

## Pulmonary prophylactic impact of melatonin and/or quercetin: A novel therapy for inflammatory hypoxic stress in rats

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The study aims to compare, through histological and biochemical studies, the effects of quercetin, melatonin and their combination in regulation of immuno-inflammatory mediators and heat shock protein expressions in sodium nitrite induced hypoxia in rat lungs. The results revealed that NaNO<sub>2</sub> injection caused a significant decrease in Hb in rats, while serum levels of TNF- $\alpha$ , IL-6 and CRP, VEGF and HSP70 were elevated compared to the control group. Administration of melatonin, quercetin or their combination before NaNO<sub>2</sub> injection markedly reduced these parameters. Histopathological examination of the lung tissue supported these biochemical findings. The study suggests that melatonin and/or quercetin are responsible for lung tissue protection in hypoxia by downregulation of immuno-inflammatory mediators and heat shock protein expressions. Pre-treatment of hypoxic animals with a combination of melatonin and quercetin was effective in modulating most of the studied parameters to near-normal levels.

*Keywords:* hypoxia, melatonin, quercetin, inflammatory mediators, heat shock protein

Exposure to hypoxia favors the increase of reactive oxygen species and modifies the levels of antioxidant substances, causing pulmonary oxidative damage, as well as establishing an inflammatory process (1). Imbalance of the redox state in lungs induced by hypoxia has been suggested to be a participant in the changes observed in lung function (1, 2). Hypoxia has an important role in triggering the vascular endothelial growth factor (VEGF), which is a signal protein produced by alveolar macrophages and other cell types. This factor stimulates vasculogenesis and angiogenesis to restore oxygen supply to tissues during hypoxia (3). However, VEGF has a deleterious role in pulmonary edema formation by increasing vascular permeability during hypoxia (4).

Oxygen homeostasis is maintained through a family of hypoxia-inducible transcription factors (HIFs), composed of a constitutively expressed HIF-1 $\beta$  subunit and a HIF- $\alpha$

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subunit (HIF-1 $\alpha$  and HIF-2 $\alpha$ ) that is tightly regulated by tissue oxygen levels (4). Abnormal activation or deregulation of hypoxia-induced transcriptional pathways may contribute to the pathogenesis of various conditions. Hypoxia, *via* the transcription factor, hypoxia-inducible factor HIF-1 $\alpha$ , induces a large number of genes encoding proteins, which participate in inflammation (5). A cross-talk between hypoxic and non-hypoxic signaling pathways, including pro-inflammatory cytokines, such as tumor necrosis factor- $\alpha$  and interleukin-1 $\beta$ , may further amplify inflammatory responses by activating HIF as well as other oxygen-sensitive transcription factors, for example, nuclear factor- $\kappa$ B (NF- $\kappa$ B) (6). Expression of heat shock proteins (HSPs) is another mechanism involved in inflammatory reactions during hypoxia. Heat shock proteins (HSPs) are a group of stress intracellular proteins whose expression increases in response to hypoxic stress which function as cytoprotectants by inhibiting various inflammatory mediators (7). However, cell damage may evoke an early release of these proteins outside the cell (8). Extracellular HSP acts as a danger signal to innate immunity and can be recognized by antigen presenting cells (APC) and their interaction subsequently triggers APC to produce the proinflammatory cytokines and activate the nuclear factor (NF)- $\kappa$ B, thus initiating the adaptive immune response and the presentation of antigens to the cytotoxic T cells (9). Thus, agents with the ability to suppress the expression of inflammatory molecules may be beneficial in reducing the risk of tissue injury and organ dysfunction during hypoxia.

Melatonin (*N*-acetyl-5-methoxytryptamine), which is secreted in the circulation from the pineal gland, is produced in small amounts in the retina, the gastrointestinal system and by leukocytes. It has protective effects against cellular injuries through its potent antioxidant and anti-inflammatory properties at both physiological and pharmacological concentrations (10). Melatonin possesses anti-angiogenic activity by inhibiting stabilization of HIF-1 $\alpha$  in HCT 116 cell lines (11). Also, it has been shown that melatonin could invert the upregulation in HSP70 gene expression in human epidermis exposed to ultraviolet radiation (12).

