Histol Histopathol (2012) 27: 1599-1610
DOI: 10.14670/HH-27.1599

http://www.hh.um.es

Histology and Histopathology
Cellular and Molecular Biology

Fluoxetine may worsen hyperoxia-induced lung damage in neonatal rats

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Summary. Fluoxetine shows controversial lung effects as it prevents pulmonary hypertension in adult rats but exposure during gestation causes pulmonary hypertension in neonatal rats. In the present study, we tested the null hypothesis that the antidepressant drug fluoxetine does not modify the development of bronchopulmonary dysplasia (BPD) in neonatal rats. Experimental categories included I: room air (controls) with daily injection of saline; II: room air with daily injection of 10 mg/kg fluoxetine, i.p., during two weeks; III: 60% oxygen with daily injection of saline; and IV: 60% oxygen with daily injection of 10 mg/kg fluoxetine, i.p., during two weeks. Hyperoxia resulted in significant reduction in alveolar density and an increase in pulmonary endocrine cells, as well as increases in muscle layer areas of bronchi and arteries. Fluoxetine treatment generated a further increase in muscularisation and did not significantly modify the hyperoxia-induced reductions in alveolar density and increases in the endocrine cells. In hyperoxia, Real-Time PCR showed a lower pulmonary expression of vascular endothelial growth factor (VEGF) with no significant changes in the expression of matrix metalloproteinases (MMP) 2 and 12. Fluoxetine did not affect VEGF or MMP-2 expression but it significantly increased MMP-12 mRNA in both normoxic and hyperoxic groups. Zymographic analysis of MMP-2 activity in bronchoalveolar fluid showed a significantly reduced MMP-2 activity in hyperoxia, while fluoxetine treatment restored MMP-2 activity to levels comparable with the normoxic group. In conclusion, our data show that fluoxetine may worsen bronchial and arterial muscularisation during development of BPD and may up-regulate MMP expression or activity.

Key words: Matrix metalloproteinases, Broncho-pulmonar dysplasia, VEGF

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Introduction

Selective serotonin reuptake inhibitors (SSRI), such as fluoxetine and paroxetine, are probably the antidepressants most commonly used for depressive disorders in pregnancy and following childbirth (Fleschler and Peskin, 2008). Plasma levels of fluoxetine and norfluoxetine in infants at birth are reportedly 65% and 72%, respectively, of their mothers' levels, the estimated half-lives in the newborn being 5 days for fluoxetine and 14 for norfluoxetine (Lattimore et al., 2005). There is also a risk of infants developing high serum levels of fluoxetine as a result of their mothers using the drug while breastfeeding (e.g., Weissman et al., 2004).

Bronchopulmonary dysplasia (BPD) is the most common chronic lung disease of prematurity. The severe, classic form of BPD was strongly correlated with oxygen toxicity and mechanical injury; milder forms are mainly seen nowadays in small premature infants surviving after prolonged mechanical ventilation, and relate more to immaturity, perinatal infection and inflammation, persistent ductus arteriosus, and disrupted alveolar and capillary development (Jobe and Bancalari, 2001; Bancalari et al., 2003). BPD mainly results in impaired alveolar growth and dysmorphic vascular architecture (Thebaud and Abman, 2007). Many animal models of BPD have been proposed, mainly involving

exposure to hyperoxia of premature baboons or neonatal rats and mice in the early postnatal period. Hyperoxia disrupts postnatal alveolar development, leading to smaller numbers of enlarged and simplified alveoli, thicker septa, and an increase in alveolar macrophages (e.g., Dauger et al., 2003; Balasubramaniam et al., 2007). Experimental hyperoxic models of BPD also cause changes in microvascular development and thickening of the medial muscle layer of arteries, with pulmonary hypertension (e.g., Jones et al., 1984; Koppel et al., 1994). The pulmonary effects of fluoxetine exposure in the first postnatal period and/or during development of BPD have not been investigated, but fluoxetine has different and sometimes contrasting effects on lung vasculature, depending on developmental stage. For instance, fluoxetine exposure during gestation causes foetal pulmonary hypertension, right ventricle hypertrophy and increased medial thickness of pulmonary arteries (Fornaro et al., 2007). Conversely, in adult rats, SSRIs prevent pulmonary hypertension induced by hypoxia (Marcos et al., 2003) or monocrotaline (e.g., Guignabert et al., 2005; Zhai et al., 2009; Li et al., 2011). The aim of the present work was therefore to assess the effects of postnatal fluoxetine exposure in a rat model of BPD, with particular reference to pulmonary vasculature, in order to ascertain whether fluoxetine exposure worsens hyperoxia-induced vascular changes, as in the foetal period, or whether it has therapeutic effects, as in adulthood.

