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The protective effect of melatonin in lungs of newborn rats exposed to maternal nicotine

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Abstract

We investigated possible healing effects of melatonin (MEL) on biochemical and histological changes in the lungs of rat offspring caused by exposure to nicotine (NT) *in utero*. Pregnant rats were divided randomly into five groups. The SP group was treated with physiological saline. The EA group was treated with ethyl alcohol. The MEL group was treated with MEL. The NT group was treated with NT. The NT + MEL group was treated with NT and MEL. At the end of the study, the biochemistry and histopathology of lung tissue of the offspring were examined. Reduced alveolar development and increased numbers of alveolar macrophages and mast cells were observed in the NT group compared to the SP, EA and MEL groups. We also found increased malondialdehyde (MDA) levels and decreased total glutathione (GSH) levels in the NT group. Application of MEL ameliorated the histological and biochemical damage caused by NT. The number of alveoli was greater in the NT + MEL group than in the NT group. Also, the increased numbers of alveolar macrophages and mast cells resulting from exposure to NT were decreased following MEL treatment. We found that MEL caused a significant decrease in the level of MDA. Maternal exposure to NT caused significant structural and biochemical changes in the lungs of the offspring and administration of MEL ameliorated the changes.

Key words: histopathology, lung, maternal, melatonin, nicotine, oxidative stress, rats

Prenatal and early postnatal exposure to tobacco smoke causes a wide range of adverse health effects including spontaneous abortion, low birth weight, premature delivery and perinatal complications (Faiz and Ananth 2003, Hammoud et al. 2005, George et al. 2006, Fantuzzi et al. 2007, Hogberg and Cnattingius 2007). Nicotine (NT), an important component of tobacco smoke (Guan et al. 2003), passes readily across the placenta and enters the fetal circulation (Luck et al. 1985). NT in the fetal circulation passes through the amniotic fluid and is absorbed by fetal skin (Onuki et al. 2003). The NT concentration in the blood of the fetus is similar to the NT concentration in the blood of the mother. Breast fed babies are exposed to greater NT concentration than in the mother's plasma (Maritz and Harding 2011).

Fetal exposure to maternal NT during pregnancy and lactation inhibits the growth of fetal and neonatal lungs (Fergusson et al. 1981, Lodrup et al. 1997, Hofhuis et al. 2003). NT binds to the nicotinic acetylcholine receptors; when these are activated, the Ca^{++} flow to the intracellular region increases (Leonard and Bertrand 2001, Hogg et al. 2003). Increased intracellular Ca^{++} concentration inhibits organelle function and signaling pathways, which leads to an overproduction of oxygen radicals that cause oxidative stress (Zhao and Reece 2005). Consequently, NT increases the likelihood of lung diseases by disturbing the oxidant-antioxidant balance in the lungs (Kalpana and Menon 2004). Therefore, fetal exposure to maternal NT during pregnancy and lactation disturbs the

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oxidant-antioxidant balance in both mother and offspring (Onuki et al. 2003), which impairs lung development (Maritz and Burger 1992).

Melatonin (MEL) stabilizes cell membranes and inhibits oxidative damage caused by lipid oxidation. MEL is a potent antioxidant, a direct free radical scavenger and an indirect stimulator of endogenous antioxidant enzymes. MEL administered to pregnant women passes readily through the placenta and enters the fetal circulation; it also passes readily into breast milk (Rezzani et al. 2006). Therefore, MEL may reduce free radical damage to cell membranes, organelles and the nucleus (Reiter et al. 2000, Okatani et al. 2000).

We investigated the histological and biochemical changes in the lungs of rat offspring exposed to NT during pregnancy and lactation, and evaluated the possible protective effects of MEL on these changes.

Material and methods

Animals and experimental procedure

Our study was approved by the Animal Research Ethical Committee of Inonu University, Malatya, Turkey, and we followed the National Institutes of Health (Washington, DC) *Guide for the Care and Use of Laboratory Animals*.

Male and female 200–250 g Wistar albino adult rats were obtained from the Experimental Animals Breeding and Research Center of Inonu University. Rats were housed in individual cages in a well ventilated room at 21° C with a 12 h light:12 h dark cycle. Animals were provided standard rat chow and tap water *ad libitum*. Pregnant rats were obtained by mating overnight with a sexually experienced male. Pregnancy was confirmed by vaginal smear and embryonic day zero was determined by the presence of sperm in a vaginal smear.

