



Published in final edited form as:

*Vascul Pharmacol.* 2009 ; 51(2-3): 190–197. doi:10.1016/j.vph.2009.06.002.

## 2-Methoxyestradiol Attenuates Bleomycin-Induced Pulmonary Hypertension and Fibrosis in Estrogen-Deficient Rats

Stevan P. Tofovic, Xinchun Zhang, Edwin K. Jackson, Hong Zhu, and Gordana Petrushevka  
Center for Clinical Pharmacology, Department of Medicine, University of Pittsburgh School of Medicine

### Abstract

Pulmonary hypertension (PH) is a common and life-threatening complication of pulmonary fibrosis. Estradiol (E2) is protective in experimental PH, and its non-estrogenic metabolite 2-methoxyestradiol (2ME) prevents the development and retards the progression of monocrotaline-induced PH in male and female rats. However, the effects of E2 and 2ME on pulmonary fibrosis and associated PH have not been examined. Therefore, we compared the growth-inhibitory effects of E2 and 2ME in human lung fibroblasts (hLFs) and pulmonary vascular smooth muscle cells (hPASCs), and we investigated the effects of estrogen deficiency and 2ME on bleomycin-induced pulmonary fibrosis and PH. Intact and ovariectomized (OVX) female Sprague Dawley rats were administered intratracheally either saline or bleomycin (15 IU/kg), and a subset of OVX bleomycin-treated rats received 2ME (10 µg/kg/h) for 21 days. Estradiol had only limited inhibitory effects on growth in hPASCs and no effect in hLFs, whereas 2ME exhibited strong and concentration-dependent (1–10 µM) antimitogenic effects in both cell types. Bleomycin caused lung injury/PH (significantly increased lung and right ventricle (RV) weights, RV peak systolic pressure (RVSP), and RV/left ventricle+septum ratio (RV/LV+S); caused medial hypertrophy and adventitial widening of pulmonary arteries; induced marked focal/diffuse fibrosis with diffuse infiltration of inflammatory (ED1+) cells; and resulted in 30% mortality). OVX exacerbated the disease and increased mortality (to 75%); whereas 2ME tended to reduce mortality (55.5%) and in surviving animals reduced RVSP and RV/LV+S ratio, and attenuated vascular remodeling, pulmonary inflammation and fibrosis. This study suggests that 2ME may have protective effects in bleomycin-induced PH and fibrosis. Further investigation of 2ME in pulmonary fibrosis and PH is warranted.

### Keywords

Pulmonary fibrosis; pulmonary hypertension; estradiol; estradiol metabolites

### INTRODUCTION

Pulmonary fibrosis is a penultimate consequence of chronic interstitial lung disease from various etiologies and it is characterized by a limited response to available therapies and poor

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Corresponding Author: Stevan P. Tofovic M.D., Ph.D. FAHA FASN Center for Clinical Pharmacology University of Pittsburgh School of Medicine 100 Technology Drive, Suite 450 Pittsburgh, PA 15219 FAX +412-648-1837 Phone +412-648-3363 E-mail:mailto:tofovic@dom.pitt.edu.

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prognosis. Pulmonary hypertension is common in patients with pulmonary fibrosis and its presence has a significant adverse impact on survival (Lettieri et al., 2006). Mortality rates for pulmonary fibrosis are increasing (34% in the last 15 years), and importantly the rate of increase is twice as high in women than men (Olson et al., 2007). However, little is known regarding the effects of gender and estrogens in development of lung fibrosis and associated pulmonary hypertension.

Among patients with systemic sclerosis (SSc, scleroderma), pulmonary hypertension is a serious complication and frequent cause of death (Proudman et al. 2007). Notably, in women with SSc menopause significantly increases the risk for development of disease (Scorza et al. 2002), whereas hormone replacement therapy prevents the development of PH (Beretta et al., 2006). It seems also that pregnancy and estrogens may influence the development of disease and never-pregnant women with SSc are at higher risk for developing PH (Arlett et al. 2002). Importantly, both estradiol and its non-estrogenic metabolite 2-methoxyestradiol (2ME) are present in high concentrations in women during the last trimester of pregnancy (Ball and Knuppen 1990; Tofovic et al., 2007), suggesting the notion that estrogens may attenuate the development and retard the progression of PH in patients with pulmonary fibrosis.

2ME is a major E2 metabolite which is the product of the sequential hydroxylation and methylation of E2 by the enzymes cytochrome P450 and catechol-O-methyltransferase. 2ME is not only a potent antimitogen in various cancer cells (Pribluda et al. 2000), but also inhibits proliferation of cardiovascular cells, including rat aortic smooth muscle cells, endothelial cells, cardiac fibroblasts and glomerular mesangial cells, and these effects are mediated by estrogen receptor-independent mechanisms (Dubey et al. 2004).

*In vivo*, 2ME retards the progression of PH in male rats (Tofovic et al., 2005a), and in female animals 2ME prevents the exacerbation of PH and eliminates the mortality in ovariectomized rats with MCT-induced PH (Tofovic et al., 2006). These beneficial effects are associated with marked inhibition of vascular remodeling and inflammation. 2ME also reduces acute lung injury and attenuates the development of PH and pulmonary vascular remodeling induced by the rodenticide alpha-naphthylthiourea (Tofovic et al., 2005b), and in the constricted-aorta rat model, 2ME inhibits pressure-independent vascular remodeling (Tofovic et al., 2003). Furthermore, 2ME and its metabolic precursor 2-hydroxyestradiol have direct (pressure-independent) inhibitory effects of cardiac remodeling and fibrosis in rats with isoproterenol-induced cardiac hypertrophy (Tofovic et al. 2008).

Because of the aforementioned considerations, we hypothesized that 2ME has protective effects and that estrogen deficiency exacerbates pulmonary fibrosis and associated pulmonary hypertension. To test our hypothesis we used bleomycin-induced lung fibrosis and pulmonary hypertension rat model. Although this model exhibits some features of human form of interstitial pulmonary fibrosis, it also has clear limitations (Gharaee-Kermani et al., 2005) that should be taken in consideration when assessing the clinical relevance of therapeutic responses in this model. The results of the present study suggest that 2ME is antimitogenic in human lung fibroblasts and pulmonary artery vascular smooth muscle cells and has beneficial effects in experimental pulmonary fibrosis and associated pulmonary hypertension.

## MATERIALS AND METHODS

### 1. Growth inhibitory effects of estradiol and 2ME

Cryopreserved human pulmonary artery smooth muscle cells (hPASMCs; Cascade Biologics, Inc.) were cultured in M231 supplemented with 10% FCS and 2 mM glutamax (Invitrogen). Cryopreserved human lung fibroblast (hLFs; Cell Applications, Inc., San Diego, CA) were cultured in DMEM (Invitrogen) supplemented with 10% FCS and 2 mM glutamax. Cells were

plated in a 75 cm<sup>2</sup> culture flask (Falcon) and incubated at 37° C with 5% CO<sub>2</sub>. Twenty four-hours later, cells were rinsed and provided fresh medium and thereafter fresh medium was provided every three days.