As regards possible mechanisms of fluoxetine action, the pulmonary neuroendocrine cell (PNEC) population and expression of vascular endothelial growth factor (VEGF) and matrix metalloproteinases (MMPs) were also evaluated. No data on the effects of fluoxetine on PNEC populations are available. However, changes may be hypothesised, as the pharmacological action fluoxetine is also exerted on PNEC, which is the major lung source of serotonin. The number of PNECs has been reported to increase as the foetus grows, peaking at birth and during the neonatal period, and diminishing thereafter (e.g., Cutz et al., 1985; Porzionato et al., 2008). Changes in the numbers of PNECs and their peptide levels have been reported in infants with BPD (e.g., Johnson et al., 1985; Cullen et al., 2002) and in rats exposed to hyperoxia (Shenberger et al., 1997).

Modifications in the expression of VEGF and MMPs have been reported in clinical (Lassus et al., 1999; Bhatt et al., 2001; Danan et al., 2002) and/or experimental (Hosford and Olson, 2003; Hosford et al., 2004; Remesal et al., 2009) BPD and after exposure to SSRIs (Warner-Schmidt and Duman, 2007; Kubera et al., 2009). MMPs are involved in remodelling the extracellular matrix and, through their actions in processing growth factors, receptors and cytokines, they also play a role in inflammation, cell proliferation and angiogenesis. Instead, many proinflammatory mediators, growth factors, hormones and reactive oxygen species may increase the expression of various MMPs. In the present study, we considered MMP-2, which degrades the main components of the basement membrane (gelatin, elastin, fibronectin and type IV collagen), and MMP-12, which is mainly involved in elastin degradation.

Materials and methods

Animals and experimental procedures for hyperoxia exposure

Female wild-type Sprague-Dawley rats (Harlan, Udine, Italy) and their offspring were housed and handled in accordance with the guidelines of the Helsinki Declaration and the recommendations of the Italian public health authorities. The study was conducted on male or female rat pups kept together with their nursing mother in clear polished acrylic chambers, where oxygen and CO_2 were continuously monitored (BioSpherix, OxyCycler model A84XOV, Redfield, NY). The animals were maintained under standardized conditions of light (alternate 12-hour cycles of light and dark, starting with light at 08.00 hours) at a room temperature of 22°C, and a humidity level of 50%. After term gestation, the pups were randomly distributed between the following four experimental groups: 1) neonatal rats (n=10) raised in ambient air for 2 weeks and given saline solution (NaCl 0.9%) once a day intraperitoneally from postnatal day 1 to postnatal day 14 (21% + Saline); 2); neonatal rats (n=11) raised in ambient air for 2 weeks and treated with fluoxetine hydrochloride (Fluoxetine ≥99.9% [Sigma, St. Louis, MO, USA] solution dissolved in NaCl 0.9% w/v at a final concentration of 40 μg/100 μl) at a dose of 10 mg/kg given once a day intraperitoneally (21% + Flu); 3) neonatal rats (n=10) exposed to 60% oxygen for 2 weeks and given saline solution (NaCl 0.9%) once a day intraperitoneally (60% + Saline); and 4) neonatal rats (n=18) exposed to 60% oxygen for 2 weeks and treated with fluoxetine hydrochloride as described for group 2 (60% + Flu). The fluoxetine dose used in this study is known to result in rat plasma drug concentrations similar to the human therapeutic range (Stevens and Wrighton, 1993; Liu et al., 2005; Fornaro et al., 2007). The nursing dams were rotated every one or two days to prevent any negative effects of hyperoxia on nursing. The pups' body weight was measured daily. On postnatal day 14, the animals were euthanized with an overdose of tiletamine-zolazepam (Zoletil®) and xylazine (Rompun®). Bronchoalveolar fluid (BALF) was collected from the animals in each group after instilling 0.5 ml of 0.9% NaCl solution into the trachea through a 0.5 F catheter (Vygon Corporation); the fluid was centrifuged at 1500 rpm for 10 minutes and then stored at -80°C. Lung samples for molecular studies were also taken from all rats and frozen at -80°C. The animals' tracheas were then cannulated and 4% neutral buffered formalin was insufflated overnight at a pressure of 25 cm H_2O. The lungs were then removed *in toto* and fixed in buffered formalin, serially dehydrated in rising concentrations of

ethanol, and embedded in paraffin.

