). Smoke induced lung injury decreased with resveratrol and theophylline administrations compared to carrier administrations ($p< 0.05$). Neither serum TNF-α nor lung histopathology scores of rats treated with resveratrol and theophylline were different ($p> 0.05$). **Conclusion:** Resveratrol and theophylline were found to have similar effects on histopathological findings and cytokine expression, therefore could be considered for treatment of chronic obstructive pulmonary disease. However, comprehensive long-term clinical studies are needed.

Key Words: Pulmonary disease, chronic obstructive; resveratrol; theophylline; tumor necrosis factor alpha (36-68)

ÖZET Amaç: Bu çalışmanın amacı, pasif sigara dumanına maruz bırakılan kronik bronşitli farelerde teofilin ve resveratrolün anti-inflamatuvar etkilerinin karşılaştırılmasıdır. **Gereç ve Yöntemler:** Toplam 25 Wistar-Albino faresi beş gruba ayrıldı. Pasif sigara dumanına 20 hafta süreyle maruz bırakıldıktan sonra dört grup fareye sırasıyla dimetilsülfoksid içinde çözünmüş resveratrol, demetilsülfoksid, serum fizyolojik içinde çözünmüş teofilin ve serum fizyolojik periton içine tedavi amaçlı olarak verilmiştir. Son grup sağlıklı kontrollerden oluşmuştur. Deney sonunda farelerden alınan kan örneklerinde serum tümör nekroz faktör-alfa (TNF-alfa) düzeylerine bakıldı, akciğer dokusu örnekleri ışık ve elektron mikroskobunda incelendi. **Bulgular:** Serum TNF-alfa değerleri resveratrol ve teofilin verilen farelerde kontrol grubuna göre daha düşük düzeyde bulundu ($p< 0.05$). Sigara dumanına bağlı akciğer hasarı resveratrol ve teofilin verilen farelerde çözücü verilen farelere göre daha az olmuştur. Resveratrol ve teofilin grupları arasında ne serum TNF-alfa seviyelerinde ne de akciğer histopatoloji skorlarında farklılık saptanmamıştır ($p> 0.05$). **Sonuç:** Resveratrol ve teofilinin histopatolojik değişiklikler ve sitokin salımı üzerine etkileri benzer bulunmuştur, bu nedenle kronik obstrüktif akciğer hastalığının tedavisinde kullanılabilecekleri düşünülmektedir. Ancak, bu konuda büyük ve uzun süreli çalışmalara ihtiyaç bulunmaktadır.

Anahtar Kelimeler: Akciğer hastalığı, kronik obstrüktif; resveratrol; teofilin; tümör nekroz faktörü alfa (36-68)

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Chronic obstructive pulmonary disease (COPD) is characterized by chronic and progressive airflow limitation which is associated with inhalation of noxious particles and gases, especially smoking. These chronic irritants provoke an abnormal inflammatory response in the respiratory tract which induces the parenchymal destruction and causes several damages of the defense and repair mechanisms. Consequently, characteristic pathophysiological changes in COPD occur, which are parenchymal destruction (emphysema) and airway obstruction (obstructive bronchitis).[1,2]

Several inflammatory cells increase in the airways in COPD. The degree of contribution of these inflammatory cells in the development and progression of COPD is not fully understood yet. Macrophages are thought to be the principal inflammatory cells leading to pulmonary inflammation. Neutrophils and T-lymphocytes are also increased in the airways in COPD, and thought to be responsible for the progression of inflammation and parenchymal destruction.[3-5] These inflammatory cells produce and secrete several cytokines and chemotactic factors some of which have been already proven to have several roles in the tissue destruction and permanent inflammation. The most studied cytokines in the pathogenesis of COPD are interleukin-8 (IL-8), tumor necrosis factor alpha (TNF-α) and LTB4.[6,7]

An effective anti-inflammatory treatment that suppresses the airway inflammation and prevents the disease progression in COPD is not yet available. However, for better understanding of pathogenesis of COPD, several new anti-inflammatory agents have gained attention.[7] Resveratrol (trans-3,5,4'-trihydroxystilbene) is an extract of red wine and thought to have several anti-inflammatory and antioxidant properties.[8-10] It suppresses the cytokine secretion from macrophages in corticosteroid-unresponsive COPD patients.[8] The exact mechanism of this effect has not been elucidated yet. Theophylline is a drug in methylxanthine class, and has been used in the treatment of COPD for years. It is a non-selective inhibitor of phosphodiesterase enzyme type III and IV through increasing the level of cAMP in the smooth muscle cells and leading to bronchodilatation. It also suppresses the secretion of adenosine, prostaglandins and TNF-α via the inhibition of phosphodiesterase type IV isoenzyme, which is suggested as the anti-inflammatory properties of theophylline.[11] This study was conducted to compare the anti-inflammatory effects of theophylline and resveratrol in chronic bronchitis-induced rats by exposure to passive cigarette smoke, as reflected by serum TNF-α level and lung injury score.