Pregnant rats were assigned randomly into five groups. The SP group (n = 3) of pregnant rats received only physiological saline intraperitoneally (i.p.) during pregnancy from 1 to 21 days gestation and during lactation until postnatal day 21. The EA group (n = 3) received only 10% v/v aqueous ethanol i.p. during the study period. Melatonin was dissolved in a small amount of ethanol, then diluted with physiological saline; the final concentration of ethanol was 10%. The MEL group (n = 3) received 10 mg/kg MEL i.p. for the same period (Baykan et al. 2008, Baydas et al. 2007, Kim et al. 2004). The NT group (n = 5) received 3 mg/kg NT (nicotine tartrate; Sigma, Darmstadt, Germany) to obtain plasma NT levels comparable to moderate smokers (Gunes et al. 2011). The NT + MEL group (n = 5) received 3 mg/kg NT and 10 mg/kg MEL i.p. during the study period.

All injections (SP, EA, MEL and NT) were carried out i.p. once/day using a volume of 0.5 ml for the duration of experimental period. We used 21 offspring in the each of the SP, EA and MEL groups, and 35 offspring in the NT and NT + MEL groups. At the end of study, the 22-day-old offspring were sacrificed using a high dose of an anesthesia mixture consisting of 10% alfamine and 2% alfazyne (3440 AB; Alfasan International B.V. Woerden, Holland) and the lung tissues were removed quickly.

Histological and histochemical analyses

Lung tissues were excised and fixed in 10% neutral formalin solution (D-89,555; Sigma Aldrich Chemie GmbH, Steinheim, Germany), dehydrated through 80, 95% and absolute ethyl alcohol (Sigma Aldrich), cleared in xylene (Sigma Aldrich) and embedded in paraffin (Lab Vision Corp., Thermo Scientific, Fremont, CA). Sections were cut at 4 µm, mounted on slides and stained with hematoxylin and eosin (H & E) (KGaA, 64271; Merck, Darmstadt, Germany) (Prophet et al. 1992) for general lung structure, periodic acid-Schiff (PAS) (KGaA, 64271; Merck) (Prophet et al. 1992) to demonstrate alveolar macrophages and toluidine blue (Sigma Aldrich) (Smit and Pretorius 2007) to demonstrate mast cells.

We assessed lung injury in 10 randomly selected fields on each section and scored for two tissue injury criteria: emphysema and increased interstitial tissue. Semiquantitative scoring of each variable used the following scale: 0, normal tissue; 1, injury involving < 25% of the total area; 2, injury involving 25–75% of the total area; 3, injury involving > 75% of the total area (Ozdemir et al. 2016). Ten randomly selected areas of each section were examined. We used 21 offspring in each of the SP, EA and MEL groups, and 35 offspring in the NT and NT + MEL groups. Also, the number of alveoli was counted in 10 randomly selected fields to determine alveolar development using a modification of the method of Deng et al. (2012). The alveolar macrophages and mast cells also were counted in 10 fields (Ozdemir et al. 2016). Histological evaluations were performed using a Leica Q Win Plus Image Analysis System (Leica Micros Imaging Solutions Ltd., Cambridge, UK).

Immunohistochemical analysis

Sections were deparaffinized with xylene, then passed through 80, 95% and absolute ethanol and washed in tap water, then placed in citrate buffer, pH 6.0, antigen retrieval solution, boiled in a pressure cooker for 20 min and cooled to room temperature for 20 min. Sections were washed with phosphate-buffered saline, pH 7.4 (PBS). Endogenous peroxidase activity was blocked by incubating the sections in 0.3% (v/v) aqueous hydrogen peroxide solution for 15 min at room temperature, after which sections were washed in PBS. Nonspecific antigen binding sites then were blocked with protein block (ready to use Ultra V Block; Lab Vision Corp.). Monoclonal mouse anti-smooth muscle actin (anti-α-SMA) antibody (MS-113-P0; NeoMarkers, Lab Vision) diluted 1:500 with ready to use Large Volume UltrAb Diluent Plus (Lab Vision Corp.) was applied for 30 min at room temperature. After rinsing with PBS, sections were incubated with biotinylated secondary antibody (ready to use biotinylated goat anti-polyvalent; Lab Vision Corp.) and streptavidin peroxidase (Lab Vision Corp.) for 10 min at room temperature. Staining was visualized using the chromogenic substrate 3-amino-9-ethylcarbazole (AEC) (Lab Vision Corp.), counterstained with hematoxylin and mounted on glass slides. Sections were mounted with aqueous mount medium (Large Volume Vision Mount; Lab Vision Corp.) (Durak et al. 2017).