For cell proliferation assay, hPASMCs and hLFs (5,000 cells/well) were grown in 24-well tissue culture plates (Nunc). The growth of starved hPASMCs was stimulated with 2.5% FCS and Smooth Muscle Cell Growth Supplement (Cascade Biologics, Inc) (containing human basic fibroblast growth factor (bFGF 2 ng/ml), human epidermal growth factor (0.5 ng/ml), heparin (5 ng/ml), insulin (5 µg/ml) and BSA (0.2 µg/ml)), and in the presence or absence of the tested agents. For hLFs, cells were treated with M200 (phenol red free) containing 2.5% FCS and 1% Low Serum Growth Supplement (LSGS; Cascade Biologics) (containing hydrocortisone (0.5 µg/ml), human epidermal growth factor, (5ng/ml), bFGF (1.5 ng/ml) and heparin (5 µg/ml) and bFGF (6 ng/ml)), in the presence or absence of the tested agents. The cells were detached with 0.025% trypsin/EDTA (Sigma) at 5–7 days. The Quick Cell Proliferation Assay Kit (BioVision Research Products, Mountain View, CA) was used for quantification of cell proliferation and viability. Briefly, the assay is based on the cleavage of the tetrazolium salt WST-1 to formazan by cellular mitochondrial dehydrogenase. The activity of dehydrogenase correlates with cell proliferation, and formation of formazan dye is quantified by multi-well spectrophotometer by measuring the absorbance of the dye at 440 nm.

## 2. Animal studies

Forty-three female Sprague Dawley rats weighing 200–250 g were obtained from Charles River Laboratories (Wilmington, MA). Animals were fed rodent chow (Pro Lab RHM 3000 rodent diet, PMI Nutrition, Inc, St Louis, MO), had free access to water, and were housed at 22° C, 12-hour light cycle, and 55% relative humidity. All experiments were carried out in accordance with the University of Pittsburgh Institutional guidelines for animal welfare, and the Animal Care and Use Committee approved experimental protocols.

A subset of animals (n=27) underwent bilateral ovariectomy using the flank approach, whereas the remaining animals (n=16) were sham operated. To confirm that the ovaries were successfully removed, uterus weight was measured at autopsy. To produce pulmonary fibrosis, both intact and OVX animals were randomly assigned to receive under halothane anesthesia either an intratracheal injection of bleomycin (Sigma, St Louis, MO; 5 mg/kg/0.3 ml saline; Bleo and OVX-Bleo groups) or 0.3 ml saline (Control and OVX groups). A subset of animals (n=9) were implanted ALZET osmotic pumps (2ML-4, Durect Corporation, Cupertino, CA) delivering 2ME (10 µg/kg/h; Steraloids, Inc., Newport, RI). The effects of 2ME on bleomycin disposition are unknown, and it is possible that 2ME interferes with bleomycin disposition. The half-life of bleomycin in rodents ranges between 30 minutes and two hours (Lazo and Pham, 1984). Therefore, to avoid possible interference with bleomycin disposition, pumps were implanted 8 hours after bleomycin administration.

## 3. Acute Hemodynamic measurements

Twenty-one days after administration of bleomycin, animals underwent surgery and were instrumented for measurement of right ventricular peak systolic pressure (RVPSP) as described previously (Tofovic et al., 2005a, 2006). Briefly, rats were anesthetized with Inactin (90 mg/kg i.p.), and a PE-240 polyethylene catheter was inserted into the trachea to facilitate breathing. A PE-50 catheter was inserted into the left carotid artery and connected to a digital blood pressure analyzer (BPA, Micro-Med. Inc., Louisville, KY) for continuous measurements of systolic, diastolic and mean arterial blood pressures and heart rate. The rats were then mechanically ventilated (Harvard Rodent Ventilator, Model 683, Harvard Apparatus, MA) using constant respiratory rate (50/min) and tidal volume (1.0 ml). Next, the thorax was opened, and the right heart was punctured with a 23-gauge needle attached to a PE-50 line and Heart

Performance Analyzer (HPA-200  $\tau$ , Micro-Med. Inc., Louisville, KY). After a 30-minute stabilization period, RVPSP was recorded for 20 minutes at 1-minute intervals.

#### 4. Morphometric measurements and immunohistochemical studies

Animals were euthanatized by anesthetic overdose and heart and lungs were dissected and weighed. The ratios of wet weights of heart and lung to body weight (BW) were calculated (H/BW and LV/BW, respectively). The right ventricle (RV) free wall was separated from the left ventricle and the septum (LV+S) to determine the wet weight, the RV to body weight ratio (RV/BW), the LV+S to body weight ratio (LV+S/BW), and the RV to LV+S weight ratio (RV/LV+S, Fulton Index).

The lungs were removed from the chest cavity en block with the trachea and perfused *via* the trachea with a fixative solution (10% neutral-buffered formalin) at a pressure of 25 cm H<sub>2</sub>O. Perfused tissue samples were immersed in the fixative for 24 to 72 hours for subsequent light microscopy and immunohistochemistry. Tissue samples were embedded in paraffin blocks for light microscopy. Five-micron serial tissue sections from formalin-fixed, paraffin-embedded lungs were dewaxed and stained with H&E and Masson's trichrome for histological and morphometric assessment (to identify inflammatory cells, connective tissue and collagen deposition). Lung tissues sections were examined by light microscopy and were scored in blinded fashion.

The number of interstitial monocytes/macrophages was studied using a polyclonal anti-ED1 antibody (Serotec, Raleigh, NC). This antibody specifically stains positive the cytoplasm of alveolar and interstitial macrophages. Nonspecific staining was assessed by replacing the primary antibody with affinity-purified, nonimmune, rabbit IgG (R & D Systems). Sections were washed and developed further according to the directions of the manufacturer (Dako, Carpinteria, CA) using an LSAB2 kit, which contained a second antibody linked to avidin and peroxidase-conjugated to biotin.

To study vascular remodeling of small pulmonary arteries, arterial wall smooth muscle cells were stained using a mouse monoclonal anti-smooth muscle- $\alpha$  actin antibody at a dilution of 1/100 (Lab Vision, Fremont, CA). The measurements of media thickness, and media and adventitia surface were conducted using an Image Analyzing System (Diagnostic Instruments, Inc., Sterling Heights, MI) that included SPOT RT Camera installed on NIKON Eclipse 50 light microscope and specialized computer software program (SPOT Software, Version 4.1). Measurements were done ( $\times 20$  magnification) on ten cross sectioned pulmonary artery branches with 50–250-micron diameters. The peripheral lung fields were located at approximately equal distances from the pleural lining. Only vessels with an approximately circular profile (cross-sectional cuts) were studied. For each blood vessel, two rectangular diameters and their four respective media were measured. The media % index was calculated as  $2 \times \text{media/diameter} \times 100$  and is presented as average of four media measurements for a single vessel. Since bleomycin induced a significant widening of the adventitia, corrected media index was calculated using the diameter that includes only media + lumen measurement. For each blood vessel, two rectangular diameters and their four respective media were measured, and averages of four individual values of media thickness and media % index were calculated.