Lung histology and morphometric analysis

For each rat, two 4-μm sections of the lungs were stained with haematoxylin and eosin (H&E), and two sections were stained with Masson trichrome. Alveolarisation was analysed in the H&E-stained sections by performing a radial alveolar count (RAC) according to the Emery and Mithal method (1960), as revised by Cooney and Thurlbeck (1982a,b), and also by direct alveolar counting. RAC analysis was performed in 2 sections: a perpendicular line was drawn from the center of a respiratory bronchiole to the edge of the acinus, as defined by a connective tissue septum or the pleura, and the number of alveoli intersected was counted. For RAC, all respiratory bronchioles in the sections analysed were considered. At the same time, the number of alveoli/mm^2 was also assessed by counting them directly in the same two sections at a magnification of 5X. All assessments were conducted with a Leica DM 4000B microscope (Leica, Solms, Germany) integrated with a camera (Leica DFC 280).

The bronchial and arterial muscle layers were analysed on sections stained with Masson trichrome according to Fabris et al. (2001) and Fornaro et al. (2007). The internal elastic lamina was used to mark the internal arterial perimeter, and the external elastic lamina was chosen as the external arterial perimeter. Muscle layers were quantified by measuring the medial area (external minus internal arterial area, in mm^2), normalised to a given diameter to avoid bias due to heterogeneity in arterial and bronchial diameters between the experimental groups. All morphometric analyses were performed by an investigator blinded to the nature of the experimental groups.

Right ventricular hypertrophy

Hearts were removed intact and fixed in buffered formalin. The atria were removed and the right ventricular free wall was separated from the left ventricle and septal walls. The right ventricular hypertrophy index (Fulton's index) was calculated as the ratio obtained from the weight of the right ventricular free wall divided by the sum of the weights of the septum and the left ventricular free wall [RV/(LV+S)] (Fulton et al., 1952; Fornaro et al., 2007).

Anti-protein gene producto 9.5 (PGP9.5) immunohistochemistry

Lung sections were hydrated gradually and were incubated in 0.03% hydrogen peroxide in deionised H_2O, to eliminate endogenous peroxidase activity and enhance antibody penetration in the tissue. Antigen unmasking was performed with 10 mM sodium citrate buffer, pH 6.0, at 96°C for 30 min. Sections were incubated for 30 min in blocking serum (0.04% bovine serum albumin (A2153, Sigma-Aldrich, Milan, Italy) and 0.5% normal goat serum (X0907, Dako Corporation, Carpinteria, CA, USA) to eliminate unspecific binding. Sections were then incubated for 1 hour at room temperature with a rabbit polyclonal antibody against PGP9.5 (Ab1761, Millipore) diluted 1:500 in PBS. Primary antibody binding was revealed by incubation with anti-rabbit/mouse serum diluted 1:100 in blocking serum for 30 min at room temperature (DAKO® EnVision + TM Peroxidase, Rabbit/Mouse, Dako Corporation, Glostrup, Denmark) and developed in 3,3'-diaminobenzidine for 3 min at room temperature. Lastly, sections were counterstained with haematoxylin. Sections incubated without primary antibodies showed no immunoreactivity, confirming the specificity of the immunostaining.

Polymerase chain reaction

Total RNA was extracted from lung samples stored at -80°C with TRIzol reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). The samples were further treated with DNase I (DNA-*free,* Ambion, Austin, TX, USA) to exclude DNA contamination. RNA quality was assessed by formaldehyde agarose gel electrophoresis. Total RNA (100 ng) was added to a reaction mixture containing 100 ng of random nanomers (Stratagene, La Jolla, CA, USA), 1X cDNA first-strand buffer, 500 mM of DTT, 0.4 U/μL of RNase inhibitor, 1 mM each of deoxyribonucleoside triphosphate (dNTP) and 0.75 U/μL of Superscript II reverse transcriptase (Invitrogen). Negative RT (no enzyme) and no-template controls were also included. The RT thermal cycle was 25°C for 10 min, 50°C for 45 min, and 85°C for 5 min.

A quantitative real-time PCR assay was also performed with SYBR Green I (Applied Biosystems) as fluorescent intercalation dye in double-stranded DNA during the amplification cycles. The housekeeping glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as internal standard. Assays were performed in 96 multi-well PCR plates covered with optical tapes in the Applied Biosystems 7500 Real-Time PCR System, in a final volume of 25 μl containing 10 ng of cDNA, 12.5 μl of Power Master Mix 2X (Applied Biosystems), 5 μM of VEGF or MMP-2, or MMP-12 or GAPDH forward and reverse primers, and water. The sequences of the primers used were as follows:

VEGF: d-ATGACGAGGGCCTGGAGTGTG
r-CCTATGTGCTGGCCTTGGTGAG
MMP-2: d-TGCCATCCCTGATAACCTGGAT
r-CTCCTTCAGACTTTGGTTCTCG
MMP-12: d-AGACCCAAAAGAGAACCAACG
r-TTCTGGCTGAAGGTTTCTGAG
GAPDH: d-TGAGGACCAGGTTGTCTC
r-ACAGCGTCGAATCCTTTGAG

The reaction was subjected to denaturation at 95°C for 10 minutes, followed by 40 cycles of denaturation at 95°C for 30 seconds, annealing, and elongation at 60°C

for 1 minute. The levels of mRNA expression were assessed by relative quantitative real-time PCR in each lung from the experimental animals and normalised to GAPDH. The comparative Ct method (ΔΔCt) was used to quantify gene expression, and the relative quantification was calculated as 2-ΔΔCt. Gene expression levels in animals raised in room air were assumed to be 100%, and the VEGF and MMP levels in the other samples were calculated in relation to this value.

Zymographic analysis of MMP-2 gelatinase activity

Zymographic analysis of BALFs was carried out as described previously (Slongo et al., 2002). Equal amounts of BALF in sample buffer (4% SDS, 125 mM Tris-HCl pH 6.8, 20% glycerol and 0.05% bromophenol blue) were resolved in non-reducing conditions on 10% SDS-PAGE gels copolymerised with 0.1% gelatin. After electrophoresis, gels were washed for 30 min in 2.5% Triton X-100 at room temperature to remove SDS, equilibrated for 30 min in collagenase buffer (50 mM Tris, 200 mM NaCl, 5 mM $CaCl_2$ and 0.02% Triton X-100, pH 7.4) and incubated overnight with fresh collagenase buffer at 37°C. After incubation, gels were stained in 0.1% Coomassie Brilliant Blue R-250 30% MetOH/10% acetic acid for 1 hour and destained in 30% MetOH/10% acetic acid. Clear bands on the blue background represented areas of gelatinolysis. Digestion bands were analysed with ImageJ software (http://rsb.info.nih.gov/ij/).

Statistics

Results are expressed as mean values ± standard deviation (SD). Statistical analysis was performed with one-way ANOVA and Tukey's multiple comparison test. A $P<0.05$ was considered statistically significant. Statistical calculations were conducted with Prism 3.0.3 (GraphPad Software Inc., San Diego, CA, USA).

Results

Growth

No statistically significant differences in body weight were seen between the different experimental groups (21% + Saline: 20.6±2.0 g; 21% + Flu: 20.5±2.3

Table 1. Lung morphometry parameters in the four experimental groups.

	21% + Saline	21% + Flu	60% + Saline	60% + Flu	
RAC					
Mean ± SD	15.54±3.801	16.09±2.449	13.71±2.258	14.31±2.662	
[Median]	[15.70]	[16.30]	[12.90]	[14.50]	P>0.05
(95% CI)	(12.82-18.25)	(14.45-17.74)	(12.09-15.33)	(12.98-15.63)	
Alveoli/mm^2					
Mean ± SD	39.87±7.325	39.23±6.796	29.87±5.486	30.35±7.252	21%+Saline and 21%+Flu
[Median]	[38.25]	[39.50]	[29.45]	[30.50]	vs
(95% CI)	(34.63-45.10)	(34.67-43.80)	(25.94-33.79)	(26.74-33.95)	60%+Saline (P<0.05) and 60%+Flu (P<0.01)
Bronchial muscle layer					
Mean ± SD	2697±621	3699±529	4305±867.7	5474±1137	60%+Saline vs 21%+Saline (P<0.01)
[Median]	[2424]	[3638]	[4256]	[5836]	60%+Flu vs 21%+Saline; 21%+Flu (P<0.001)
(95% CI)	(2253-3142)	(3343-4054)	(3685-4926)	(4908-6039)	and 60%+Saline (P<0.01)
Arterial muscle layer (<50 μm)					
Mean ± SD	1568±819.4	1680±751.7	1938±653.8	1739±643.9	
[Median]	[1322]	[1442]	[1932]	[1571]	P>0.05
(95% CI)	(982.1-2154)	(1175-2185)	(1471-2406)	(1419-2060)	
Arterial muscle layer (50-100 μm)					
Mean ± SD	1377±397.1	3185±491.3	3203±839.5	4783±906.2	21%+Saline vs 21%+Flu; 60%+Saline
[Median]	[1317]	[3241]	[3159]	[4970]	and 60%+Flu (P<0.001)
(95% CI)	(1093-1661)	(2855-3515)	(2602-3803)	(4333-5234)	60%+Saline and 21%+Flu vs 60%+Flu (P<0.001)
Right Ventr. Hypertr. Index					
Mean ± SD	0.26±0.03	0.29±0.03	0.33±0.05	0.38±0.04	60%+Saline vs 21%+Saline (P<0.001)
[Median]	[0.25]	[0.28]	[0.33]	[0.37]	60%+Flu vs 21%+Saline; 21%+Flu (P<0.001)
(95% CI)	(0.24-0.28)	(0.27-0.31)	(0.29-0.37)	(0.36-0.39)	and 60%+Saline (P<0.05)
PNEC/100mm					
Mean ± SD	10.70±2.879	10.29±2.289	16.30±5.293	15.00±3.308	60%+Saline vs 21%+Saline;21%+Flu (P<0.01)
[Median]	[11.25]	[10.50]	[17.00]	[14.50]	60%+Flu vs 21%+Saline (P<0.05)
(95% CI)	(8.640-12.76)	(8.753-11.83)	(12.51-20.09)	(13.36-16.64)	and 21%+Flu (P<0.01)