MATERIAL AND METHODS

ANIMALS

The present study was performed in accordance with the Guiding Principles for the Care and Use of Laboratory Animals; all procedures were approved by the ethics committee of Kocaeli University. Male Wistar-Albino rats, weighing 250-300 g, were used in this study (n=35). The rats were kept under standard conditions (stainless-steel cages, 18-21°C, 55-60% relative humidity, and 12 hours light/dark cycles). Standard chow in tablet form and water were available ad libitum. All animals were free from infections.

Rats were exposed to passive cigarette smoke in order to develop COPD for 20 weeks[12] and were decapitated 24-hour after the last injection of agents and solvents; 4 ml of blood was taken intracordially for the measurement of serum TNF-alpha levels. Rat lung tissues were dissected after intracardiac perfusion.

SMOKE EXPOSURE MACHINE

A special smoke exposure machine based on Chen definition and Walton modification was prepared in Kocaeli University, Experimental Medicine Research Laboratory.[13] Briefly, the machine was consisted of three separated chambers connected to each other. The first chamber was generation chamber where smoke was generated, the second one was residence chamber where smoke was diluted and the last one was exposure chamber where rats were exposed to smoke. The fans between the chambers were used to conduct smoke to one

chamber to another, worked in a speed of 5.2 l/min. Standardized nicotine and tar included cigarette (LM, Philip Morris) were used for exposure for 20 weeks, two hours daily.

EXPERIMENTAL GROUPS

The rats were divided into five groups with seven rats in each to be administered intraperitoneally with 20 mg/kg resveratrol (Sigma-Aldrich, Italy) dissolved in 1 ml dimethylsulfoxide (DMSO) (Group 1), 1 ml DMSO (Sigma-Aldrich, Italy) (Group 2), 14 mg/kg theophylline (Sigma-Aldrich, Italy) dissolved in 1 ml physiological saline (PS) (Group 3), and 1 ml PS (Group 4). The group not exposed to smoke exposure included healthy rats and served as control. All injections were performed every day at 09:00 am after development of COPD, and continued for 21 days. The control rats were kept in same machine for 2 hours daily with clean air in order to expose similar stress.

HISTOPATHOLOGICAL EVALUATION

Light Microscopic Evaluation

After perfusion of 2.5% glutaraldehyde, lung tissue specimens were dehydrated in a graded series of ethanol (70%, 80%, 90% and 100%), cleared in toluene and embedded in paraffin. Sectioned paraffin blocks at a 5 μm thickness were stained with hemotoxylen & eosin and evaluated under Olympus BH2 photomicroscope (Tokyo, Japan). Light microscopic results were scored in four histopathological categories: 1) dilatation of respiratory tract, 2) infiltration of inflammatory cells, 3) proliferation of respiratory epithelium and 4) vascular congestion. The first three categories were scored from 0 to 3 while 0 indicated no pathology and 3 indicated the most severe damage, using the semi-quantitative scale and the last category was scored from 0 to 1 with respect to absence or presence of vascular congestion. The total histopathological score of the lung was calculated as the sum of the scores given for each criterion.[14]

Electron Microscopic Evaluation

After the perfusion of 2.5% glutaraldehyde, lung tissue specimens were post-fixated with 1% osmium tetroxide (0.1 M, pH 7.2), dehydrated in a graded series of ethanol (70%, 80%, 90% and 100%), cleared in toluene and embedded in Epon812 for 24 hours at 60°C. Semi-thin sections (1 μm) were cut, stained with toluidine blue and viewed under the light microscope for proper orientation. Tissues were then thin sectioned (60 nm), stained with uranyl acetate and lead citrate and evaluated under JEOL 1200 SX transmission electron microscope.[15]

SERUM TNF-ALPHA MEASUREMENT

Blood samples were taken intra-cordially into dry tubes. After centrifugation, serum was aspirated and TNF-α was measured using the ELISA method (Biosource Rat TNF-alpha kit, CA, USA). The curve for TNF-α ranged from 0 to 1000 pg/ml.

STATISTICAL ANALYSIS

One way ANOVA and Bonferroni's test as a post hoc test was used for multiple comparisons among experimental groups and between the control and experimental groups (SPSS, version 13.0, Chicago, IL). The results were expressed as mean ± standard error (SE). A p value less than 0.05 was considered significant.

RESULTS

LIGHT AND ELECTRON MICROSCOPY FINDINGS

There was dilatation in alveolar duct and respiratory bronchioles, proliferation of alveolar epithelial cells, inflammatory cell infiltration and vascular congestion in rats received DMSO and PS. The number of type 2 pneumocytes and surfactant levels were increased in both groups (Figure 1 and 2).