Quercetin is a flavonoid present in many vegetables, fruits, and beverages; it possesses a broad range of pharmacological properties, including anti-inflammatory, anti-proliferative and anti-oxidative (13). A previous study demonstrated that quercetin can elicit protective effects in different lung injury models (14, 31). At the molecular level, quercetin inhibits pulmonary oxidant stress in lung epithelial cells through inducing heme oxygenase-1 (HO-1) and *via* modulating the expression of antioxidant genes (14).

Although both melatonin and quercetin show different pharmacological mechanisms in modulating several pathological conditions, their modulating mechanisms for the expression of different types of inflammatory proteins causing lung tissue damage during hypoxia, particularly when used in combination, are still unclear. The present study aims to explore the effects of quercetin, melatonin or their combination in regulation of inflammatory protein expressions including VEGF, immuno-inflammatory mediators and HSP70 in hypoxic lung induced by sodium nitrite in rats.

## EXPERIMENTAL

### Chemicals

All chemicals used in the study were of high analytical grade, products of Sigma-Aldrich (USA) and Merck (Germany).

### *Animals and treatments*

Fifty adult male Wistar albino rats weighing 170–200 g were used in the study. Rats were obtained from the Experimental Animal House, Faculty of Pharmacy, King Saud University, Saudi Arabia. Animals were housed in special clean cages and maintained under standard conditions (12-h light/dark cycle, temperature 20–22 °C and 60 % humidity). For acclimatization, rats were fed the standard rat pellet chow with free access to tap water *ad libitum* for one week before the experiment. The animal experimental protocol was approved by the Experimental Animal Ethics Committee of the King Saud University, Faculty of Pharmacy (Riyadh, Saudi Arabia). After one week of acclimatization, the rats were randomly divided into five groups of ten rats each. Group 1 served as the control, group 2 – NaNO<sub>2</sub>-treated animals, group 3 – NaNO<sub>2</sub>-treated animals injected with melatonin (200 mg kg<sup>-1</sup>, *i.p.*), group 4 – NaNO<sub>2</sub>-treated animals injected with quercetin (200 mg kg<sup>-1</sup>, *i.p.*), group 5 – NaNO<sub>2</sub>-treated animals injected with a combination of melatonin (200 mg kg<sup>-1</sup>, *i.p.*) and quercetin (200 mg kg<sup>-1</sup>, *i.p.*). NaNO<sub>2</sub> (60 mg kg<sup>-1</sup>) was administered as a single dose subcutaneously. Melatonin and quercetin were administered 24 hours before NaNO<sub>2</sub> injection. One hour after NaNO<sub>2</sub> injection, the rats were sacrificed under ether anesthesia; blood samples were collected and divided into two parts. One part was collected into tubes containing heparin for hemoglobin determination. The other part was allowed to coagulate and was centrifuged for serum separation and used for serum biochemical analysis. After blood collection, the lungs were removed and placed in phosphate-buffered 10 % formalin overnight for fixation and histopathological examination.

### *Determination of Hb*

Hb was determined colorimetrically using Drabkin's reagent according to the method after Kjeldsberg (15).

### *Biochemical serum analyses*

Interleukin-6 (IL-6) was measured using a highly sensitive rat enzyme-linked immunosorbent assay (ELISA) kit (IBL International GmbH, Germany) following the manufacturer's instructions. Tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) was measured using the ELISA assay kit following instructions supplied by the manufacturer (DuoSet kits, R&D Systems, USA). C-reactive protein (CRP) was estimated using the immunonephelometric assay (Dade Behring N Latex High Sensitivity CRPTM mono assay) on a Behring Nephelometer II analyzer (Dade Behring Diagnostics Inc., USA). The level of VEGF was assayed by colorimetric, quantitative, sandwich ELISA (R&D Systems, UK) at 492 nm, following the manufacturer's instructions. A sandwich rat HSP70 ELISA kit was used for quantitative determination of HSP70 (Kamiya Biomedical, USA).