Collagen fibrosis was assessed on Mason's Trichrome stained lung sections by the same image analyzing system on 10 low-power random fields ( $\times 10$ ) using the region area measurement option and expressed as percent from a calibrated 1,000,000  $\mu^2$  microscopic field area.

## 5. Statistical Analysis

Statistical analyses were performed using the Number Cruncher Statistical software program (Kaysville, Utah). Group comparisons were performed by a one- or two- factor analysis of variance (1F-, 2-F ANOVA), followed by post-hoc comparison using the Fisher=s LSD test. The probability value of  $p < 0.05$  was considered statistically significant. All data are presented as mean  $\pm$  S.E.M.

## RESULTS

The growth inhibitory effects of estradiol and 2ME in human pulmonary artery smooth muscle cells (hPASMCs) and human lung fibroblasts (hLFs) are presented in Figure 1. In hPASMCs at physiological (1–10 nM) and low pharmacological (100 nM) concentrations, E2 had no effects on cell growth stimulated by 2.5% FCS and growth factor-supplement, but exhibited modest (–20 and –40%) antimitogenic effects at high concentrations (1 and 10  $\mu$ M; Figure 1A). No effects on hLF growth were detected when cells were exposed for 5 days to increasing concentrations (1 nM–10  $\mu$ M) of E2 (Figure 1B). In contrast, 2ME exhibited strong and concentration-dependent antimitogenic effects in both hPASMCs and hLFs.

The data examining the effects of 2ME on bleomycin-induced pulmonary hypertension and fibrosis in estrogen-deficient rats are presented in Figures 2– 7 and Table 1. The intratracheal instillation of bleomycin induced severe lung injury, substantial weight loss and premature deaths that occurred between days 3 and 16 of the experiment. The body weight of rats that received saline intratracheally increased with time, and weight gain was significantly greater in OVX animals (Day 21:  $317 \pm 11$  and  $346 \pm 7$ g, Cont and OVX group, respectively). In animals receiving bleomycin, there was no body weight gain during the 3-week study period (data not shown), and there was no significant difference in body weight among the three diseased experimental groups (Bleo, OVX-Bleo and OVX-Bleo-2ME). Bleomycin increased lung weight, and animals exposed to bleomycin had a doubling of lung weight compared to animals that received saline intratracheally. Importantly, ovariectomy further increased lung weight, and 2ME prevented further increases in lung weight in OVX-Bleo rats (Figure 2 upper panel, OVX-Bleo+2ME group). The differences in lung weight correlated with signs of microvascular leakage and severity of fibrosis (*infra vide*). Intratracheal instillation of bleomycin induced pulmonary hypertension, ovariectomy further increased the RVPSP (Figure 2, lower panel) and treatment with 2ME prevented further increases in RVPSP in OVX animals with intratracheally instilled bleomycin (OVX-Bleo vs. OVX-Bleo+2ME group,  $p < 0.05$ , Fisher post hoc comparison). Bleomycin-treated animals that died prematurely developed both right and left ventricular hypertrophy and OVX-Bleo group had the most severe cardiac hypertrophy (Figure 3, Day 3–16). Twenty-one days into treatments, the bleomycin-induced isolated RV hypertrophy (i.e., Fulton index, RV+S/RV ratio) was exacerbated in diseased OVX animals, and 2ME reduced the RV hypertrophy in OVX-Bleo rats (Figure 3, Day 21).

Animals that died in the first week had no fibrosis, but rather expressed marked congestion, edema, and inflammatory-cell infiltration (Figure 5J), and these alterations were multifocal in nature in intact females treated with Bleo. More severe changes were detected in OVX-Bleo rats, and these animals had diffuse (rather than multifocal) exudative, inflammatory and proliferative reactions. Animals that died between Day 8 and Day 16 had significant interstitial fibrosis, and the Bleo and Ovx-Bleo groups did not differ in regard to the extension of fibrosis (Figure 4A). In contrast, in animals that survived, Masson-trichrome staining of formalin-fixed tissue revealed greater and denser amounts of collagen deposition in lungs from Ovx-Bleo rats compared to Bleo group (Day 21; Figure 4A, Figures 5 G & H). Treatment with 2ME eliminated the exacerbation of fibrosis due to ovariectomy (Figures 4A and 4I). In fibrotic areas significant number of cells stained positive for smooth muscle  $\alpha$ -actin indicating myofibroblastic



proliferation and semi-quantitative assessment suggested reduced myofibroblastic proliferation in animals treated with 2ME.

Bleomycin-induced pulmonary fibrosis and hypertension detected on Day 21 were accompanied by significant vascular remodeling (Figure 4B, Table 1), and ovariectomy increased medial hypertrophy and adventitial widening (Table 1). In OVX-Bleo rats, treatment with 2ME markedly inhibited vascular remodeling, and OVX-Bleo+2ME rats had lesser vascular remodeling and adventitial widening than even intact diseased animals (Bleo group; Table 1).

Intratracheal instillation of bleomycin and the subsequent lung injury were also associated with significant inflammatory responses as evidenced by the presence of a large number of ED1+ cells (Figures 4C and 5L). The inflammatory response was even more pronounced in animals that died prematurely. Importantly, OVX exacerbated inflammation in all animals, 2ME markedly reduced the number of ED1+ cells, and the intensity of inflammation in OVX-Bleo+2ME group was even smaller than intact females treated with bleomycin (Bleo group). Finally, the more severe lung injury (fibrosis, inflammation, PH) in the OVX-Bleo group was associated with increased mortality, and treatment with 2ME tended to reduce the augmented mortality due to ovariectomy (Figure 6).

## DISCUSSION

Bleomycin induces dose-dependent lung parenchymal injury and fibrosis in both humans and animals. In rodents, bleomycin-induced lung fibrosis exhibits certain features of the interstitial pulmonary fibrosis (IPF) in humans, and it is the most commonly used model to study the pathogenesis and treatment of IPF. Even so, it also has clear limitations (the rapidity of its development, its self-limiting nature, and the severity of the associated inflammation) that should be considered when discussing the clinical relevance of therapeutic interventions in this model (Gharaee-Kermani et al., 2005).