Ten randomly selected fields were scored for each section to evaluate the amount of fibrosis based on α-SMA expression. A numerical scoring scale was used to quantify the α-SMA expressing cells as follows: 0, no α-SMA-positive cells in the alveolar septa; 1, unclear α-SMA-positive cells in the alveolar septa; 2, single or small groups of α-SMA-positive cells in the alveolar septa; 3, widespread α-SMA-positive cells in the alveolar septa (Gulasi et al. 2016).

Tissue homogenates

Tissues were homogenized using a PCV Kinematica Status homogenizer (PCV Kinematica Status homogenizer, Lucerne, Switzerland) in ice-cold PBS and sonicated with three cycles of 20 sec sonication, then a 40 sec pause on ice in an ultrasonifier (Branson sonifier 450; Branson Ultrasonics Corp., Danbury, CT). The homogenate was centrifuged at 15,000 × *g* for 10 min at 4° C; the cell-free supernatant was subjected immediately to enzyme assay.

Malondialdehyde (MDA)

Analysis of lipid oxidation was performed as described by Buege and Aust (1978), with a minor modification. The reaction mixture was prepared by adding 500 µl homogenate (0.25 g cell aggregate/500 µl PBS) into 2 ml reaction solution consisting of 15% trichloroacetic acid, 0.375% thiobarbituric acid and 0.25 N aqueous HCl 1:1:1 (w/w/v) and heated at 100° C for 20 min. The mixture was cooled to room temperature, centrifuged at 10,000 × g for 10 min and the absorbance of the supernatant measured at 532 nm. The MDA standard was 1,1,3,3-tetramethoxypropane. MDA results were expressed as nmol/g wet tissue in the homogenate.

Glutathione (GSH)

GSH was assayed using the procedure of Theodorus et al. (1981). The formation of 5-thio-2-nitrobenzoate is followed spectrophotometrically at 412 nm. The following reagents were used: 123 mM phosphate buffer, pH 7.2; 6.3 mM EDTA; 3.5 mM 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB); 4 mg/ml β-nicotinamide adenine dinucleotide phosphate (NADPH)) and 6 U/ml glutathione reductase (GR). Ten microliters of supernatant was added to the reaction mixture. The formation of 5-thio-2-nitrobenzoate was followed spectrophotometrically at 412 nm for 5 min at intervals of 30 sec. The amount of GSH in the extract was measured as nmol/mg protein using a commercial GSH as the standard (Sigma Aldrich).

Statistical analysis

Data were analyzed using the SPSS software program for Windows, version 15.0 (SPSS Inc., Chicago, IL). The data were expressed as either medians or means ± SD depending on the overall variable distribution. The normality of the distribution was confirmed using the Shapiro-Wilk test. The normally distributed data were analyzed by one-way ANOVA followed by the Tukey *post hoc* test. The non-normally distributed data were compared by the Kruskal-Wallis H test. When significant differences were determined, multiple comparisons were carried out using the Mann-Whitney U test with Bonfernoni correction. Results were considered statistically significant at $p \leq 0.05$.

Results

Histopathology

In sections stained with H & E, the alveoli, bronchi, bronchioles and pulmonary interstitium appeared

normal in the EA, MEL and SP groups; alveoli were patent with an open side in these sections (Fig. 1A–C). By contrast, sections of the NT group exhibited emphysematous changes, such as enlarged airspaces and thinning of alveolar septa. Also, NT group lungs exhibited areas of increased interstitial tissue (Fig. 1D, E). Interstitial tissue and emphysematous changes were significantly greater in the NT group compared to the SP, EA and MEL groups ($p < 0.05$). In the NT group, the size and shapes of alveoli were irregular. The number of alveoli was significantly decreased in the NT group compared to the SP, EA and MEL groups ($p < 0.05$) (Fig. 2).

MEL treatment decreased the severity of lung injury caused by NT. Lung sections of the NT + MEL group were similar to those of the SP, EA and MEL groups, except for slightly increased interstitial tissue (Fig. 1F). Histological changes were decreased significantly in the NT + MEL group compared to the NT group ($p < 0.001$). The mean number of alveoli was significantly greater in the NT + MEL group compared to NT group ($p = 0.012$, Fig. 2). Histopathological scoring for each group is summarized in Table 1.