### *Histopathological examination*

Small pieces of the lung were fixed in 10 % buffered formalin at 4 °C for 24 hours and processed to prepare 5- $\mu$ m thick paraffin sections. These sections were stained with hematoxylin and eosin (H&E) to detect pathological changes due to hypoxia and the effect of certain drugs on these changes.

### Statistical analysis

Data were analyzed by comparing the values for different treatment groups with the values of individual controls. Results were expressed as mean  $\pm$  SD. Significant differences among the values were analyzed using the one-way analysis of variance (ANOVA) followed by Bonferroni's test post-ANOVA.

## RESULTS AND DISCUSSION

Fig. 1 shows the effect of melatonin and/or quercetin on blood Hb concentration in the control and NaNO<sub>2</sub>-induced hypoxia in experimental groups. In line with previous studies (16), the present work demonstrated that induction of hypoxia in rats by NaNO<sub>2</sub> caused a decrease in blood Hb level (57.9 %) compared to normal animals ( $p \leq 0.001$ ). This result may indicate the development of anemia in rats in response to hypoxia. The decrease in Hb level may be attributed to a decrease in the red blood cell count in response to NaNO<sub>2</sub>. A previous preliminary study reported a significantly reduced red blood cell count in rats exposed to NaNO<sub>2</sub> toxicity (13). Nitrite in toxic doses has been reported to induce hemic hypoxia. The underlying mechanism of such effect is mainly due to increased methemoglobin formation (17). Some authors have reported that NaNO<sub>2</sub> can induce oxidative damage through free radical generation, which stimulates the oxidation of ferrous ions in oxyhemoglobin to form methemoglobin and erythrocyte lysis (18). We have found that administration of melatonin and/or quercetin to hypoxic rats significantly ameliorated the reduced level of blood Hb compared to hypoxic untreated animals ( $p \leq 0.001$ ). In comparison with the control, the changes were 23.6, 14.3 and 13.2 %, respectively. RBCs are highly exposed to oxygen and can be a site for radical formation under pathological conditions, resulting in their destruction. In this study, the administration of melatonin alone showed significant

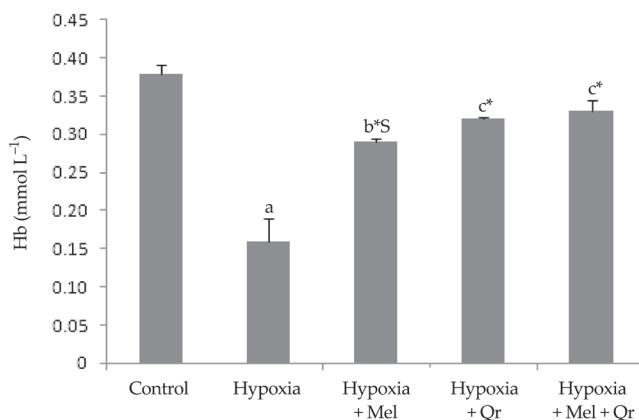


Fig. 1. Effect of melatonin (Mel) and/or quercetin (Qr) on blood hemoglobin (Hb) in the control and hypoxia treated groups. Data are expressed as mean  $\pm$  SD ( $n = 10$ ). Significant difference compared to the control group: <sup>a</sup> $p \leq 0.001$ , <sup>b</sup> $p \leq 0.01$ , <sup>c</sup> $p \leq 0.05$ ; compared to the hypoxia group: \* $p \leq 0.001$ , compared to the combination group (hypoxia + Mel + Qr): <sup>s</sup> $p \leq 0.05$ , using ANOVA, followed by Bonferroni as a post-ANOVA test.

improvement in the Hb levels compared to hypoxic untreated rats ( $p \leq 0.001$ ), which is in agreement with a previous study that reported that melatonin leads to a significant increase in the hemoglobin level and erythrocyte count in healthy humans (19). Melatonin is actively taken up into erythrocytes under oxidative stress and is consumed in the defense of the cell, delaying Hb denaturation and release of hemin (20). Also, the protective effect of quercetin may rely on its antioxidant and anti-inflammatory properties. A previous study reported that quercetin could prevent the decrease in nuclear translocation of Nrf2, a key regulator of the antioxidant response, and the increase in reactive oxygen species levels by inhibiting enhancement of NADPH oxidase and xanthine oxidase activities (21).