In resveratrol group, dilatation of alveolar duct was seen less prominently, with a decreased the inflammatory cell infiltration and vascular congestion. Respiratory epithelial cells were relatively normal in morphology. Electron microscopic findings were comparable with healthy controls where type 2 pneumocytes and surfactant levels were normal (Figure 3). Total histopathological score was 3.2 ± 0.2 in resveratrol group, which was not different from healthy controls (2.0 ± 0.5), whereas it was significantly lower than DMSO and PS groups (7.2 ± 1.2 and 8.2 ± 0.6 respectively, $p<0.05$) (Figure 4).

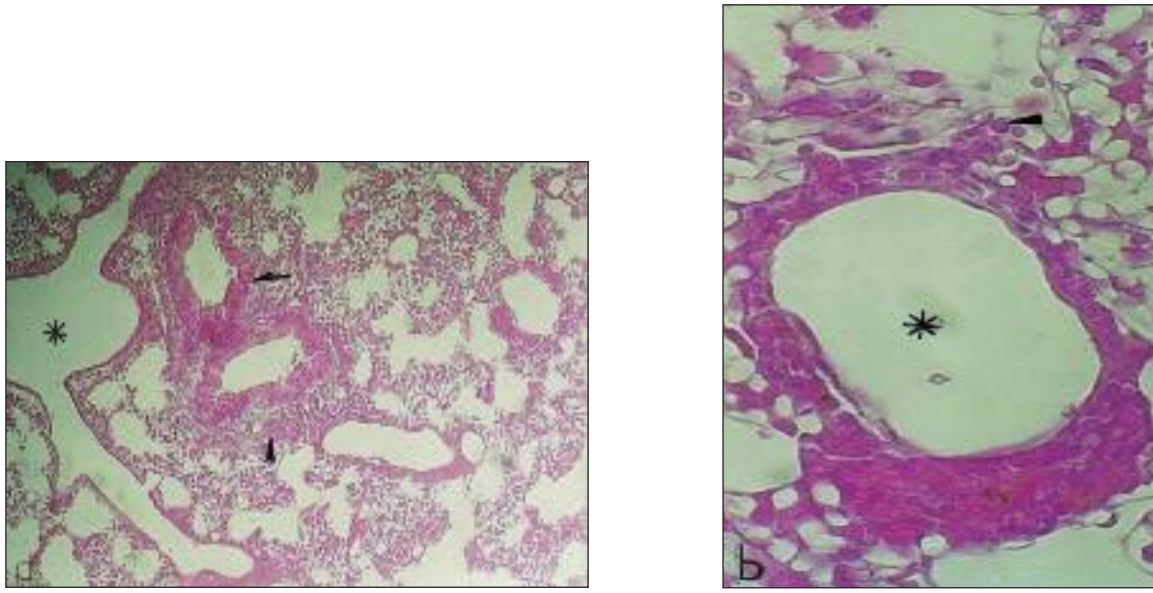

FIGURE 1a, b: Alveolar duct dilatation (*), respiratory epithelial proliferation (→) and inflammatory cell infiltration (►) (H&E, 1a:X100; 1b:X400).

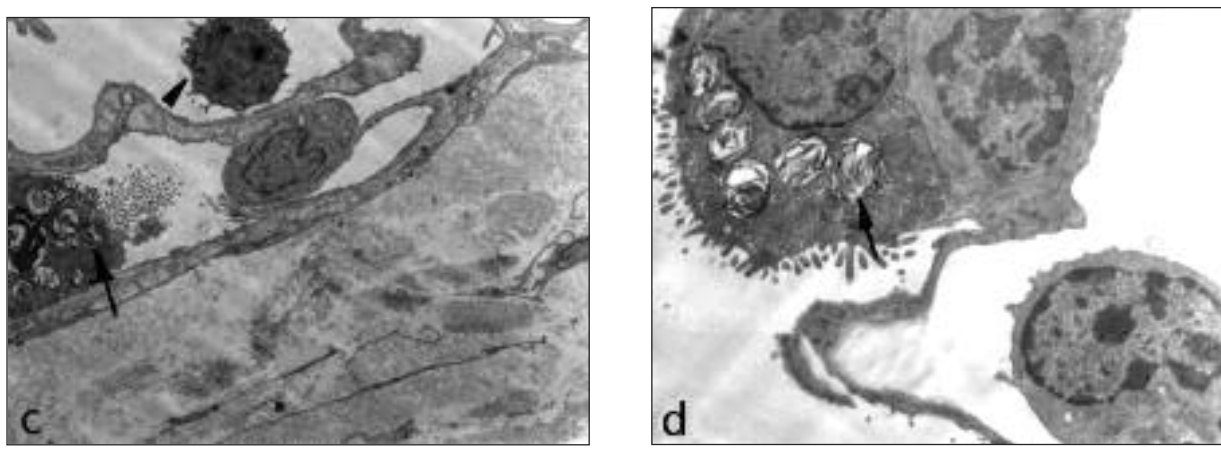

FIGURE 1c, d: Increase in surfactant deposition (→), increase in the number of macrophages (►) and irregular alveolar-capillary barrier (*) (1c:X3000, 1d:X5000).