An important finding of the present study is that estradiol and its downstream metabolite 2ME differ significantly in regard to their antimetagenic effects in cell lines involved in pulmonary fibrosis and vascular remodeling, i.e., in hLFs and hPASCs. The present study shows that estradiol has no effects on growth of hLFs in concentrations up to 10  $\mu$ M. To our knowledge, this is the first investigation of E2 in adult hLFs. Previous studies in fetal lung fibroblasts demonstrate an antimetagenic effect of estradiol only at high pharmacological concentrations (>5  $\mu$ M) (Kondo et al., 1983). Also, previous studies of cellular growth in fibroblasts from various origins reveal both stimulatory and inhibitory properties of E2 (Dubey et al. 2004; Tomaszewski J et al., 2003). The variable effects of E2 may reflect the phenotypic heterogeneity among fibroblasts, including their differentiation (particularly during growth and repair) into myofibroblasts.

Our results also show that estradiol has only modest antimetagenic effects in hPASCs. This is in contrast to the well established antimetagenic effects of E2 in vascular smooth muscle cells from systemic arteries (Dubey et al, 2004; Espinosa et al., 1996). Nonetheless, it is plausible that E2 may have different anti-remodeling effects on systemic and pulmonary blood vessels that phylogenetically are from different origins. Along these lines, E2 stimulates proliferation of rat PASCs in cell culture, has no effect on growth in intact canine pulmonary artery segments, and stimulates growth of PASCs in segments when the endothelium is removed (Farhat et al., 1992).

In contrast to E2, 2ME in a concentration-dependent manner inhibits growth of hLFs and hPASCs. This is not surprising since 2ME is a more potent antimetagen than E2 in cardiac fibroblasts, vascular smooth muscle cells and glomerular mesangial cells (Dubey et al.,

2004). In the order of potency 2ME>2HE>E2, E2 and its metabolites inhibit the proliferation of hepatic stellate cells and collagen synthesis by these fibroblast-like cells (Liu et al., 2004). Hepatic stellate cells, together with vascular smooth muscle cells, cardiac fibroblasts, and glomerular mesangial cells belong to the pericyte family. During growth or tissue injury and repair, these cells display properties of myofibroblasts and are the most important source for collagen, fibronectin, and other extracellular matrix proteins. It is notable that in bleomycin-treated animals in fibrotic areas, a significant number of cells stained positive for smooth muscle  $\alpha$ -actin, indicating myofibroblastic proliferation. Importantly, the present study suggests (using semi-quantitative assessment) reduced myofibroblastic proliferation in animals treated with 2ME.

The present study demonstrates that ovariectomy exacerbates pulmonary fibrosis and pulmonary vascular remodeling and hypertension, suggesting that E2 is protective in pulmonary hypertension due to lung fibrosis. These effects are consistent with previous reports in pulmonary hypertensive rats (Farhat et al. 1993, Tofovic et al. 2006). The effects of ovariectomy on pulmonary fibrosis are also in accordance with previous studies showing that ovariectomy increases lung collagen, airway smooth muscle thickening and cardiac hypertrophy in wild-type females and aggravates the pathology in relaxin-gene knockout female mice (Lekgabe et al., 2006) and that ovariectomy has fibrogenic effects, whereas E2 suppresses fibrotic responses in models of liver fibrosis (Liu et al., 2004; Yasuda et al., 1999). However, the present study contradicts a recent report that OVX ameliorates and exogenous E2 exacerbates bleomycin-induced pulmonary fibrosis in female Fisher rats (Gharaee-Kermani et al., 2005b). The reason for the discrepancy between the two studies is unknown, but there are several factors that may account for the different outcomes. First, there is significant strain difference among rodents with regard to sensitivity to pneumotoxins. Thus, compared to Sprague Dawley (SD) rats, Fisher rats are less sensitive (mortality) to the pneumotoxin monocrotaline and they develop a less severe inflammatory and vascular remodeling response (Pan et al., 1993). Notably, the present study shows that bleomycin produces a 3–9 times greater inflammatory response (i.e., fold increase in ED1+ cells) in SD rats than in intact and OVX female Fisher rats (Gharaee-Kermani et al., 2005b). Second, in OVX Fisher rats, there are significant plasma levels of E2 (that are twice higher than levels in SD rats), bleomycin augments the levels of E2, and E2 supplementation in Fisher rats produces plasma levels of E2 only observed in Sprague Dawley rats during pregnancy. Notably, high physiological concentrations of E2 enhance the activity of the pro-inflammatory transcription factor NF $\kappa$ B (Hirano et al., 2007), and the differences in baseline inflammatory response together with high levels of E2 may be the reason that in Fisher rats E2 is pro-inflammatory rather than anti-inflammatory (Straub, 2007).

In the present study bleomycin induced influx of macrophages into injured lungs, and ovariectomy exacerbated the inflammatory response in the lungs injured by bleomycin. This is not surprising because estrogen deficiency is associated with increases in cell adhesion molecule expression (Speyer et al., 2005), E2 inhibits the expression of cell adhesion molecules in macrophages (Frazier-Jessen et al., 1995), and ovariectomy increases whereas E2 suppresses adherence of monocytes in estrogen-deficient rats (Kurokawa et al., 2007).

Treatment with 2ME reduces both early and late inflammatory responses in diseased, estrogen-deficient animals. This remarkable effect of 2ME on inflammatory cells (Figure 5N) is consistent with our previous studies in male and OVX female rats with MCT-induced PH (Tofovic et al., 2005a, 2006). Although the mechanism of the effect of 2ME on lung macrophages is unclear, there is cumulating evidence that 2ME may have anti-inflammatory properties. Along these lines, in vivo when used in doses (30–300  $\mu$ g/kg/day) similar to that (240 $\mu$ g/kg/day) used in the present study, 2ME inhibits the adherence of monocytes to endothelium in estrogen deficient rats (Kurokawa et al., 2007), attenuates the basal or TNF-

alpha induced migration of inflammatory cells (Issekutz et al., 2008) and reduces the expression of inflammatory transcription factor NFκB (Zhang et al., 2007). *In vitro*, 2ME reduces TNF-alpha induced increases in monocyte chemoattracting protein 1 (Seeger and Mueck, 2006), and inhibits posttranscriptional activity of NFκB (Takata et al., 2005). Interestingly, 2ME not only triggers a significant decrease in cytoskeletal/structural genes (tubulin, actin), but also down-regulates the expression of genes that regulate the activity of TNF-alpha, neutrophil activation and chemotaxis (Chauhan et al., 2003). These findings raise the possibility that in addition to antimitogenic effects, 2ME may have anti-inflammatory properties. This would also suggest that the anti-inflammatory effects of E2 may be mediated, at least in part, by its downstream metabolite, and that the inhibition of the inflammatory response may also explain the antifibrotic effects of 2ME. Sufficient evidence exists to suggest that alveolar macrophages play an important role in pulmonary fibrosis. During bleomycin-induced lung injury alveolar macrophages produce large quantities of the pro-fibrotic cytokine TGF-β and fibronectin (Khalil et al., 1993).

