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Original Research Article

Evaluation of antioxidant and anti-inflammatory effects of red raspberries on chronic nicotine-induced damage: an experimental study

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ABSTRACT

Background: As an addictive substance, nicotine has been recognized as a risk factor to induce oxidative tissue damage, which is a main risk factor for development of lung-related diseases. Red raspberries (Rubus idaeus L.) have been used for the treatment of several tissue damage for decreasing oxidative stress and inflammation. For this purpose, authors aimed to investigate the antioxidant and anti-inflammatory effects of raspberry on nicotine-induced lung damage in rats.
Methods: 32 male Sprague–Dawley rats included in present study. The rats were divided into the following four groups, with eight rats in each group: control, raspberry 100 mg/kg, nicotine 5 mg/kg, nicotine + raspberry 100 mg/kg treated (concomitant nicotine and raspberry extract) groups. The assessment of lung structure was made on light and stereo investigator microscope, immunohistochemical analysis was performed by determining anti-Caspase-3 immunostaining.
Results: The lung of the nicotine applied group exhibited emphysematous air spaces, massive congestion areas, disrupted alveoli, thickened septa and inflammatory cell infiltration. Much improvement was observed in the raspberry-treated group despite the presence of mild residual changes. Moreover, loss of massive congestion areas and decreased Caspase-3 level were detected in the raspberry-treated group.
Conclusions: Our results indicate that the raspberry extract attenuate the pathological changes of nicotine in the lung rats through antioxidative and anti- inflammatory mechanisms.

Keywords: Lung injury, Nicotine, Oxidative stress, Raspberry extract

INTRODUCTION

Smoking is the most common method of consuming tobacco. Smoking cigarettes precipitates a major health risk. Cigarette smoking has been related with various diseases including lung, cardiovascular, and cancer. Nicotine has obtained special attention among over the 6000 molecular species present in the cigarette smoking which directly and indirectly affects cellular metabolism. The detrimental effects of nicotine have been extensively investigated in various experimental and clinical studies, consequently cardiovascular, respiratory, endocrine and reproductive system damage has been reported via this study.[1] Nicotine is absorbed through the epithelium of the lung and result in damage of the lung endothelial barrier. Dose-related harmful pulmonary effects of nicotine cause acute lung inflammation, a process involved in releasing of mediators, oxidant stress, and the mobilization of inflammatory cells into tissue, and reduced lung endothelial cell proliferation.[2,3] Studies reported the

development of emphysematous changes, pulmonary congestion and edema, hemorrhage into lung parenchyma due to use of nicotine.[4] Chronic nicotine exposure cause the chronic lung disorders as well as chronic obstructive pulmonary disease and lung cancer.[5,6]

The mechanism of nicotine which is associated with lung damages is not completely described. Dose-dependent negative effects of nicotine inhalation is the induction of inflammation, a process characterized by the release of soluble mediators, oxidative stress. As 70-80% of nicotine is converted to cotinine in the liver, nicotine induced cytokines production reported in liver injury.[6] Due to presence of nicotinic receptors in lung tissue inflammatory processes involve production of a variety of cytokines.[3] This promotes tissue remodeling, including alterations in lung structure and function. Nicotine disrupts the antioxidant defense mechanisms and also induces oxidative stress by increasing the level of lipid peroxidation (LPO). Changing balance between antioxidant capacity and oxidative stress cause production of reactive oxygen species (ROS) and reactive nitrogen species (RNS).[7] Chronic nicotine administration decreas levels of glutathione (GSH) and superoxide dismutase (SOD) and this evidence points to the involvement of oxidative stress.[8] Nicotine generates intracellular oxidative stress and chronic oxidative stress has been related to lung diseases. Under oxidative stress conditions, pulmonary epithelial cells increase their antioxidant defense mechanisms, produce and release free radical scavengers.