FIGURE 1: Light and electron microscopic photomicrographs of lung tissue obtained from the physiologic saline group.

In theophylline group, alveolar duct dilatation and inflammatory cell infiltration were still seen, with a decreased respiratory epithelial cell proliferation. There was increased type 2 pneumocytes and surfactant levels, surfactant accumulation in the alveolar sac and vascular congestion in electron microscopy (Figure 5). Total histopathological score for theophylline group was greater than healthy controls (5.0 ± 0.7 *vs.* 2 ± 0.5, $p<0.05$). Moreover, it was lower than that for DMSO (7.2 ± 1.2, $p>0.05$) and PS groups (8.2 ± 0.6, $p<0.05$). There was difference for total histopathological score between resveratrol and theophylline groups (Figure 4, Tables 1 and 2).

SERUM TNF-α LEVEL

Serum TNF-α levels were lower for resveratrol and theophylline groups compared to DMSO and PS groups. There were no significant differences between healthy controls and theophylline and resveratrol groups (Figure 6, Table 3).

DISCUSSION

In this study, we have compared the anti-inflammatory effects of two novel anti-inflammatory agents in smoke-induced inflammatory changes in rats, and found that both resveratrol and theophylline suppressed the TNF-α levels and reversed the

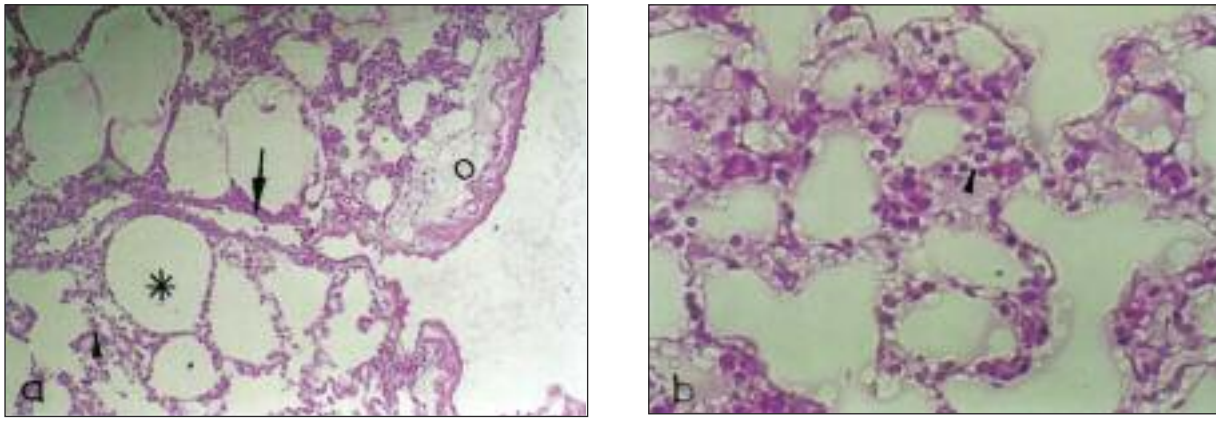

FIGURE 2a, b: Alveolar duct dilatation (*), respiratory epithelial proliferation (→), inflammatory cell infiltration (►) and arterial wall edema (o) (H&E, 1a:X100; 1b:X400).

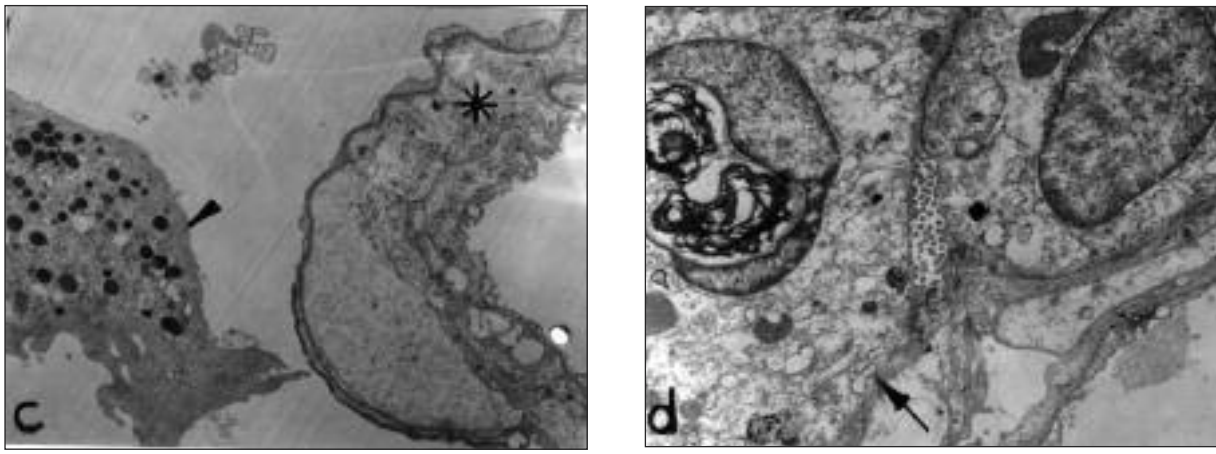

FIGURE 2c, d: Capillary wall edema (*), increase in surfactant deposition and number of the type 2 pneumocytes (→), increase in the number of macrophages (►) (1c:X4000, 1d:X5000).