In the present study, 2ME significantly inhibited cardiac remodeling and this effect was most likely due to reduced pulmonary hypertension. However, it is worth mentioning that 2-ME may have direct effects on RV function and remodeling. In this regard, 2ME inhibits proliferation of cardiac fibroblasts (Dubey et al., 2004) *in vitro*, and *in vivo* has direct, pressure-independent, anti-fibrotic and anti-remodeling effects in both right and left ventricles in rats with isoproterenol-induced cardiac hypertrophy (Tofovic et al. 2008). This suggests that the overall beneficial effects of 2ME may extend beyond its effects on elevated pressure and remodeling in pulmonary arteries, and may be due in part to its direct anti-remodeling effects in the right ventricle. Further studies are needed to explore this possibility.

In summary, this study suggests that, in contrast to estradiol, 2ME is antimitogen in cells involved in pulmonary vascular remodeling and fibrosis. The presented data suggest strong anti-inflammatory, anti-fibrotic and anti-remodeling effects of 2ME in a model of pulmonary fibrosis and hypertension and warrant further investigation of the role of female hormones and their metabolites and analogs in pulmonary fibrosis and associated pulmonary hypertension.

## Acknowledgments

This work was supported by NIH grant # HL080560-01A and presented in part at American Thoracic Society International Conference in San Diego, May 2006.

## ABBREVIATIONS

PH, Pulmonary hypertension; E2, 17β-estradiol; 2ME, 2-methoxyestradiol; OVX, ovariectomy; RV, right ventricle; RVPSP, right ventricular peak systolic pressure; LV+S, left ventricle plus septum; ANTU, alpha-naphthylthiourea; hLFs, human lung fibroblasts; hPASMCs, human pulmonary artery smooth muscle cells.

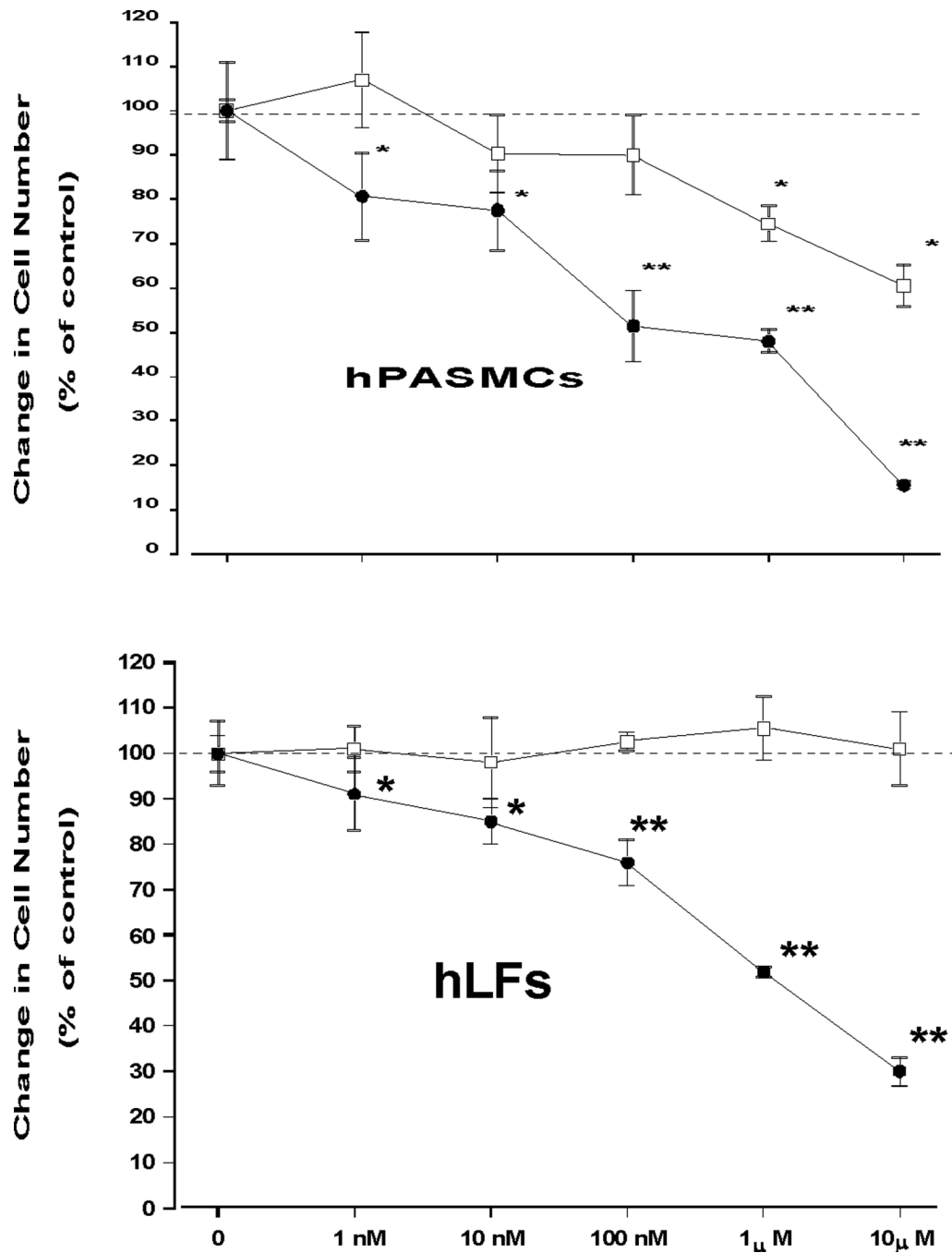
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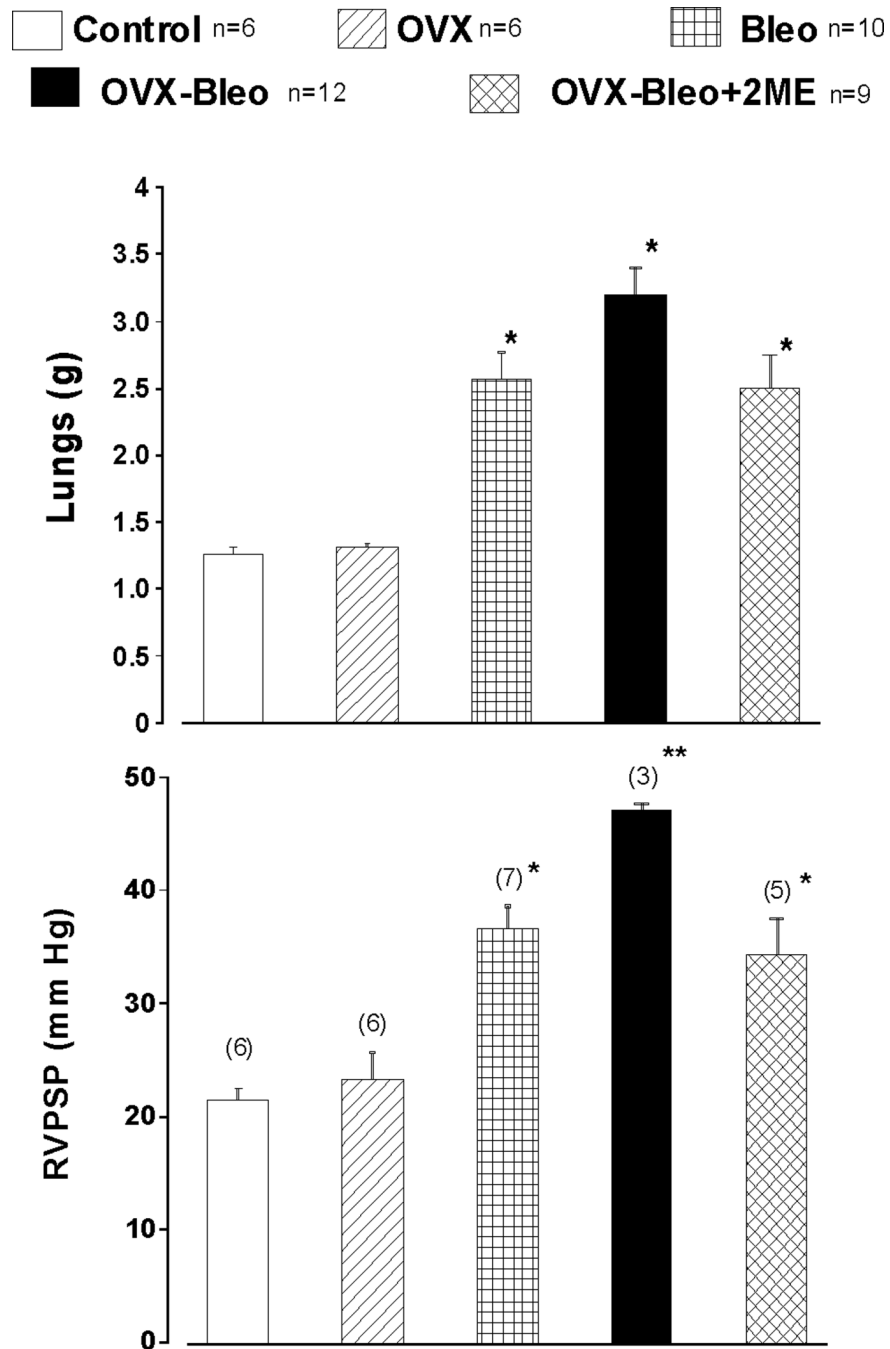