Among the genes regulated *via* the HIF-1 $\alpha$  pathway under hypoxic conditions are the highly conserved HSPs, which act as cellular chaperones for proteins misfolded by cellular stress (22). In this study, HSP70 was significantly higher in the hypoxia induced group compared to the control (Fig. 2,  $p \leq 0.001$ ) with a percentage change of 83.1 %. This finding was in agreement with the recent study of Tsuchida *et al.* (23), who suggested that under hypoxia the HIF-1 $\alpha$ -induced HSP70 overexpression in chondrocytes enhanced the metabolic activity of chondrocytes and chondroprotective effects.

Administration of melatonin and/or quercetin before NaNO<sub>2</sub> injection markedly reduced the dramatic increase in HSP70 levels compared to NaNO<sub>2</sub> untreated animals ( $p \leq 0.001$ ). Percentage changes from normal were 20.8, 22.6 and 9.4 %, respectively. Pretreatment of NaNO<sub>2</sub> hypoxic animals with the combination of melatonin and quercetin was more effective in modulating most of the studied parameters to near-normal levels than each agent individually. Moreover, it has been reported that melatonin effectively prevented acidification of cytosol, causing significant downregulation of HSP70 in melatonin-incubated skin samples (24).

In line with the previous study (3), the vascular endothelial growth factor (VEGF) was significantly increased after hypoxia (32.9 % change from normal) and administration of melatonin and/or quercetin ameliorated the hypoxic effect (Fig. 3,  $p \leq 0.001$ ) by 8.3, 8.3 and 6.9 % from normal, respectively. This may be due to the activation of HIF-1 in hypoxia,

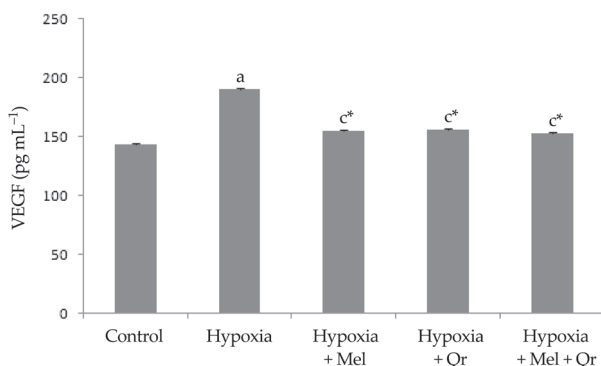


Fig. 2. Effect of melatonin (Mel) and/or quercetin (Qr) on serum VEGF in the control and hypoxia treated groups. Data are expressed as mean  $\pm$  SD ( $n = 10$ ). Significant difference compared to the control group: <sup>a</sup>  $p \leq 0.001$ , <sup>c</sup>  $p \leq 0.05$ ; compared to the hypoxia group: <sup>\*</sup>  $p \leq 0.001$ ; compared to the combination group (hypoxia + Mel + Qr): <sup>§</sup>  $p \leq 0.05$ , using ANOVA, followed by Bonferroni as a post-ANOVA test.

resulting in increased expression of a number of genes encoding proteins such as erythropoietin (EPO), vascular endothelial growth factor (VEGF) and inducible nitric oxide synthase (iNOS), which increase tissue oxygenation. These factors allow adaptation to hypoxia, which is directed toward increasing tissue perfusion and oxygenation and hence overcoming the initial hypoxic insult. Melatonin suppressed HIF-1 $\alpha$  transcriptional activity, leading to reduction in different angiogenic processes such as VEGF expression (20).