Berries are a rich in phenolic compounds (phenolic acids, flavonoids, such as anthocyanins and flavonols, and tannins) and ascorbic acid.[9] Red raspberries (Rubus idaeus L.) belong to family Rosaceae and contain the best dietary sources of bioactive compounds.[10] Plants such as berries rich in anthocyanins have been shown to exhibit anti-inflammatory, antioxidant and antitumor activities. Raspberries possess high antioxidant capacity due to significant amoun of anthocyanins and ascorbic acid.[11,12] Phenolic compounds belong to a wide and heterogeneous group of chemical components that possess one or more aromatic rings and tend to donate an electron or a hydrogen atom to a free radical. So, raspberries have relevant in vitro and in vivo antioxidant activities due to can scavenge free radicals.[13] Antioxidants serve to counterbalance the effect of oxidants and protect the body from cellular damage.[14] Recent studies demonstrated that raspberry supplementation decreased oxidative stress and inflammation, and protected against cardiovascular and renal damage.[15,16] Studies identified that inflammation decreased in response to raspberry treatment after ~1 mo.[17] Red raspberry extract significantly reduced inflammation, bone resorption, soft tissue swelling, and osteophyte formation in collagen-induced arthritis rat model.[18]

In a gastritis model, researches confirm decreased measures of inflammation and increased endogenous antioxidant defenses enzymes in animals treated with raspberries extract.[19] Therefore, Authors investigated whether intake raspberries extract affects the nicotine induced lung damage in an animal model and antioxidan, anti-inflammatory effects of raspberry evaluated by histopathological, immunohistochemical and stereological analyses.

METHODS

Sprague-Dawley male rats about 245-260g weight, 8-12 week-old were purchased from Ataturk University Animal Care and Research Unit. Before carrying out the experiment thirty-two adult male albino rats were acclimatized in the laboratory for a period of two weeks. The sample size was based according to the resource equation method.[20, 21] The rats were kept in cages with a temperature around (23°±2°) with 12 hours light and 12 hours dark cycle. All animals received humane care according to the criteria National Institutes of Health guide for the care and use of Laboratory Animals. The study was approved by Ataturk University Animal Ethical Committee (ID: 2019/1-10). The rats were observed daily for signs of morbidity and mortality.

Study design

Rats were allowed access standard rat chow (Bayramoglu Feed and Flour Industry Trading Corporation Erzurum, Turkey) and tap water *ad libitum* along the experiment. At the beginning of the study all animals' (n= 32), age was approximately 2-3 months, were divided randomly into four groups (Table 1).

Table 1: Experimental study design.

Group	Rat numbers
Control	8
Raspberry 100 mg/kg	8
Nicotine 5 mg/kg	8
Nicotine+Raspberry 100 mg treated	8

Group I as control group (n=8) didn't received any drugs except the drugs used for anesthesia. Group 2 was determined as raspberry control group (n=8). Raspberry extract dissolved in the ethanol and was given intraperitoneally once/day in a dose of 100 mg/kg for 4 weeks.[22,23] Group 3 was determined as nicotine control group (n=8).

This group was injected with nicotine hydrogen bitartrate subcutaneously at a dose of 5 mg/kg body weight daily for 4 weeks.[24] Group 4 was determined as nicoitne 5mg/kg + raspberry 100mg/kg treated group (n=8). This group received daily subcutaneous injections of nicotine 5 mg/kg and intraperitoneal raspberry extract were administered following the nicotine injection for 4 weeks. At the end of the experiment, all rats were anesthetized and sacrificed after 4 weeks.

Chemical

Nicotine hydrogen bitartrate (Sigma Aldrich Co., St. Louis, Mo) was dissolved in 0.9% saline and neutralized to PH 7.2 with NaOH. The treatment was given at a dose of 5 mg/kg subcutaneously once daily for 5 days per week for 4 weeks.[2,4,24] The selection of time schedule was based on previous studies which indicate nicotine injection leads to oxidative stress and tissue injury.[25,26]

Preparation of the raspberry extract

Raspberry extract ([Rubus idaeus L.] Mc Cormick, USA) was used as a nutritional support product. The product was diluted in sterile conditions with a mix of half distilled water and half ethanol to achieve a raspberry concentration of 50 mg/ml.

Histopathological analysis

The lungs were dissected and fixed in 10% neutral formaldehyde for histological evaluation. To randomize selection, the entire lung was cut starting at the superior border. After the fixation, specimens were dehydrated a graded alcohol series, cleared in xylene, embedded in paraffin and were cut 5 µm thick using a microtome (Leica RM2125RT, Germany). Preparations of sections were stained with H&E and analyzed under a light microscope with a camera attachment (Olympus microscope (BX41) with DP72 camera attachment). The findings assessed by a two blinded histologist.