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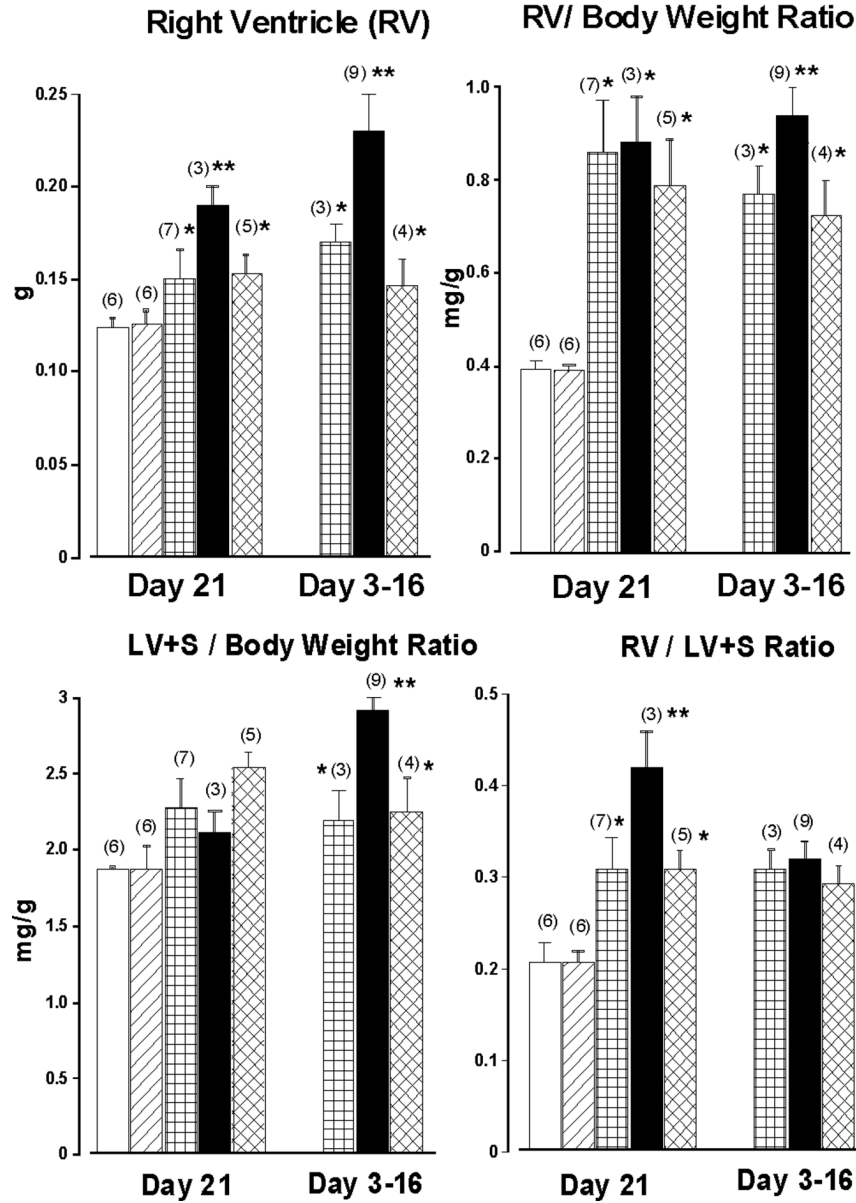
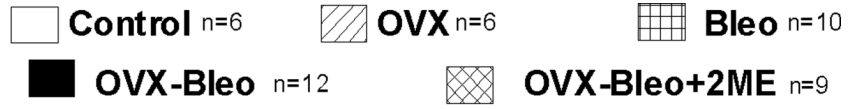
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**Figure 1.** Effects of increasing concentrations of 17β-estradiol (E2;□) and 2-methoxyestradiol (2ME;●) on cell growth in human pulmonary artery vascular smooth muscle cells (hPASMC) and human lung fibroblasts (hLF). The results are presented as the percent change from control. Values for each data point represent mean ± SEM from 2–3 separate experiments conducted in quadruplicate. p<0.05, \* - vs. control treated with FCS alone; 1 \*\* - vs. Cont & E2