FIGURE 2: Light and electron microscopic photomicrographs of lung tissue of the DMSO group.

histopathological changes observed in the lung tissue both in light and electron microscopy. To our knowledge, this is the first study comparing theophylline and resveratrol and reported similar anti-inflammatory effects of both agents.

Bronchodilator therapy is the cornerstone in COPD because of its inflammatory characteristics. Inhaled corticosteroids are known as the most potent anti-inflammatory agents in asthma; however it is suggested that different cells and inflammatory markers are responsible in the airway inflammation in COPD resistant to corticosteroids.[8] This observation leads the investigation of new anti-inflammatory agents.

Since epidemiological studies reported an inverse correlation between wine consumption and cardiovascular events, phenolic compounds such as resveratrol that is present in the skins of red grapes has became a new focus of interest.[16] It possesses anti-inflammatory, anti-oxidant and antineoplastic properties.[14,16,17] The suggested mechanisms for anti-inflammatory effect of resveratrol include the suppression of NF-KB, inhibition of arachidonic acid metabolism and activation of AP-1.[16,18]

The possible preventive effect of resveratrol on smoke-induced injury was demonstrated in thyroid tissue of smoke-exposed rats.[19] Previous studies investigating pulmonary effects of resveratrol reported inhibition of airway neutrophilia, suppression of several inflammatory cytokines such as TNF-alpha, IL-8, IL-6, GM-CSF and reversion of the histopathological changes.[8,14,20-22] This study al-

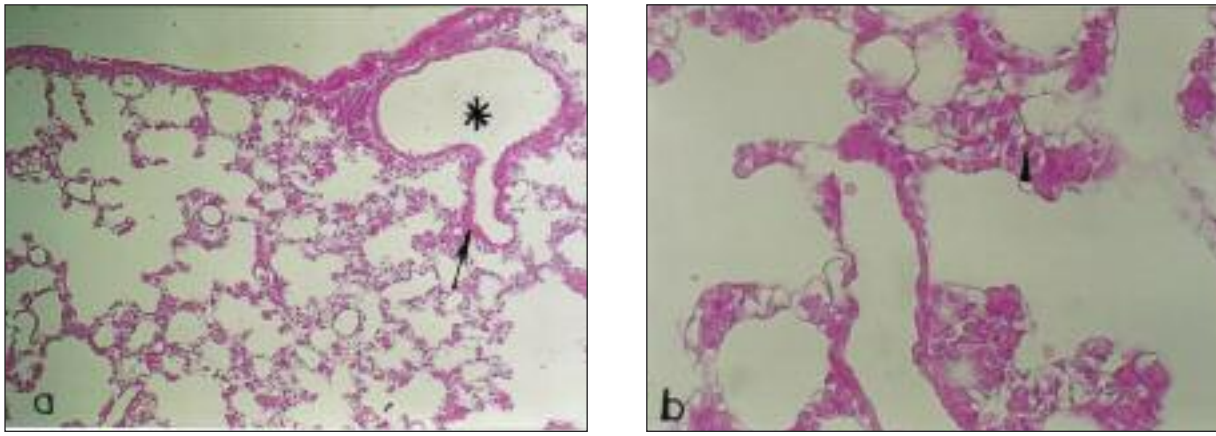

FIGURE 3a,b: Less prominent alveolar duct dilatation (*),less inflammatory cell infiltration (►), respiratory epithelial cells (→) nearly normal in morphology (H&E, 3a:X100; 3b:X400).