Immunohistochemistry (IHC) analysis

A Caspase-3 immune marker was used to indicate the oxidative activity in lung tissue. Lung tissue blocks were fixed in 3.7% formaldehyde and were subsequently embedded in paraffin. 1-3 µm sections were collected on poly-L lysine-coated slides. Primary antibodies Caspase-3 (1:100, rabbit anti-caspase 3 polyclonal antibody, ab4051; Abcam) used in a 1:80 dilution. The sections were loaded onto an Automatic IHC staining machine (Ventana Benchmark GX, USA). The secondary antibody was applied (UltraView Universal DAB Detection kit, 760–500; Ventana Medical Systems, Inc., Tucson, AZ, USA) and the counterstain performed by using hematoxylin (Ventana Medical Systems, Inc, Cat No: 5266726). Evaluation by light microscopy was carried out and photos were taken as described above.

Semi-quantitative analysis

For a quantitative estimate of the intensity of the immune reactive cell, for each section 5 micro-scopic areas were randomly selected. The arithmetic mean of the histopathological evaluation was scored semi-quantitatively. The scores were derived semi-quantitatively using light microscopy on the preparations from each animal and were reported as follows: none (0), mild (1), medium (2), severe (3), and extremely severe (4).

Stereological analysis

The mean Caspase 3 positive cell numerical density was calculated using the optic fractionator frame method via the stereo investigator system ((Stereoinvestigator 8; MBF Bioscience, Williston, VT, USA)). Measurement area was sampled systematically and randomly over the fractionator probe. Slides were examined without any randomising in their orientation and determinations of immune positive cells were applied as described previously.[27,28] The unbiased counting frame-fractionator combination is a stereological method for counting cells in tissue sections. Cells were counted using a 40x Apo objective for accurate recognition (Figure 1). The mean numerical density was estimated according to the formula given below:

$$Nv = Q/Sx\ A$$

where Nv is numerical density, Q is total markers counted, S is no. of sampling sides and A is counting frame area.

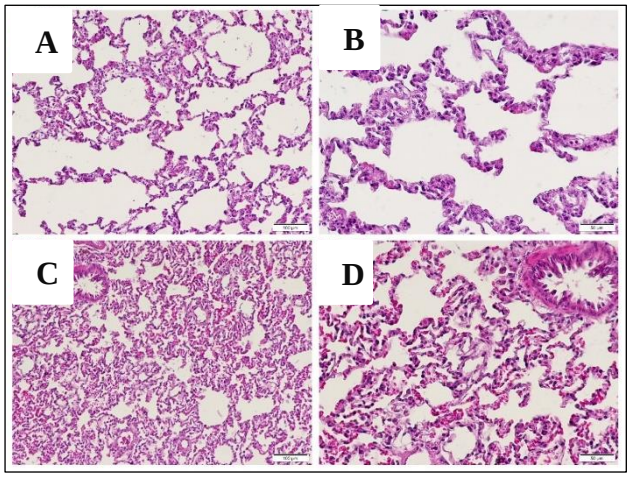

Figure 1: Light microscopic photomicrograph from lung tissue sections stained with H&E. (A-B) Control group, (C-D) Raspberry 100mg/kg group.

Statistical analysis

Statistical analysis was performed using SPSS Statistics 20.0 software (IBM Inc., Chicago, IL). Caspase 3 positive cell numerical density were expressed as means±standard deviation (SD) and analyses were performed using differences between the groups were tested using post hoc Tukey's Honest Significant Difference test (Tukey HSD test). Significance was considered when the p-value was less than 0.05.

RESULTS

Histopathological results

Light microscopy results

Lung sections of the control group revealed normal histological organization (Figure 1A, B). Last segments of respiratory portion as well as alveolar ducts, alveolar sacs and alveloli observed normally. Raspberry 100mg/kg group had normal histological structure and no pathology was detected (Figure 1C, D). Type 1 pneumocytes and type 1 pneumocytes lined the epithelium of alveoli and the alveolar septum between adjacent alveoli exhibit normal connective tissue structure that contain blood capillaries. However, emphysematous air spaces, massive congestion areas with extravasation of RBCs, which indicate increase in vascular permeability, were determined in nicotine 5mg/kg group (Figure 2A, B). In addition, disrupted alveoli, thickened septa and inflammatory cell infiltration were detected in this group along with swollen epithelial cells. The prominent finding of this group was the presence of foamy macrophages surrounding the alveoli (Figure 2A, B). In nicotine + rasberry 100mg/kg treated group histological findings were improved and the mild changes were observed in architecture of lung. Massive congestion areas were absent, thickening of alveolar septum and inflammatory cell infiltration were decreased (Figure 2C, D).