Alveolar macrophage cytoplasm was PAS positive and these cells were observed in the lumen or near the surface of alveoli in the SP, EA and MEL groups (Fig. 3A–C). In the NT group, macrophages accumulated in the alveoli and bronchiolar lumens (Fig. 4A). The number of alveolar macrophages was significantly increased in the NT group compared to the SP, EA and MEL groups ($p < 0.05$) (Fig. 5). MEL treatment decreased the number of macrophages. The number of alveolar macrophages in the NT + MEL group was lower than that of the NT group (Fig. 4A). The decreased number of alveolar macrophages in the NT + MEL group was statistically significant compared to the NT group ($p < 0.001$, Fig. 5).

Mast cells granules were stained violet by toluidine blue and were observed rarely in the connective tissue surrounding the bronchi, bronchioles and blood vessels or in the interstitium (Fig. 3D–F). The number of mast cells was increased significantly in the NT group (Fig. 4C) compared to the SP, EA and MEL groups ($p < 0.05$) (Fig. 5). We found a significant decrease, however, in the number of mast cells in the NT + MEL group (Fig. 4D) compared to the NT group ($p = 0.029$) (Fig. 5).

α-sma expression

The prevalence of α-SMA-expressing cells was scored to assess fibrosis (Figs. 6 and 7). The NT and NT + MEL groups exhibited increased α-SMA-expressing cells in the interstitial areas. Cells expressing α-SMA were found more frequently in the NT group than in the SP, EA and MEL groups and this difference was statistically significant ($p < 0.05$). The number of α-SMA-expressing cells in the NT + MEL group was significantly lower than for the NT group ($p < 0.001$). The numbers of α-SMA-expressing cells of each group are summarized in Table 1.

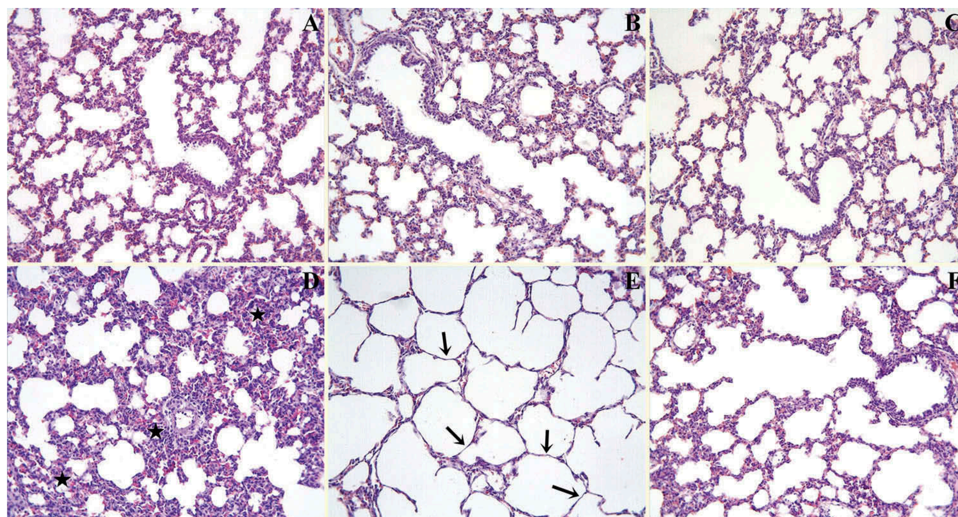

Fig. 1. Lungs of SP group (A), EA group (B) and MEL group (C) appear normal. Lungs of NT group rats show increased interstitial tissue (D, stars) and emphysema (E, arrows). Lungs of the NT + MEL group rats (F) are similar to SP, EA and MEL groups. H & E. × 20.

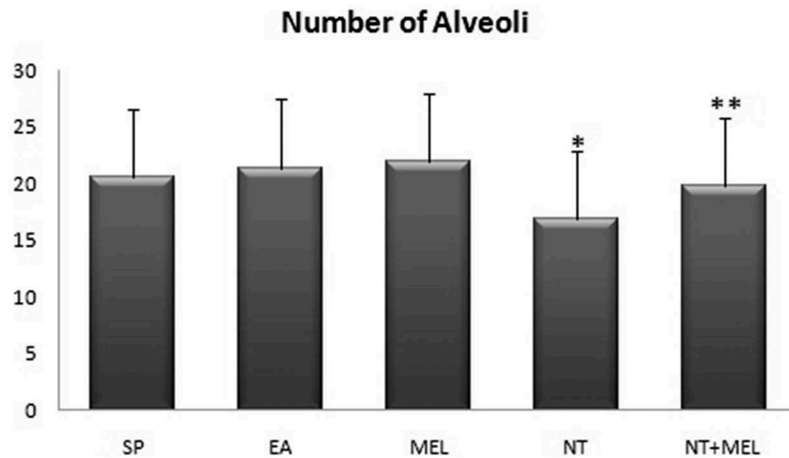

Fig. 2. Comparison of number of alveoli in rat lung. Data are means ± SD. *$p < 0.05$ vs. SP, EA and MEL groups. **$p = 0.012$ vs. NT group.