In the present study, quercetin inhibited hypoxia-induced VEGF, which was in accord with the study of Anso *et al.* (25). When cells were exposed to hypoxia in the presence of this flavonoid, HIF-1 $\alpha$  translocated to the nucleus and interacted with p300/CBP, but this complex was transcriptionally inactive. Taken together, these findings indicate that flavonoids impair VEGF transcription by an alternative mechanism that does not depend on nuclear HIF levels (26).

Furthermore, the current study revealed that rats exposed to hypoxia exhibited higher circulating levels of TNF- $\alpha$ , IL-6 and CRP compared to the controls (Figs. 3, 4 and 5, resp.,  $p \leq 0.001$ ). Deviations in the levels of these inflammatory markers from the normal level were 74.0, 57.4 and 100.0 %, respectively. These results have shown that the release of pro-inflammatory cytokines, which are the systemic inflammatory markers, is associated with hypoxia (6). Parts of our findings are consistent with other reports of increased TNF- $\alpha$  and IL-6 levels in rats subjected to hypoxia (6). When exposed to hypoxia, NF- $\kappa$ B is activated and stimulates increased production of circulating cytokines such as TNF- $\alpha$  and IL-6, which, in turn, activates signaling cascades to enhance NF- $\kappa$ B activation. Positive regulation contributes to the amplification of inflammatory responses (6).

Pre-injection of the studied agents, alone or in combination, to rats before induction of hypoxia, successfully attenuated hypoxia-induced stimulation of the inflammatory immunologic biomarkers, TNF- $\alpha$ , IL-6 and CRP. This beneficial impact of the two agents may be related to their beneficial anti-inflammatory and immunomodulatory actions. This result is confirmed by previous investigations, reporting that supplementation of either quercetin or melatonin could protect against the inflammatory responses caused by dif-

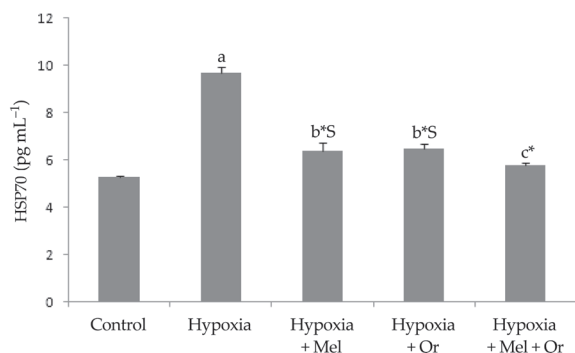


Fig. 3. Effect of melatonin (Mel) and/or quercetin (Qr) on serum HSP70 in the control and hypoxia treated groups. Data are expressed as mean  $\pm$  SD ( $n = 10$ ). Significant difference compared to the control group: <sup>a</sup>  $p \leq 0.001$ , <sup>b</sup>  $p \leq 0.01$ , <sup>c</sup>  $p \leq 0.05$ ; compared to the hypoxia group: \*  $p \leq 0.001$ ; compared to the combination group (hypoxia + Mel + Qr): <sup>s</sup>  $p \leq 0.05$ , using ANOVA, followed by Bonferroni as a post-ANOVA test.

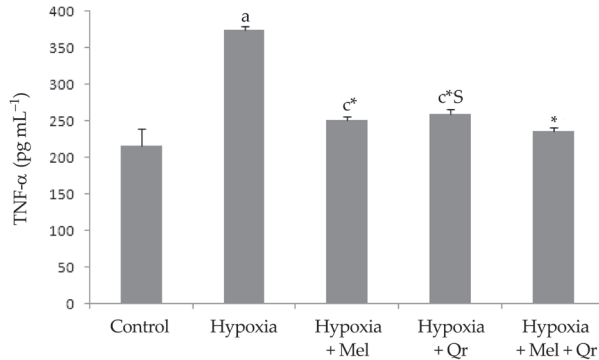


Fig. 4. Effect of melatonin (Mel) and/or quercetin (Qr) on serum TNF- $\alpha$  in the control and hypoxia treated groups. Data are expressed as mean  $\pm$  SD ( $n = 10$ ). Significant difference compared to the control group: <sup>a</sup>  $p \leq 0.001$ , <sup>c</sup>  $p \leq 0.05$ ; compared to the hypoxia group: \*  $p \leq 0.001$ ; compared to the combination group (hypoxia + Mel + Qr): <sup>s</sup>  $p \leq 0.05$ , using ANOVA, followed by Bonferroni as a post-ANOVA test.