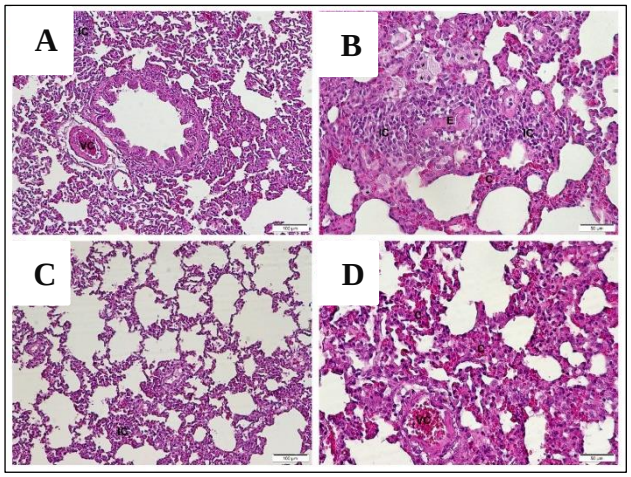

Figure 2: Light microscopic photomicrograph from lung tissue sections stained with H&E. (A-B) Nicotine 5mg/kg group, (C-D) Nicotine + Raspberry 100mg/kg treated group; (asterisk) : Foamy macrophages, E: Edema, IC: Inflammatory cells infiltration, C: Alveolar congestion, VC: Vascular congestion

Table 2: Grading of the histopathological changes in lung sections of groups.

Findings	Groups			
	Control	Raspberry	Nicotine	Nicotine+Raspberry
Normal structure of lung	++++	++++	-	-
Congested alveolar capillaries	-	-	++++	++
Congestion of the alveolar area	-	-	++++	++
Inflammatory cells infiltration	-	-	++++	++
Thickened alveolar wall	-	-	++++	++

- Absence of the change in the studied group, ++ A change not so often observed in animals of studied group, ++++ A change that often found in animals of studied group

Although massive foamy macrophages disappear in alveolar tissue, occasionally plasma cells were found in examination of nicotine + rasberry 100mg/kg treated group sections (Figure 2C, D). The histopathological changes in lungs are graded and summarized in Table 2.

Immunohistochemical (IHC) results

A Caspase-3 portein was used as the oxidative stress marker. The intensity of the immunoreaction was scored as follows: none (0), mild (1), medium (2), severe (3), and extremely severe (4).

Furthermore, mild Caspase-3 immunoposivity was detected in the control and raspberry groups (Figure 3A,B). Severe immunoposivity was detected in the nicotine group. (Figure 3C). Caspase-3 immunoposivity was apparently reduced in nicotine + raspberry 100mg/kg treated group and scored as medium (Figure 3D). The numerical density of the Caspase-3 positive cells in alveolar tissues of the nicotine 5mg/kg group were higher compared to the control group ($p<.05$) (Table 3).

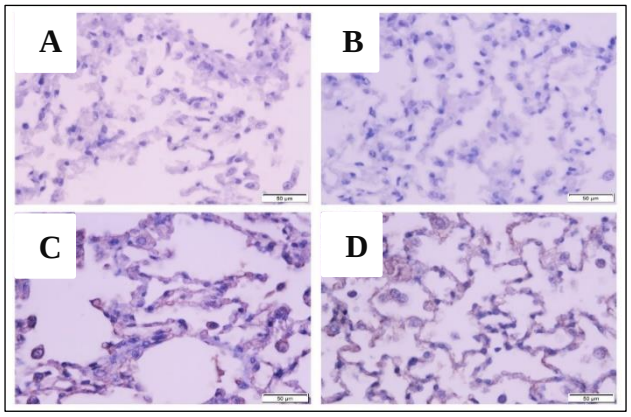

Figure 3: Light microscopic photomicrograph from lung tissue sections stained with Caspase-3. (A) Control group, (B) Raspberry 100mg/kg group, (C) Nicotine 5mg/kg, (D) Nicotine + Raspberry 100mg/kg treated group.