Table 1. Histological evaluations

Groups	Emphysema	Increased interstitial tissue	Density of α-SMA-positive cells
SP	0.0 (0.0–1.0)	0.0 (0.0–2.0)	0.0 (0.0–1.0)
EA	0.0 (0.0–1.0)	0.0 (0.0–1.0)	0.0 (0.0–1.0)
MEL	0.0 (0.0–1.0)	0.0 (0.0–1.0)	0.0 (0.0–1.0)
NT	0.0 (0.0–3.0)[a]	1.0 (0.0–3.0)[a]	1.0 (0.0–3.0)[a]
NT + MEL	0.0 (0.0–1.0)[b]	0.0 (0.0–2.0)[b]	1.0 (0.0–2.0)[b]

Data are median (min–max) values. [a]Significant increase vs. SP, EA and MEL groups ($p < 0.05$). [b]Significant decrease vs. NT group ($p < 0.001$).

MDA and GSH levels

The level of tissue MDA in the NT group was significantly higher than for the SP, EA and MEL groups ($p < 0.05$). The level of MDA in the NT + MEL group, however, was significantly lower than for the NT group ($p = 0.002$). To the contrary, the tissue GSH level of the NT group was significantly lower than for the SP, EA and MEL groups ($p < 0.05$). The tissue GSH level in the NT + MEL group was significantly higher than for the NT group ($p = 0.012$). The tissue MDA and GSH levels of each group are summarized in Table 2.

Discussion

Consistent with previous reports (Maritz 1997a, Dasgupta et al. 2012, Petre et al. 2011), we found areas where the number of alveoli were decreased and interstitial tissue was increased in the lungs of rat offspring whose mothers had been exposed to NT. During lung development, fibroblasts participate in formation of septa and alveoli (Kauffman et al. 1974). These cells are the major component of the alveolar walls and they synthesize matrix proteins that maintain the extracellular framework of alveolar structures. NT has been shown to alter the morphogenesis and differentiation of fibroblasts, which causes functional changes in the fibroblasts (Krebs et al. 2010, Rehan et al. 2005) that may in turn cause impairment of alveolus formation and a permanently decreased number of alveoli (Dasgupta et al. 2012).

We observed also emphysematous areas that were characterized by alveolar expansion that resulted from maternal NT administration. Emphysema is a permanent abnormal enlargement of air spaces distal to the terminal bronchioles owing to destruction of the alveolar septa (Maritz 1997b). Smoking has been shown to be an important risk factor for pulmonary emphysema (Snider 1989). Maritz (2002) reported that maternal exposure to NT resulted in microscopic emphysema and that the number of alveoli in emphysematous

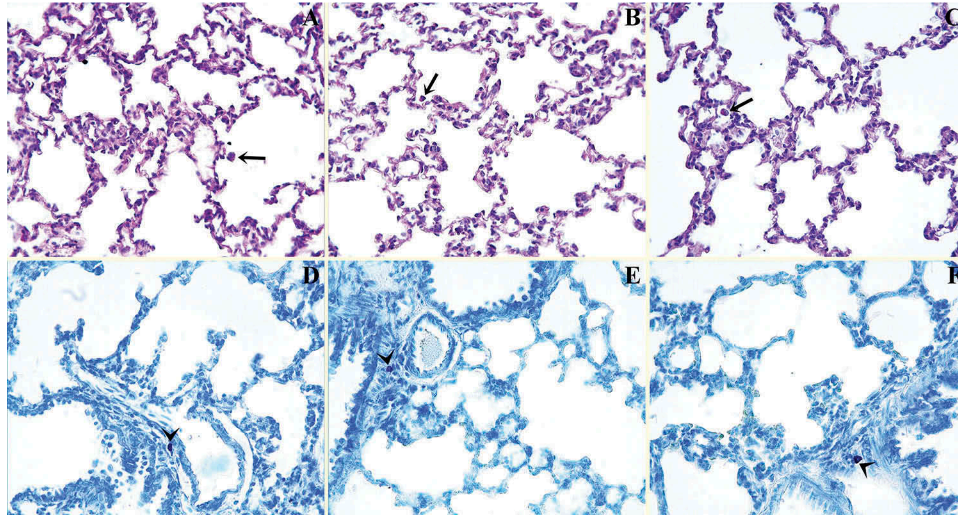

Fig. 3. Alveolar macrophages (arrows) in alveolar lumens in the SP group (A), EA group (B) and MEL group (C) in rat lung. PAS. × 400. Mast cells (arrowheads) in the connective tissue around blood vessels and bronchioles in the SP group (D), EA group (E) and MEL group (F). Toluidine blue. × 400.