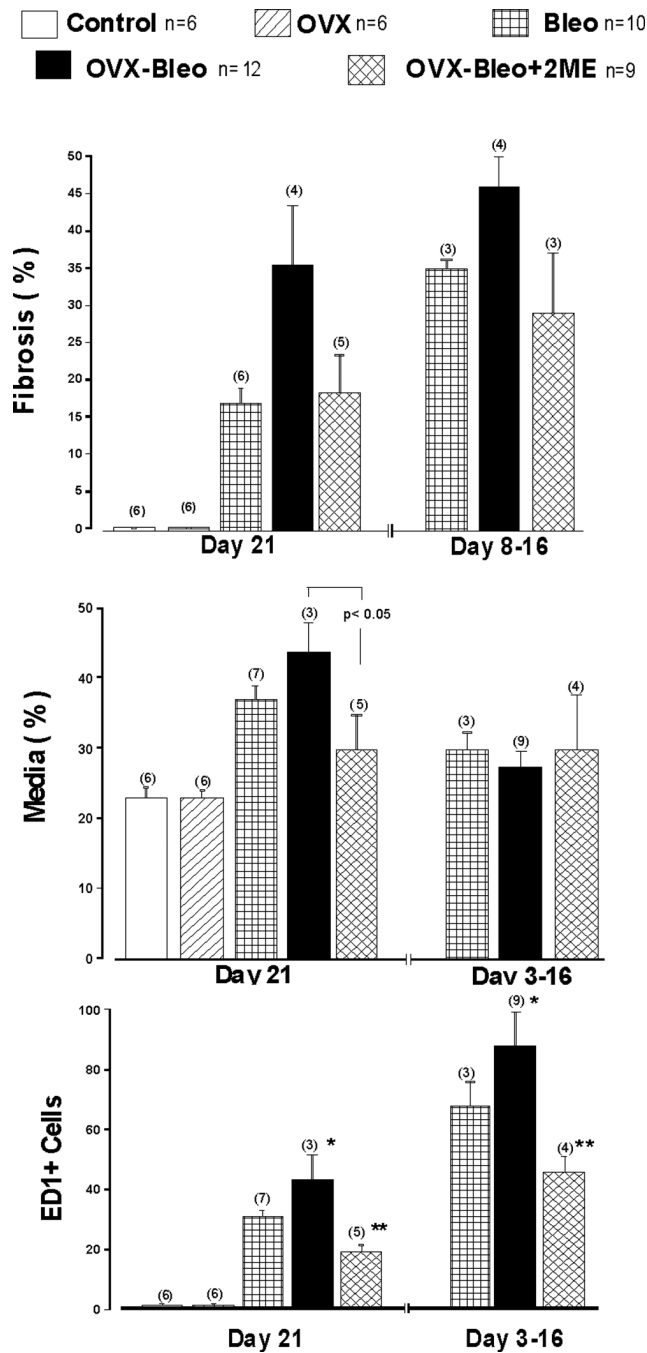


**Figure 2.** Lung weight (A) and right ventricular peak systolic pressure, RVPSP (B) in intact (Control) and ovariectomized (OVX) female rats, intact and OVX animals with bleomycin-induced pulmonary hypertension and fibrosis (Bleo and OVX-Bleo group, respectively), and OVX-Bleo rats treated with 2-methoxyestradiol (OVX-Bleo+2ME); Fisher LSD test for post-hoc comparison: \* -  $p < 0.05$  vs. respective control (Cont or OVX) \*\* -  $p < 0.05$  vs. all other groups.

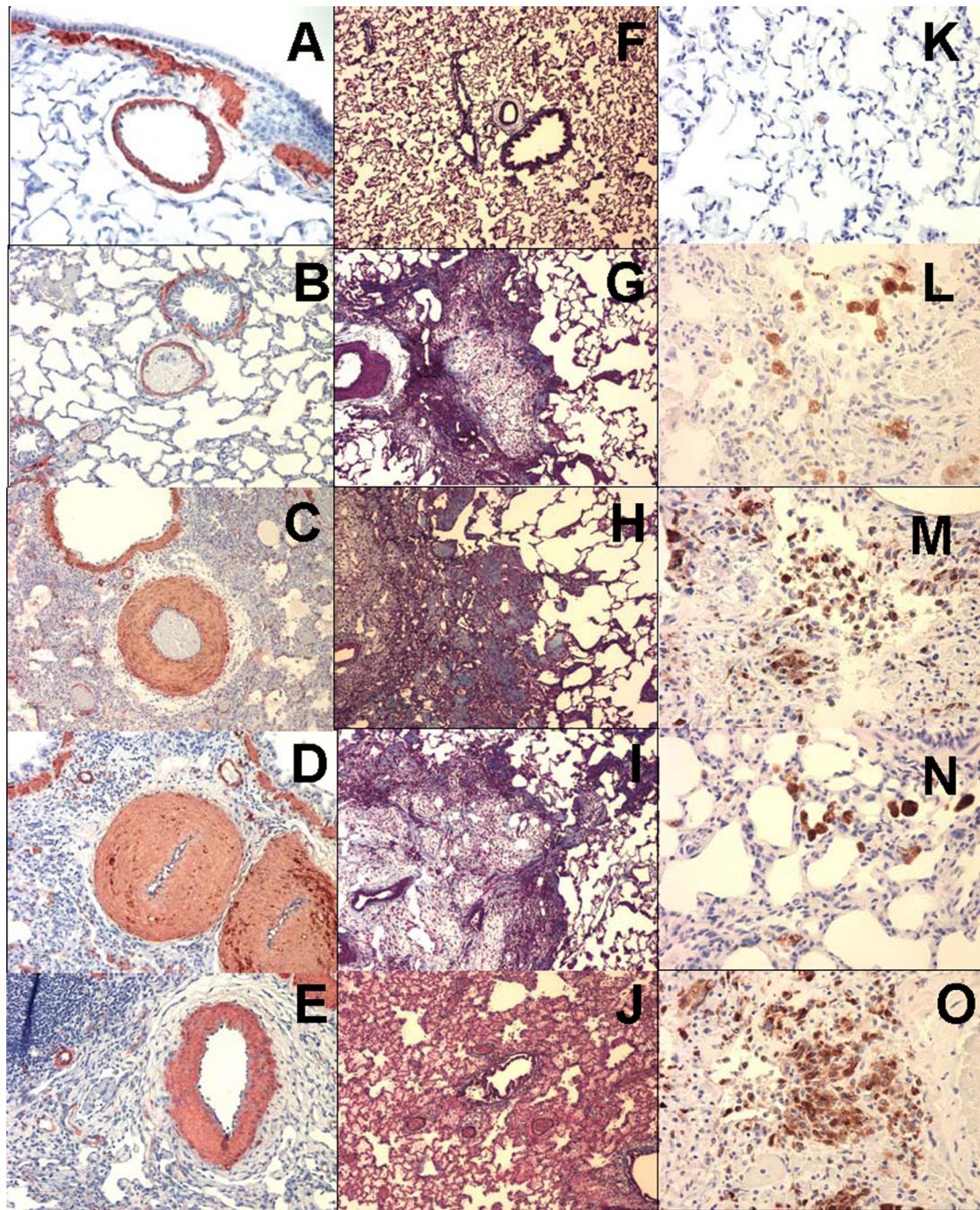


**Figure 3.** Right ventricle (RV) free wall wet weight, left ventricle plus septum (LV+S) weight and LV/LV+S ratio in intact (Control) and ovariectomized (OVX) female rats, intact and OVX female animals with bleomycin-induced pulmonary hypertension and fibrosis (Bleo and OVX-Bleo group, respectively) and OVX-Bleo rats treated with 2-methoxyestadiol (OVX-Bleo+2ME). Fisher LSD test for post-hoc comparison: \* -  $p < 0.05$  vs. respective control (Cont or OVX); \*\* -  $p < 0.05$  vs. all other groups.