Nicotine + raspberry 100mg-treated group showed less Caspase-3-positive cell numerical density than the nicotine group (p<.05) (Table 3). Conversely, statistically significant differences of Caspase-3-positive cell numerical density were not found between control and raspberry 100mg/kg groups (p>.05). (Table 3).

Table 3: Caspase 3 positive numerical density measurement (mm3) data.

Groups	+(mild)	++(medium)	+++(severe)
Control	287	272	15
Raspberry 100mg/kg	306	288	18
Nicotine 5mg/kg	348	245	103**
Nicotine+ Raspberry 100mg/kg	339	289	43*

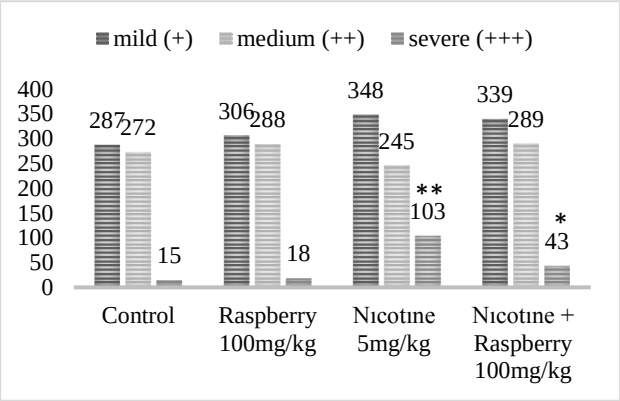

Figure 4: The numerical density of the Caspase-3 positive cells.

DISCUSSION

Cigarette smoking is considered a risk factor for lung disease chronic obstructive pulmonary disease and lung cancer.[29] It has been also associated in the pathogenesis of respiratory bronchiolitis, interstitial lung disease, idiopathic pulmonary fibrosis. Several studies demonstrated that smoking promotes lung tissue remodelling through oxidant stress, inflammation. Chronic nicotine administration has a deleterious effect on the respiratory system. Nicotine administration lead alveolar damage, congestion, edema that result increased vascular permeability. It stimulates the production and release of TGF-β1, enhances the recruitment of inflammatory cells and the production of reactive oxygen species[30]. Similar results were found histopathologically in our study. There was widespread emphysematous air spaces, collapsed alveoli with destructed interalveolar septa, loss of the alveolar epithelial cells in nicotine 5mg/kg group. Also, in the literature, several studies reported deleterious effect of nicotine administration on the lung tissue.[2,24] Gawish et al. reported many alterations in the pulmonary structures can be caused by chronic nicotine administration as thickening of interalveolar septa, destruction of alveoli wall and formation of enlarged, irregular air space.[31] In present study pulmonary tissue injury findings such as architecture changes, inflammatory cell infiltration, increase in the thickening of alveolar septa in nicotine 5mg/kg group are in agreement with El-Sokkary et al. which indicate the detrimental effects of nicotine. Additionally, nicotine induce matrix degrading proteases.[6,32] Several animal studies confirmed overexpression of matrix metalloproteinase (MMP)-1 that promotes the development of emphysema in transgenic mice.[33] In present study, large emphysematous spaces were observed in group with nicotine administration. This finding explain stimulation of extracellular matrix degradation via nicotine. Moreover, nicotine administration stimulates the production of collagen, which is the major constituent of the extracellular cell matrix. Recent study reported expression of fibronectin in lung by nicotine that stimulates lung fibroblasts to release matrix components via alpha-7 nicotinic receptors (α7 nAChRs).[32] Collagen type I is highly expressed in injured lungs as well as chronic fibrotic lung disorders.[34] Specifically, morphological airway abnormalities were detected in nicotine-treated wild type animals, which support the destruction and thickening of the interalveolar septa in nicotine control group of present study.[35] Additionally authors have observed an increment in thickening of the interalveolar septa and extensive destruction of alveolar walls in nicotine control group. Collagen deposition in lung affect the organ ”self-limiting tissue remodeling” as well as nicotine-treated fibroblasts activate monocytic cells for expression of the pro-inflammatory cytokines.[32] Increased levels of cytokines and adhesion molecules resulting in the activation of neutrophils and macrophages can lead to tissue destruction.[36] It was suggested that nicotine has the capacity to activate resident macrophages in addition to recruiting circulating macrophages.[37] In line with the findings of the mentioned study authors have find considerable amount of foamy macrophages surrounding the alveoli in nicotine treated group of present study.