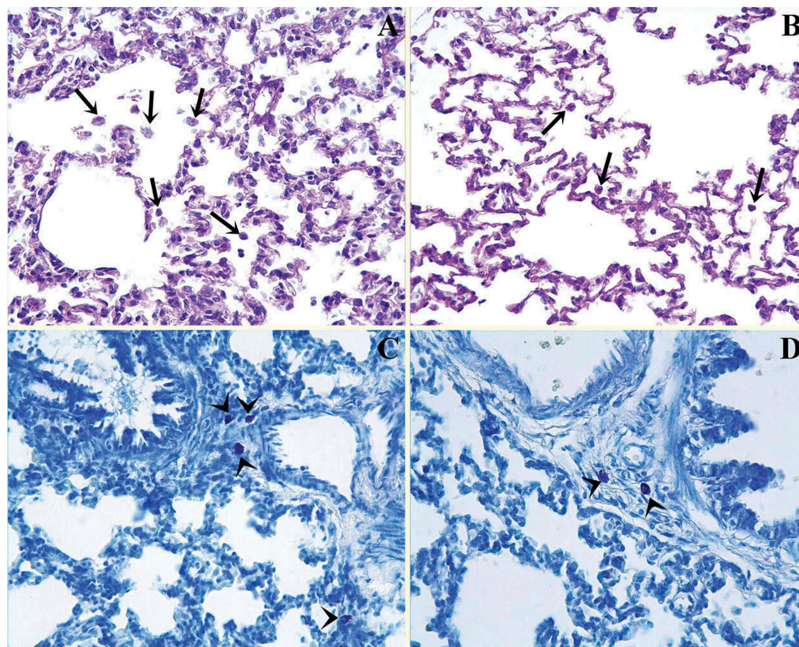

Fig. 4. Increased numbers of alveolar macrophages (A, arrows) and mast cells (C, arrowheads) in the NT group lung. PAS. × 400. Decreased number of macrophages (B, arrows) and mast cells (D, arrowheads) in the NT + MEL group compared to the NT group. Toluidine blue. × 400.

lung is less than in non-emphysematous lung. It has been reported that NT is a chemotactic agent for polymorphonuclear leukocytes (Seow et al. 1994). Both polymorphonuclear leucocytes and alveolar macrophages release proteases such as elastase (Seow et al. 1994, Finlay et al. 1997). Therefore, these cells contribute to the breakdown of the connective tissue surrounding and supporting the alveoli (Gs 1997b). We also observed that the number of alveolar macrophages was increased significantly in the lungs of rat offspring whose mothers had been exposed to NT compared to the SP, EA and MEL groups. Alveolar macrophages participate in defense and repair of lung

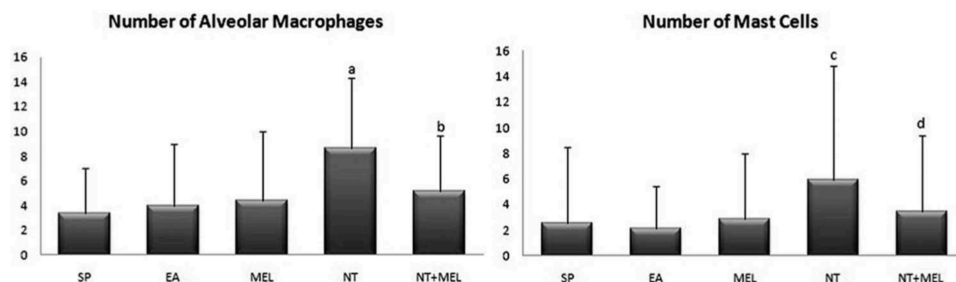

Fig. 5. Comparison of numbers of alveolar macrophages and mast cells in rat lung. Data are means ± SD, [a]$p < 0.05$ vs. the SP, EA and MEL groups, [b]$p < 0.001$ vs. the NT group, [c]$p < 0.05$ vs. the SP, EA and MEL groups, [d]$p = 0.029$ vs. NT group.