RAC: Radial Alveolar Count; PNEC: Pulmonary Neuroendocrine Cells; Flu: Fluoxetine.

g; 60% + Saline: 18.9±2.2 g; 60% + Flu: 19.1±1.6 g).

Lung histopathology and morphometric analysis

Histopathological examination of the lung sections showed an impaired alveolar development in the two hyperoxic groups by comparison with the control animals in room air. The distal airspaces were fewer in number and enlarged, with reduced septation. Patchy areas of marked interstitial thickening were also appreciable. In both normoxia and hyperoxia, there were no clear histopathological differences between the fluoxetine-treated and the saline-treated animals (Fig. 1). The results of the lung morphometric analysis are listed in Table 1. The radial alveolar count identified no statistically significant differences between the various experimental groups, but morphometric analysis of the lung alveolarisation showed a significantly lower alveolar density in the hyperoxic groups than in the normoxic animals. In both normoxia and hyperoxia, there were no statistically significant differences between the fluoxetine-treated and the correspondent saline-treated groups, indicating that fluoxetine has no direct effect on lung alveolarisation.

A morphometric analysis was also performed on the muscle layers of the bronchi and arteries. The bronchi of rats exposed to hyperoxia and treated with saline solution showed an increased muscle layer area than in the normoxia-exposed, saline-treated animals. In addition, the rats exposed to hyperoxia and treated with fluoxetine showed a significantly thicker muscle layer than in the other three groups, indicating a muscularizing effect of fluoxetine on the muscle layers of the bronchi in hyperoxic conditions (Fig. 2). Conversely, fluoxetine