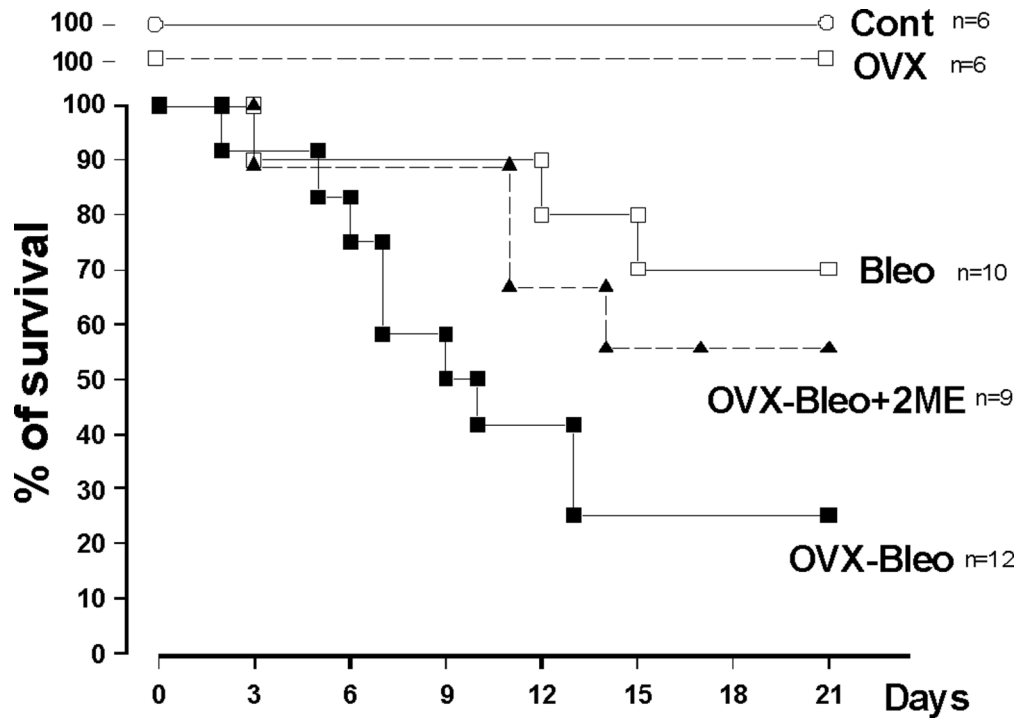




**Figure 4.** [A] Pulmonary fibrosis (Trichrome-Mason); [B] Media hypertrophy (Alpha Actin); and [C] Activated monocytes/macrophages (ED1+ cells) in the lungs of intact (Cont) and ovariectomized (OVX) controls, intact and OVX rats with bleomycin (Bleo)-induced pulmonary fibrosis and hypertension (Bleo and OVX-Bleo groups), and OVX-Bleo rats treated with 2-methoxyestradiol (OVX-Bleo+2ME); 1F-Anova,  $p < 0.001$ ; Fisher LSD test for post-hoc comparison:  $p < 0.05$ , \* - vs. Cont, OVX and OVX-Bleo+2ME, \*\* - vs. versus all other groups.



**Figure 5.** Small size pulmonary arteries (alpha actin,  $\times 20$ ) in rat lung from Control (**A**), OVX (**B**), beomycin (**C**), OVX-beomycin (**D**) and OVX-Bleo+ 2ME groups (**E**); Pulmonary fibrosis (Trichrome-Mason,  $\times 10$ ) at day 21 in rats from OVX (**F**), Bleo (**G**), OVX-Bleo (**H**) and OVX-Bleo+2ME group (**I**); Severe exudation and absence of fibrosis in OVX-Bleo rat that died at day 6 (**J**); Inflammatory cells (ED1+;  $\times 40$ ) at day 21 in rats from OVX (**K**), Bleo (**L**), OVX-Bleo group (**M**) and OVX-Bleo+2ME group (**N**) and severe inflammation in OVX-Bleo rat that died at day 13 (**O**).



**Figure 6.** Survival in intact and OVX female rats with bleomycin-induced pulmonary hypertension and fibrosis (Bleo and OVX-Bleo group), OVX-Bleo rats treated with 2-methoxyestradiol (OVX-Bleo+2ME) and respective controls (Cont and OVX group).

**Table 1**  
Vascular remodeling in control in ovariectomized rats with bleomycin-induced pulmonary fibrosis and hypertension

Group	Days into Experiment	Diameter Range	Vessels Size	Media Thickness	Media Area ( $\mu^2$ )	Adventitia Area ( $\mu^2$ )	Wall Area ( $\mu^2$ )	Wall to Lumen	Media to Lumen
Control N=6	21	53-189	103.2 ±6.0	11.0 ±0.6	2900 ±346	1612 ±207	4512 ±468	124 ±10.9	80.1 ±7.6
OVX n=6	21	52-191	100.2 ±4.7	10.4 ±0.5	2744 ±284	1247 ±123	3999 ±397	100.7 ±8.6	69.9 ±7.0
Bleomycin n=7	21	54-296	124.8* ±9.6	21.5* ±1.6	7533* ±1282	2191* ±353	9725* ±1590	304* ±40	235* ±34
OVX-Bleo N=3	21	51-299	139.7** ±16.7	30.3** ±5.0	11666** ±2859	2856** ±710	14522** ±3430	461** ±93	378** ±82
OVX-Bleo+2ME N=5	21	57-201	116.0 ±8.7	15.9 ±2.8	5468 ±1723	1616 ±369	7076 ±2031	167*** ±33	125*** ±43
Bleo n=3	3-16	68-226	154.7* ±14.7	21.0* ±2.5	7597* ±1511	2966* ±481	10563* ±1924	167* ±40	122* ±29
OVX-Bleo N=8	3-16	71-299	161.2* ±13.2	21.7* ±3.2	10100* ±2751	5105* ±962	15206* ±3357	186* ±30	121* ±23
OVX-Bleo+2ME N=4	3-16	58-286	135.1* ±11.8	22.1* ±3.1	7652* ±1856	2520 ±423	9752* ±2193	218* ±63	127* ±32

IF-Anova, p<0.05

\* - vs Control

\*\* - vs all other groups

\*\*\* - vs Bleomycin