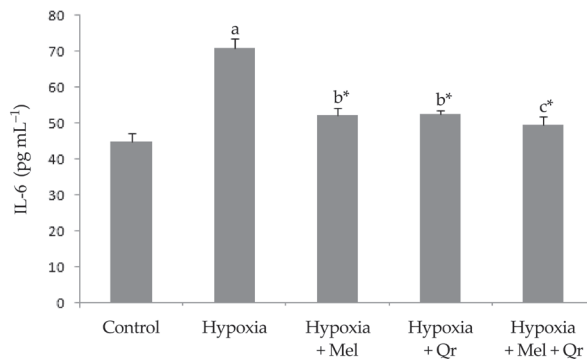


Fig. 5. Effect of melatonin (Mel) and/or quercetin (Qr) on serum IL-6 in the control and hypoxia treated groups. Data are expressed as mean  $\pm$  SD ( $n = 10$ ). Significant difference compared to the control group: <sup>a</sup>  $p \leq 0.001$ , <sup>b</sup>  $p \leq 0.01$ , <sup>c</sup>  $p \leq 0.05$ ; compared to the hypoxia group: \*  $p \leq 0.001$ , using ANOVA, followed by Bonferroni as a post-ANOVA test.

ferent pathological conditions through inhibition of the expression of these mediators (4, 13). Indeed, our results show that administration of melatonin significantly decreased the amount of inflammatory cytokines TNF- $\alpha$  and IL-6 of the hypoxic rats ( $p \leq 0.001$ ). Melatonin modulates inflammation by inhibiting the NF- $\kappa$ B nuclear erythroid 2-related factor 2 (Nrf2) cascades and downstream mediators of inflammation (27). Quercetin exerts its antioxidant properties by scavenging reactive oxygen and nitrogen species. It increases endothelial nitric oxide and induces vasodilatation through smooth muscle relaxation. It has been experimentally used in the treatment of ischemia and myocarditis (28). Experimental studies have reported that quercetin protects from ischemia-reperfusion injury through prevention of apoptosis and inhibition of the mitochondria-dependent caspase pathway in animals (29).

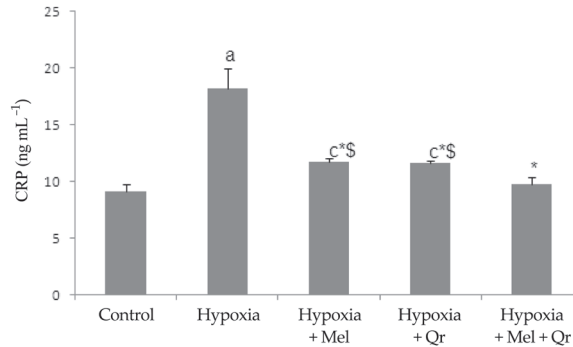


Fig. 6. Effect of melatonin (Mel) and/or quercetin (Qr) on serum CRP in the control and hypoxia treated groups. Data are expressed as mean  $\pm$  SD ( $n = 10$ ). Significant difference compared to the control group: <sup>a</sup>  $p \leq 0.001$ , <sup>c</sup>  $p \leq 0.05$ ; compared to the hypoxia group: <sup>\*</sup>  $p \leq 0.001$ ; compared to the combination group (hypoxia + Mel + Qr): <sup>§</sup>  $p \leq 0.05$ , using ANOVA, followed by Bonferroni as a post-ANOVA test.