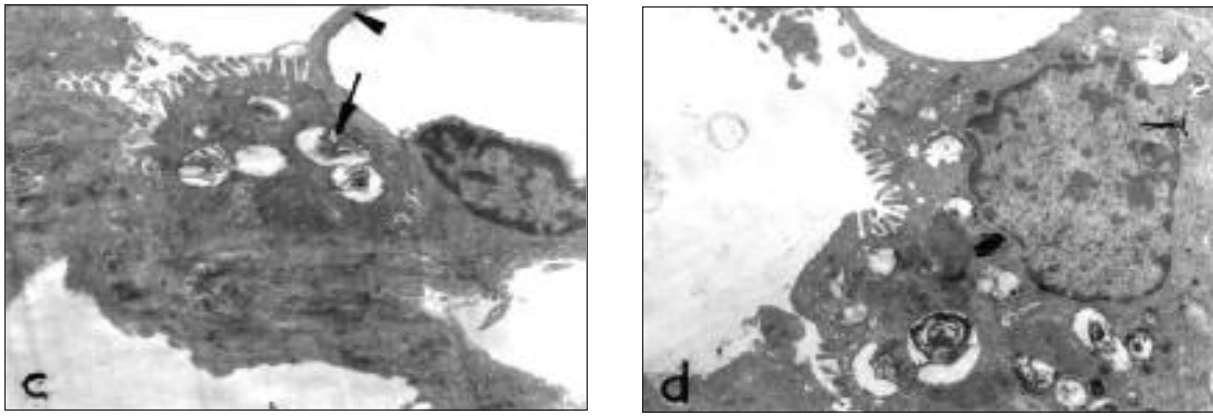

FIGURE 3c, d: Figure 3c,d; showing surfactant deposition, type 2 pneumocyte (→) and alveolarcapillary barrier (►) nearly normal in morphology (3c,d:X6000).

FIGURE 3: Light and electron microscopic photomicrographs of lung tissue in the resveratrol group.

so demonstrated significant suppression of TNF-α levels and reduction in histopathological score in the resveratrol group compared to untreated group upon smoke exposure.

Theophylline is an old drug and used as a third-line bronchodilator agent in obstructive lung disease.[11] It is also used as an anti-inflammatory agent in asthma at lower doses and reported to decrease the number of neutrophils, IL-8, TNF-alpha levels in the induced sputum of COPD patients.[23-25] This study demonstrated that theophylline reversed smoke-induced histopathological changes as well as TNF-α levels. Kaneko et al reported similar beneficial effect of theophylline and dexamethasone in the LPS-induced lung injury in guinea pigs.[26]

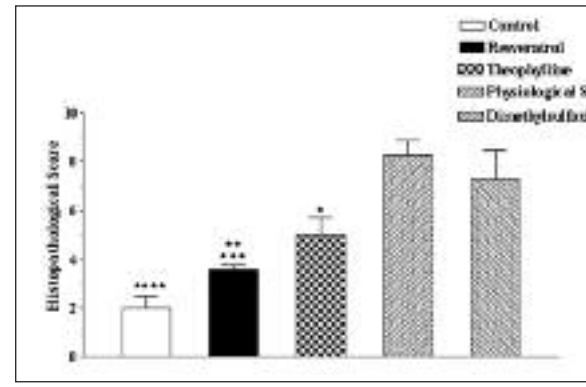

FIGURE 4: Histopathological score of the lung tissue specimen. *p<0.05 theophylline vs. physiological saline, **p<0.01 resveratrol vs. dimethylsulfoxide, ***p<0.001resveratrol vs. physiologic saline, and ****p<0.001 control vs. physiological saline and dimethylsulfoxide.

Researches have focused on the steroid resistance mechanisms and steroid activity modulation in COPD. Increased activation NF-KB in response to oxidative stress thought to be one of the important mechanisms for steroid resistance. NF-KB increases acetylation of histones and decreases the

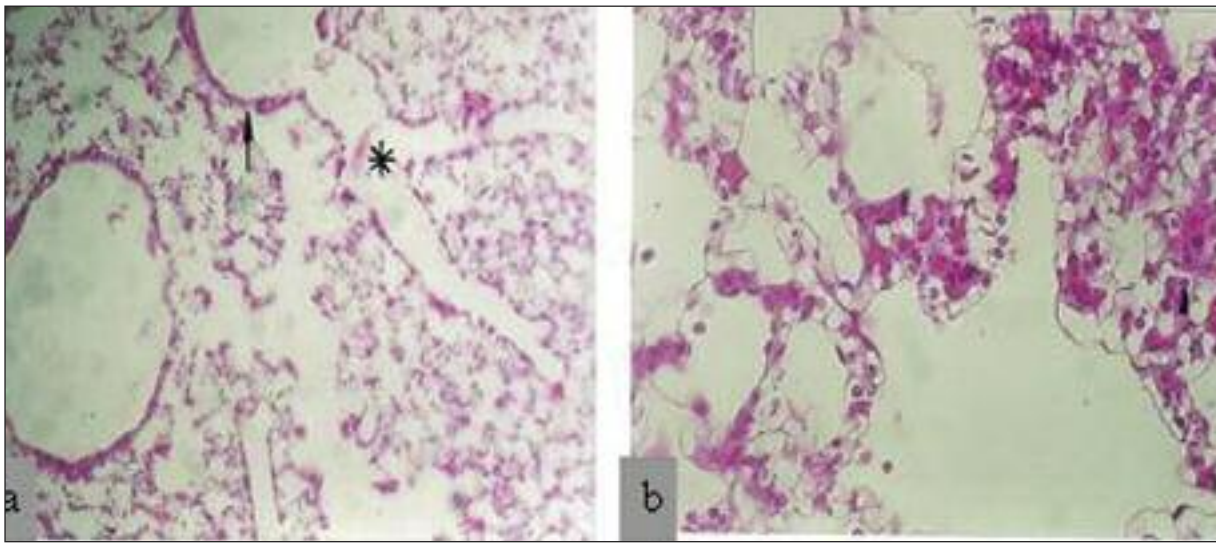

FIGURE 5a, b: Alveolar duct dilatation (*), inflammatory cell infiltration (►), less respiratory epithelial cell proliferation (→) (H&E, 5a:X100; 5b:X400).