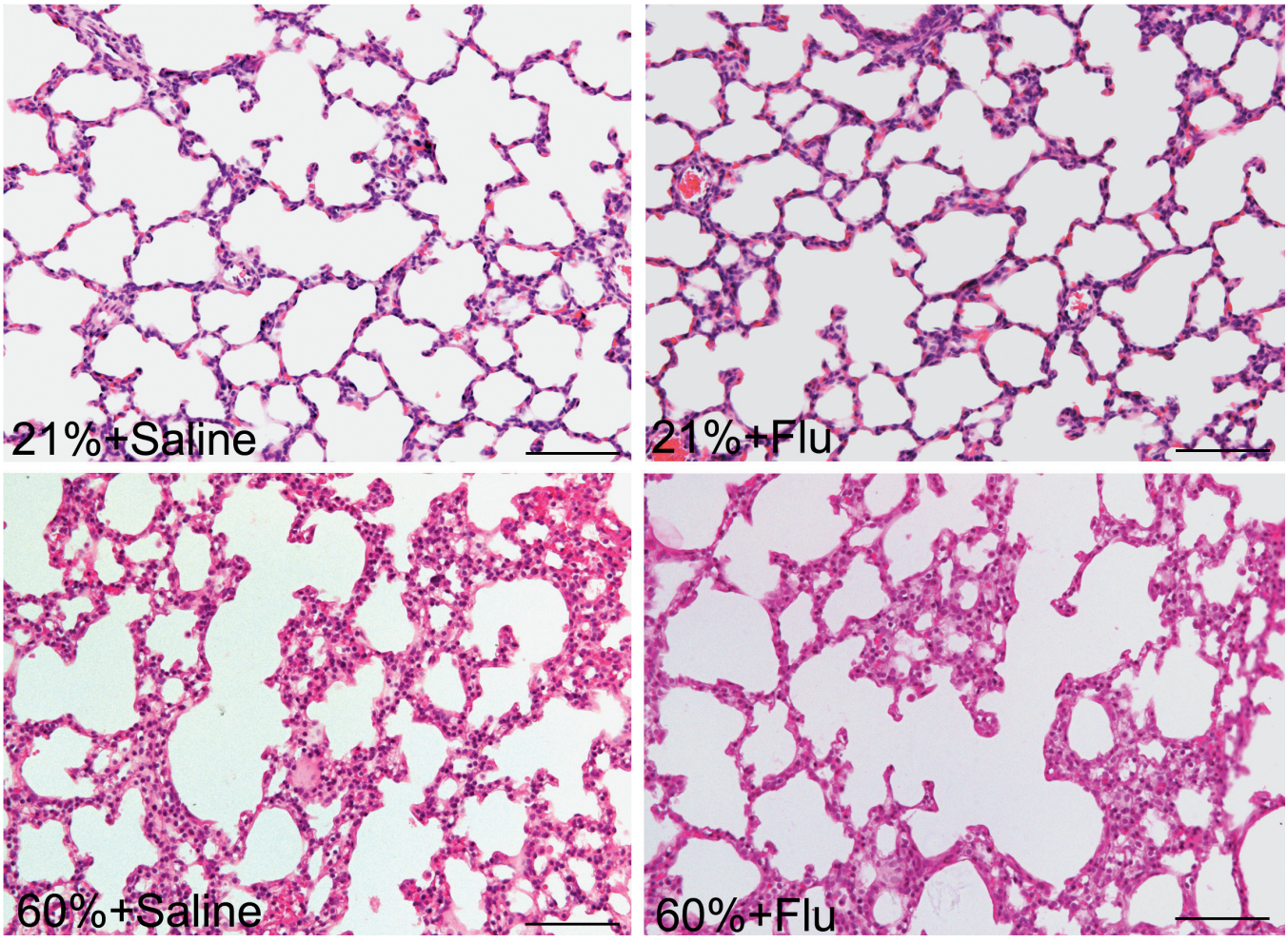

Fig. 1. Representative lung sections of the four study groups stained with haematoxylin and eosin, showing impaired alveolar development (enlarged airspaces and areas of parenchymal thickening) in hyperoxia-exposed rats irrespective of fluoxetine treatment. (21% + Saline: control animals housed in room air and treated with saline solution; 21% + Flu: animals housed in room-air and treated with fluoxetine; 60% + Saline: animals in 60% hyperoxic conditions treated with saline solution; 60% + Flu: animals in 60% hyperoxic conditions treated with fluoxetine). Scale bars: 75 μm.

exposure in normoxic rats coincided with a trend towards a thicker muscle layer, but the difference was not statistically significant.

A muscularisation effect was not clearly appreciable in arteries less than 50 μm in diameter, whereas the normoxic rats exposed to fluoxetine treatment and the animals in both the hyperoxic groups showed an increased muscle layer area in arteries 50 to 100 μm in diameter than in the normoxic saline-treated control animals. The rats exposed to hyperoxia and treated with fluoxetine also showed a statistically significant increase in the muscle layer area by comparison with hyperoxia-exposed saline-treated and normoxia-exposed fluoxetine-treated animals. The above findings indicate a muscularisation effect of fluoxetine on the muscle layers of arteries more than 50 μm in diameter in both normoxic and hyperoxic conditions (Fig. 3).

Right ventricular hypertrophy

The rats exposed to hyperoxia and treated with saline had a higher Fulton's index than the normoxic saline-treated animals. Exposure to hyperoxia and treatment with fluoxetine led to a significantly worse right ventricular hypertrophy than in the other three groups, whereas fluoxetine treatment in normoxia had no statistically significant influence on Fulton's index.

PNEC populations

PGP9.5-positive PNECs and NEBs were more numerous under hyperoxic experimental conditions than in the rats exposed to room air. In both normoxia and hyperoxia, there were no statistically significant differences between the groups treated with fluoxetine