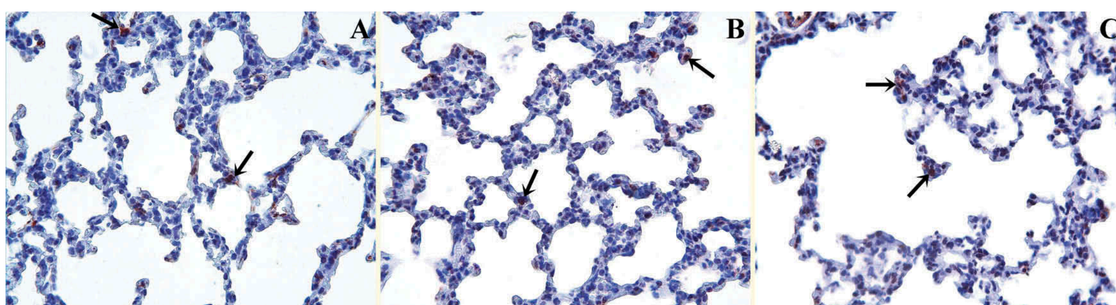

Fig. 6. α-SMA-positive cells in the interstitial area of rat lung identified by brown cytoplasmic staining. Arrows indicate α-SMA-positive cells in the SP group (A), EA group (B), and MEL group (C) of rat lung. α-SMA immunostaining. × 400.

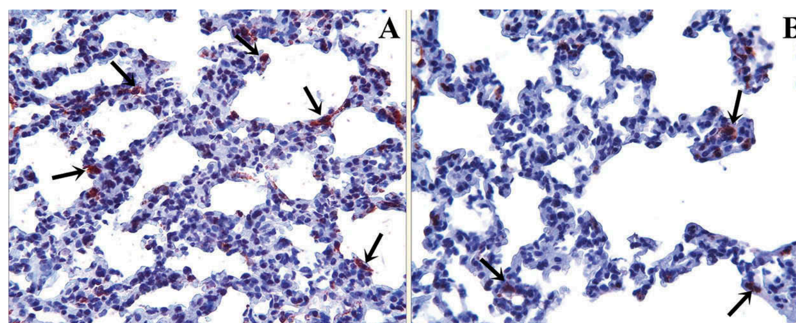

Fig. 7. Numbers of α-SMA-positive cells in rat lung. A) Increased number of cells in the NT group compared to SP, EA and MEL groups. B) Decreased number of cells in the NT + MEL group compared to the NT group. α-SMA immunostaining. × 400.

Table 2. Biochemical evaluations

Groups	MDA (nmol/g)	GSH (nmol/mg)
SP	141.94 (116.44–155.13)	4.53 (2.22–4.78)
EA	144.31 (112.25–167.28)	3.88 (2.37–4.72)
MEL	124.57 (113.32–192.31)	4.03 (2.40–6.16)
NT	291.73 (195.35–502.89)[a]	2.14 (1.75–3.18)[b]
NT + MEL	134.46 (116.33–192.89)[c]	3.17 (2.45–5.12)[d]

Data are median (min–max). [a]Significant increase vs. SP, EA and MEL groups ($p < 0.05$). [b]Significant decrease vs. SP, EA and MEL ($p < 0.05$). [c]Significant decrease vs. NT group ($p = 0.002$). [d]Significant increase vs. NT group ($p = 0.012$).

parenchyma against pathogens and environmental insults. Smoking increases the number and mobilization of alveolar macrophages in humans and experimental animals (Muller and Hirschberg 1984, Harada and Baskur 1998). The latter investigators asserted that this effect was due to the tar and NT components.

We also found that the number of α-SMA positive cells increased in the NT group compared to the SP, EA and MEL groups, which is consistent with the report by Chen et al. (2015) that maternal NT exposure increased α-SMA expression and total collagen content in the lungs of NT treated offspring. It is generally considered that fibroblasts infiltrate damaged areas caused by inflammation and epithelial degeneration and that the fibroblasts transform into myofibroblasts, which cause fibrosis. Krebs et al. (2010) reported that NT exposure impairs molecular paracrine communication between the alveolar epithelium and interstitium, which therefore causes transdifferentiation of lung alveolar interstitial fibroblasts into myofibroblasts. Myofibroblasts express α-SMA, which is a useful marker for assessing fibrosis. Pulmonary fibrosis is a widespread response to various insults to the lung, and it is characterized by an over-production of extracellular matrix. Fibrosis also can be assessed by measuring increases in transforming growth factor β1 (TGF-β1), α-SMA, or collagen (Gülaşı et al. 2016). It also has been reported that maternal exposure to NT causes pulmonary fibrosis owing to increased collagen as indicated by increased TGF-β1 expression (Sekhon et al. 2004, Chen et al. 2015).