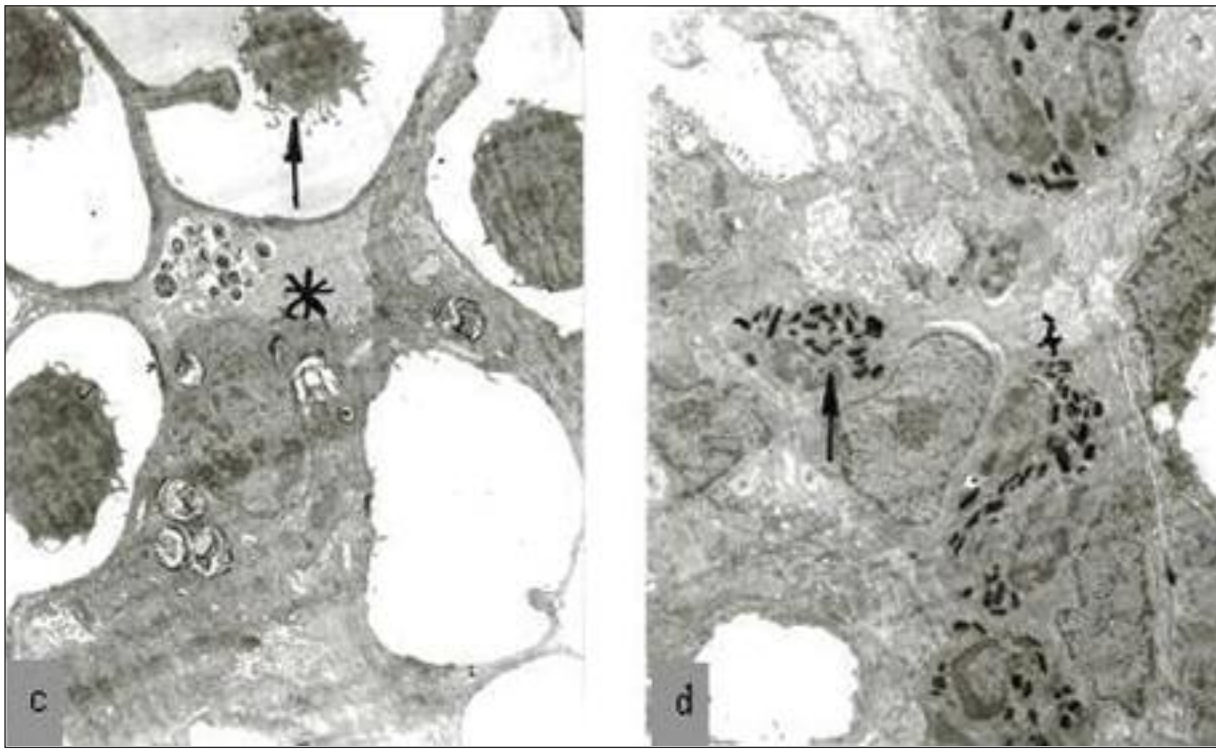

FIGURE 5c, d: Increase in the number of macrophages (5c) and eosinophils (5d) (→), increase in synthesis and deposition of surfactant in alveolar lumen (*) (5c), increase in the number of type 2 pneumocytes(*) (5c) (5c,d:X3000).

FIGURE 5: Light and electron microscopic photomicrographs of lung tissuein the theophylline group.

expression and activation of histone deacetylase (HDAC), thus up-regulates inflammatory genes and the levels of inflammatory cytokines. These smoke-induced inflammatory changes are thought to be controlled by alveolar macrophages and responsible for failure of steroid response in COPD.[27,28] Alternative anti-inflammatory therapies to suppress macrophages, NF-KB activity and up-regulate the HDAC in the lung are needed to deal inflammation in COPD and to modulate the activation of steroids. Resveratrol, theophylline and specific phosphodiesterase 4 inhibitors are consi-

TABLE 1: Comparison of histopathological scores.