Nicotine also trigger inflammatory cascade in the respiratory epithelium.[38] Inflammation is a key mechanism contributing to nicotine-induced lung injury. The inflammatory process in the lungs is characterized by the production of a variety of cytokines. Proinflammatory cytokines secreted from activated neutrophils, monocytes and macrophages of nicotine applied animals promote tissue remodelling.[39] In the present study, the immunoreactivity of Caspase-3 protein increased in nicotine group as compared to control animals which indicated DNA damage as the effect of nicotine. Nicotine induce oxidative stress abnormalities throughout the generation of ROS capable of initiating and promoting cellular oxidative damage leading the development of DNA damage. Recent study reported that nicotine administration induced oxidative stress in lung tissue by

increasing the level of ROS and RNS, while decreased the antioxidant defense mechanisms.[39] Excessive production of ROS in the nicotine applied group results in oxidative stress. Oxidative stress is described as the balance between oxidants and antioxidants in the favour of oxidants.[40] The balance between oxidant species and removal of oxidants by the antioxidant enzymes determines the ROS-mediated apoptosis signalling. The chronic exposure to nicotine with high concentrations (5 mg/kg) were caused oxidative stress.[26] Previous studies reported that oxidative stress was responsible for injury in lung tissue due to nicotine.[4] Caspase 3 responsible for regulating apoptosis and plays a key role in cell death. Elevation of Caspase-3 is an indicator of cellular damages and following apoptosis.[41] Our immunohistochemistry results showed increased protein expression levels of Caspase-3 in nicotine control group, while its expression was suppressed in the nicotine+ raspberry-treated group. Treatment with raspberry along with nicotine remarkably decreased the Caspase-3 protein expression levels suggesting that raspberry could effectively protect the lung by reducing oxidative stress.

The red raspberry (Rubus idaeus L.) is a unique berry contain a complex mixture of antioxidants such as polyphenols, carotenoids and xanthophylls, vitamin C. Raspberries possess a unique polyphenol profile due their anthocyanin and ellagitannin content. It has been proposed that anthocyanins can act as prooxidants that change cellular redox status.[42] Individual polyphenols such as anthocyanins, ellagitannins have been shown effective in preventing the proliferation of human cervical and colon cancer cells in vitro.[43,43] Raspberry ellagitannin fraction exhibited good radical scavenging activity.[45] Several in vitro cell culture, chemical assay and enzyme activity studies, animal models data indicate antioxidative and anti-inflammatory activity of red raspberry extracts/fractions and purified compounds. Recent animal studies showed decreased oxidative stress and decreased inflammation in response to raspberry treatment.[46] In rat arthritis model of inflammation, red raspberry extract significantly reduced the development of clinical signs of arthritis, soft tissue swelling.[18] Our study is in consistence with Ding at al. who reported that raspberry extracts, particularly in response to ellagitannin-rich fractions decreased oxidative stress and inflammation in cardiac or vessel tissue.[47] In the present study, histopathologic assesment revealed that performing raspberry extract 100mg/kg prevent the deterioration of the alveoli, alveolar epithelial cells from the harmful effects of nicotine. Thickened alveolar septa and inflammatory cell infiltration were improved in nicotine+raspberry 100mg/kg treated group. The protective effect of raspberry extract could be attributed to its high content of antioxidants. Our study results support the raspberry antioxidant content.

In summary, chronic nicotine exposure in rats result in pulmonary inflammation and tissue remodelling that were determined in the present study. The administration of raspberry extract followed by nicotine attenuate the pathological lung injury through antioxidative and anti-inflammatory mechanisms. As raspberry easy to obtain and low cost may be a beneficial supplement for smokers after performing randomized controlled trials to determine the antioxidant and anti-inflammatory effects of raspberries in humans.

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