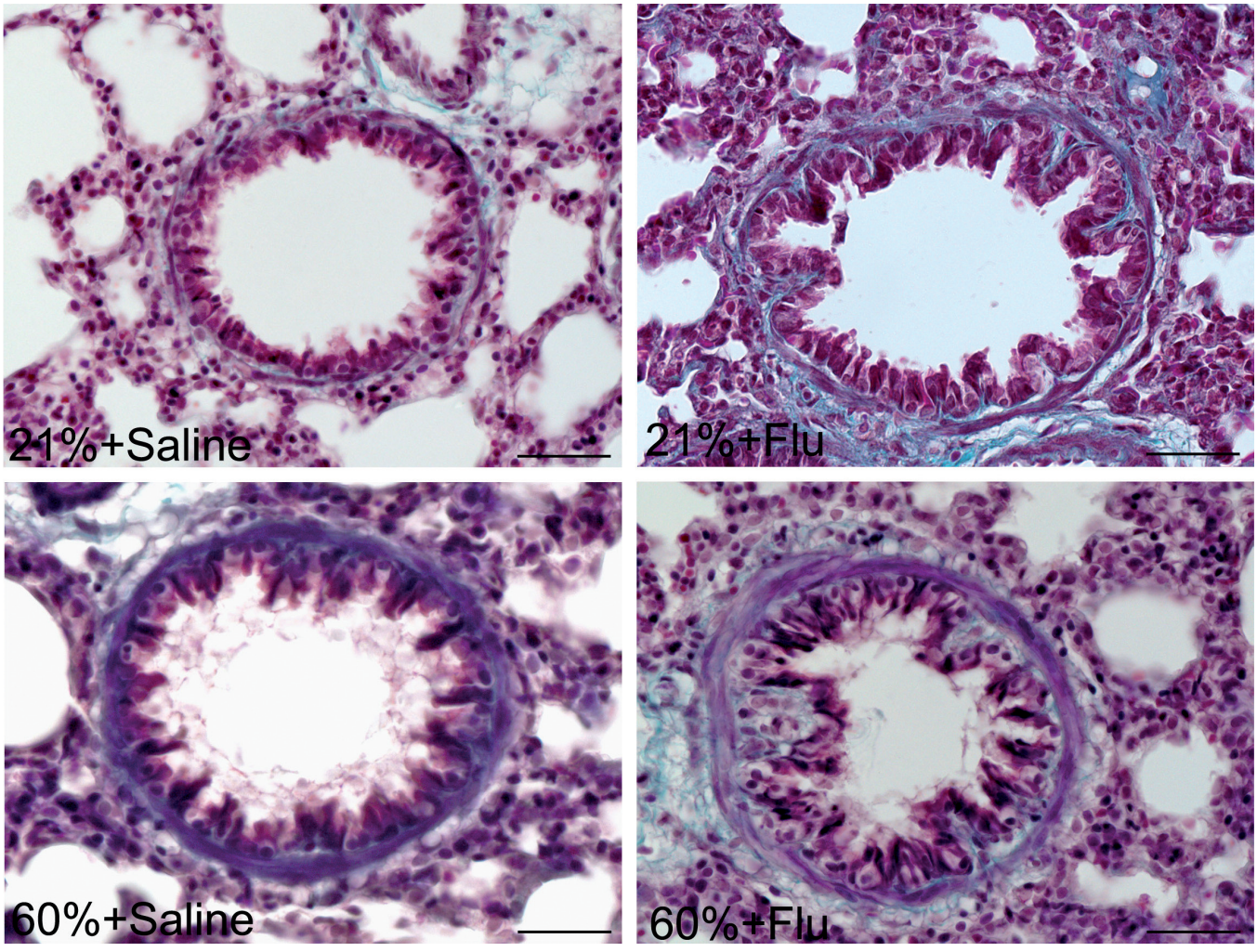

Fig. 2. Representative lung sections of the four study groups stained with Masson trichrome, showing muscle layer thickening in the bronchi of hyperoxic saline-treated animals and further increase in muscle thickness in the hyperoxic fluoxetine-treated rats. (21% + Saline: control animals housed in room air and treated with saline solution; 21% + Flu: animals housed in room-air and treated with fluoxetine; 60% + Saline: animals in 60% hyperoxic conditions treated with saline solution; 60% + Flu: animals in 60% hyperoxic conditions treated with fluoxetine). Scale bars: 37.5 μm.

and the corresponding groups given saline (Fig. 4).

VEGF mRNA expression

The results of the analysis on VEGF and MMP expression are listed in Table 2. The quantitative detection of VEGF mRNA in lung samples (using Real-Time PCR and normalizing the results to the means for the control group) indicated that VEGF expression was lower in hyperoxic experimental groups than in control rats exposed to room air. Fluoxetine treatment did not influence VEGF expression in either normoxia or hyperoxia.

Metalloproteinase expression and activity

Real-Time PCR revealed no statistically significant differences in MMP-2 mRNA content in the lung samples of the experimental groups, but zymographic analysis of MMP-2 gelatinase activity in the BALF (normalized to the mean for the control group) showed a significantly lower lung MMP-2 activity in the saline-treated animals exposed to 60% hyperoxia than in the saline- or fluoxetine-treated animals under normoxic conditions. Fluoxetine treatment was found to significantly increase lung MMP-2 activity in the animals in hyperoxic conditions, but not in normoxia.