Contrast	Mean Diff.	t	P value	95% CI
Control vs. resveratrol	-1.57	1.58	P > 0.05	-4.60 to 1.45
Control vs. theophylline	-3.00	3.01	P > 0.05	-6.02 to 0.02
Control vs. physiologic saline	-6.29	6.30	P < 0.001	-9.31 to -3.26
Control vs. dimethylsulfoxide	-5.29	5.30	P < 0.001	-8.31 to -2.26
Resveratrol vs. theophylline	-1.43	1.43	P > 0.05	-4.45 to 1.60
Resveratrol vs. physiologic saline	-4.71	4.72	P < 0.001	-7.74 to -1.69
Resveratrol vs. dimethylsulfoxide	-3.71	3.72	P < 0.01	-6.74 to -0.69
Theophylline vs. physiologic saline	-3.29	3.29	P < 0.05	-6.31 to -0.26
Theophylline vs. dimethylsulfoxide	-2.29	2.29	P > 0.05	-5.31 to 0.74
Physiologic saline vs. dimethylsulfoxide	1.00	1.00	P > 0.05	-2.02 to 4.02

TABLE 2: The histopathological score of each criterion and total score.

		Groups[1]				
Histopathological Categories	Grade	Control	Resveratrol	Theophylline	PS	DMSO
Dilatation of respiratory tract	0-3	0.6 ± 0.2	0.9 ± 0.1	1.3 ± 0.2	2.2 ± 0.2	2.1 ± 0.3
Infiltration of inflammatory cells	0-3	0.7 ± 0.1	1.1 ± 0.0	1.7 ± 0.2	2.7 ± 0.1	2.1 ± 0.4
Proliferation of respiratory epithelium	0-3	0.4 ± 0.1	0.9 ± 0.1	1.3 ± 0.2	2.5 ± 0.2	2.0 ± 0.5
Vascular congestion	0-1	0.3 ± 0.1	0.7 ± 0.0	0.7 ± 0.1	0.8 ± 0.1	1.0 ± 0.0
Total histopathological score	10	2.0 ± 0.5[a]	3.6 ± 0.2[b, c]	5.0 ± 0.7[d]	8.2 ± 0.6	7.2 ± 1.2

1PS = Physiologic Saline; DMSO = Dimethylsulfoxide

[a]p<0.001 control vs. physiological saline and dimethylsulfoxide; [b]p<0.001resveratrol vs. physiologic saline;
[c]p<0.01 resveratrol vs. dimethylsulfoxide; [d]p<0.05 theophylline vs. physiological saline.

dered as future promising agents at this point of view.[27-29]

These therapies also might have a role to modulate steroid activity in COPD. Theophylline has shown to restore steroid responsiveness via activation of HDAC in alveolar macrophages of COPD patients.[30] It is reasonable to expect similar modulator effect with resveratrol treatment. However, no clinical studies have investigating this effect of resveratrol yet. Clinical studies are needed to determine the exact role of resveratrol in COPD treatment and development of resveratrol analogues with high bioavailability are needed for future clinical studies.

In conclusion, COPD has been defined as an inflammatory disease recently; however an effective anti-inflammatory agent is needed to be identified. Since this study showed similar anti-inflammatory effects of theophylline and resveratrol, it is suggested that both medications might be used as alternative anti-inflammatory therapies in COPD patients either as principal agents or as modulators of steroid activity.

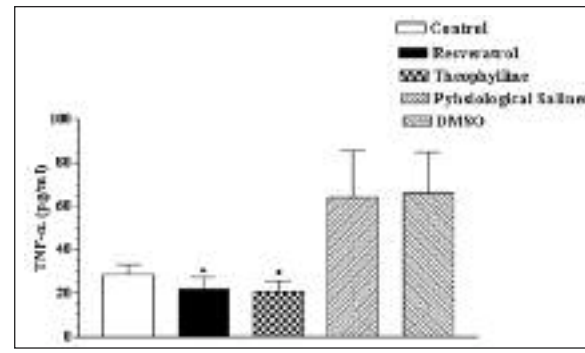

FIGURE 6: Serum TNF-alpha levels of the study groups. *p<0.05 Resveratrol and theophylline vs. physiological saline and DMSO.

TABLE 3: Comparison of serum TNF-α levels.

Contrast	Mean Diff.	t	P value	95% CI
Control vs. resveratrol	7.12	0.38	P > 0.05	-31.20 to 45.43
Control vs. theophylline	8.04	0.43	P > 0.05	-30.28 to 46.35
Control vs. physiologic saline	-35.02	1.87	P > 0.05	-73.34 to 3.29
Control vs. dimethylsulfoxide	-37.17	1.98	P > 0.05	-75.48 to 1.150
Resveratrol vs. theophylline	0.92	0.05	P > 0.05	-37.40 to 39.24
Resveratrol vs. physiologic saline	-42.14	2.25	P < 0.05	-80.46 to -3.85
Resveratrol vs. dimethylsulfoxide	-44.28	2.36	P < 0.05	-82.60 to -5.97
Theophylline vs. physiologic saline	-43.06	2.30	P < 0.05	-81.38 to -4.74
Theophylline vs. dimethylsulfoxide	-45.20	2.41	P < 0.05	-83.52 to -6.89
Physiologic saline vs. dimethylsulfoxide	-2.141	0.11	P > 0.05	-40.46 to 36.17

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