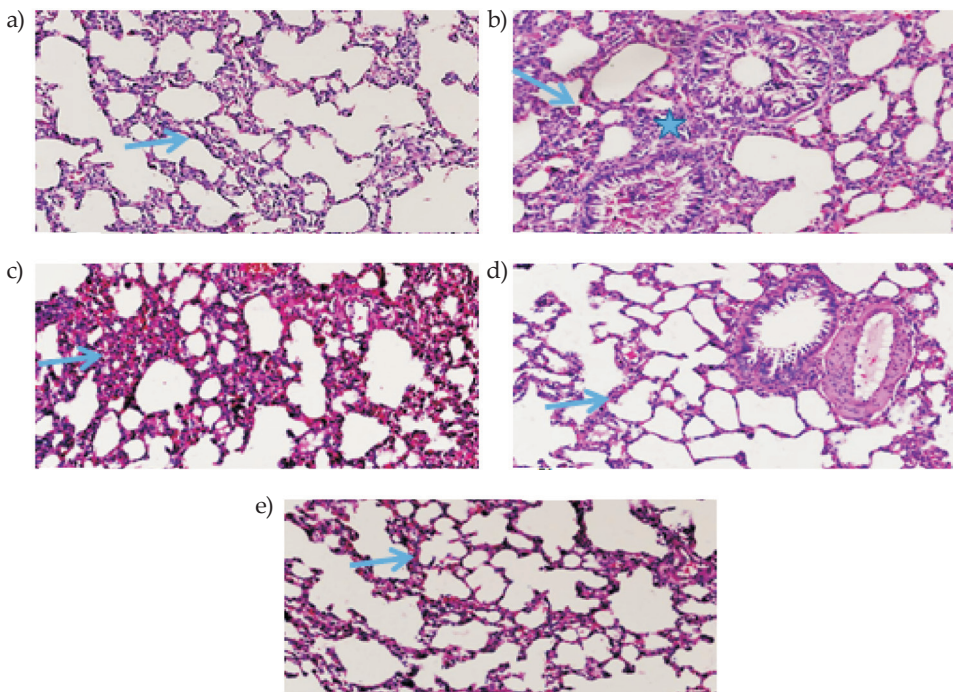


Fig. 7. Light photomicrographs of rat lung stained with hematoxylin and eosin (scale bar: 100  $\mu$ m), representing: a) normal lung alveoli and septae (arrow), b) section of lung from a rat exposed to hypoxia showing marked thickening of interalveolar septa (arrow) and alveoli and foci of inflammatory cellular infiltration (star), c)–e) sections of lung from a rat exposed to hypoxia receiving melatonin, quercetin and a combination of both, respectively.



Extensive variations were observed during the histopathological study of rat lungs. Administration of NaNO<sub>2</sub> caused degeneration of the alveoli and thickening of interalveolar septae in addition to focal cellular infiltration (Figs. 7a-e). Similar observation was found in another study during CCl<sub>4</sub> administration to rat lungs (30). Treatment with melatonin or quercetin showed marked improvement of the alveoli and alveolar septae and a marked decrease of cellular infiltration, especially in rats that received a combination of melatonin and quercetin. This beneficial effect of both melatonin and quercetin was previously documented in animals that underwent experimental lung damage (31). Greater improvement of pathological changes is obvious in rats that received co-treatment of both drugs. This result may predict the beneficial synergistic protective impact of the combination of melatonin and quercetin against lung damage induced by hypoxia.

### CONCLUSIONS

The obtained results have proven that melatonin and/or quercetin are likely to be responsible for lung tissue protection against hypoxic lung damage in rats through their downregulation of immuno-inflammatory mediators and heat shock protein expressions. The combination of melatonin and quercetin was more effective in modulating most of the studied parameters to near-normal levels than each agent individually. Therefore, the current findings may have important implications on the development of a new therapeutic strategy aimed at manipulating melatonin and quercetin in combination as a supplement for prophylaxis from hypoxic lung injury.

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