We found a marked increase in the number of pulmonary mast cells in the NT exposed group compared to the SP, EA and MEL groups, which is consistent with an earlier report of increased mast cell number in lungs and skin of smokers (Erbagci and Erkilic 2002). Mast cells originate from bone marrow and participate in allergic, immunologic and inflammatory reactions (Dvorak et al. 1994). Studies in vitro indicate the presence of nicotinic acetylcholine receptors, such as α-7, α-9 and α-10, on cell membranes of mast cells (Mishra et al. (2010), Sudheer et al. (2006). Kageyama-Yahara et al. (2008) reported that NT causes pulmonary mast cell activation through these membrane bound receptors.

Previous studies indicate that NT causes production of reactive oxygen radicals that result in oxidative damage. Increased amounts of reactive oxygen radicals cause increased oxidation and depletion of antioxidants (Nasr-Esfahani and Johnson 1992, Baykan et al. 2008, Gunes et al. 2008). We found that NT caused both increased MDA levels and decreased GSH levels in rat offspring exposed to NT *in utero*. NT increases the possibility of lung diseases by disturbing the oxidant-antioxidant balance in lungs (Kalpana and Menon 2004).

Biological compounds with antioxidant properties contribute to protection of cells and tissues against the deleterious effects of reactive oxygen species and other free radicals. MEL is a strong antioxidant, direct free radical scavenger and an activator of antioxidant enzymes (Rezzani et al. 2006); it also exhibits antifibrotic effects (Yu et al. 2016). We observed that the histological and biochemical damage in the lungs of rat offspring caused by maternal NT use was reduced significantly following MEL administration. Therefore, histological changes including increased interstitial tissue and emphysema in lungs exposed to NT were decreased significantly in the NT + MEL group. We also found a significant increase in the number of alveoli following MEL administration to NT treated animals. Slominski and Prunski (1993) and Roth et al. (1997) reported that MEL affects the cell cycle, and its effects depend on its concentration and cell type. Danilova et al. (2004) reported that the cell proliferation rate increased with the appearance MEL receptor expression in zebrafish embryos. We suggest that MEL reduced the loss of alveoli caused by NT exposure due to its strong oxygen radical scavenger properties and positive effects on embryonic cell cycle. Ekthuwapranee et al. (2015) reported that MEL may enhance cell proliferation by affecting the cell cycle. Therefore, we suggest that the increased number of alveoli in the NT + MEL group may be related to the proliferative effect of MEL. We found a marked increase in the number of α-SMA positive cells in the NT exposed group compared to the SP, EA and MEL groups. Consistent with our results, Crespo et al. (2015) demonstrated that MEL caused a significant decrease in a-SMA expression in the liver of mice treated with carbon tetrachloride. The antifibrotic effects of MEL have been documented (Czechowska et al. 2015, Zhao et al. 2014). Jang et al. (2013) reported that MEL exerts its antifibrotic effect by inhibiting profibrogenic cytokine production (Crespo et al. 2015).

We found that the NT-induced increase in the numbers of alveolar macrophages and mast cells was decreased significantly by MEL administration. This is consistent with reports that MEL exhibits anti-inflammatory and immunoregulatory properties (Qiao et al. 2002, Li et al. 2005, Mayo et al. 2005). Swindle et al. (2002) reported that reactive oxygen radicals produced by macrophages play an important role in mast cell activation. Consequently, we suggest that the effect of MEL on macrophages and

mast cells is a result of its oxygen radical scavenger properties and immunoregulatory functions.

We found that MDA levels were increased following exposure to NT, but that the increase was reduced significantly by MEL administration. This effect can be attributed to inactivation of free radicals and stimulation of enzymes that synthesize other antioxidants (Schenker et al. 1998). Our findings are consistent with earlier reports that increased MDA owing to lipid oxidation was decreased significantly following MEL administration (El-Sokkary et al. 2007, Thamtan et al. 2015). It also has been reported that MDA levels increased in heart tissues of rat offspring exposed to NT, but that MEL administration significantly reduced this increase (Baykan et al. 2008). We found that MEL exhibits beneficial effects on the adverse histological and biochemical changes in the lungs of neonatal rats exposed to NT *in utero*. We believe that the positive effects of MEL are due to its antioxidant properties.

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