Quantitative Real-Time PCR of MMP-12 mRNA in lung samples did not show statistically significant changes in saline-treated animals after hyperoxia exposure. A significantly higher MMP-12 mRNA content was found in rats given fluoxetine treatment, in both normoxic and hyperoxic conditions, fluoxetine treatment giving rise to higher MMP-12 expression

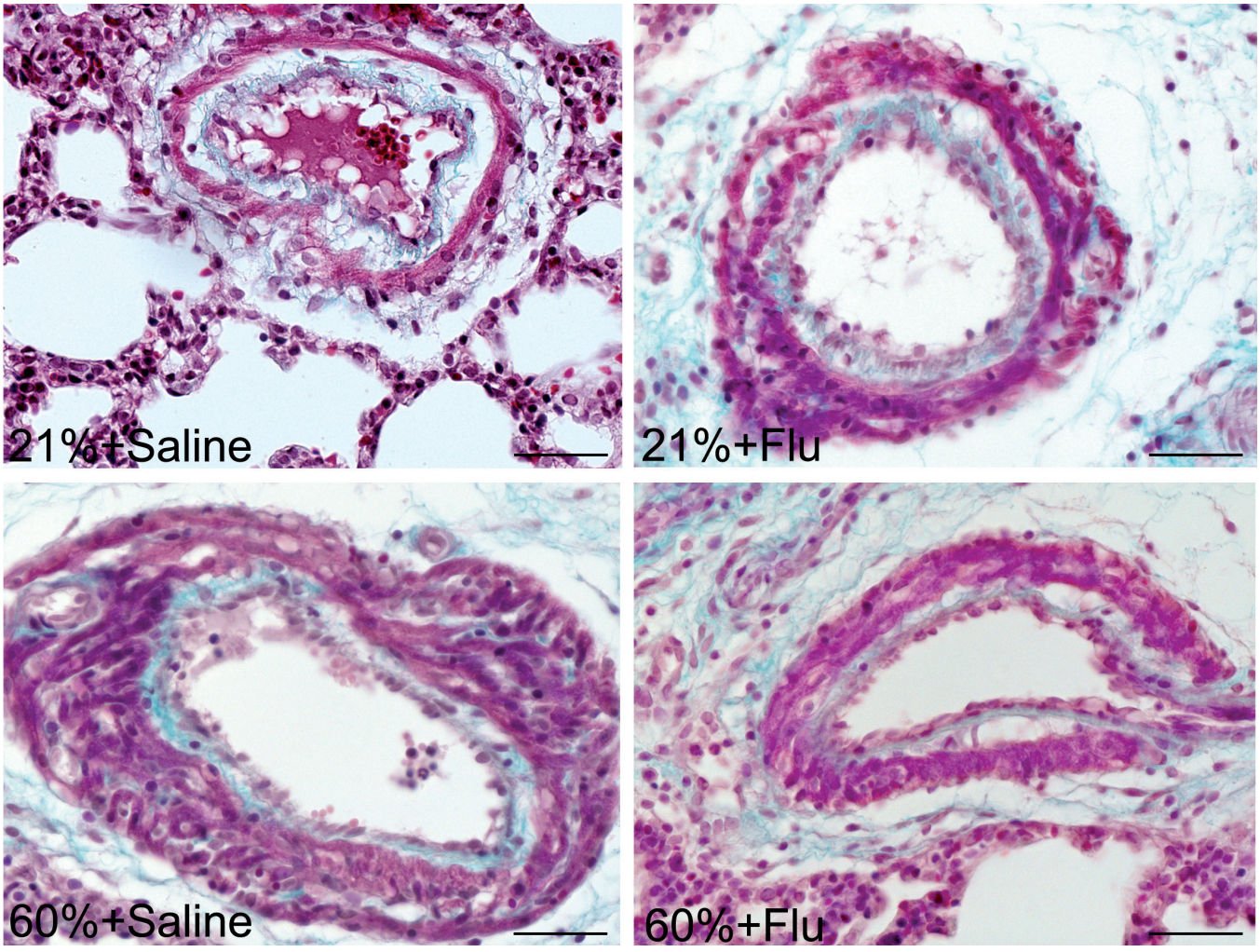

Fig. 3. Representative lung sections of the four study groups stained with Masson trichrome, showing muscle layer thickening in arteries 50-100 μm in diameter of normoxic fluoxetine-treated and hyperoxic saline-treated animals. Note a further increase of the muscle layer area in hyperoxic fluoxetine-treated rats. (21% + Saline: control animals housed in room air and treated with saline solution; 21% + Flu: animals housed in room-air and treated with fluoxetine; 60% + Saline: animals in 60% hyperoxic conditions treated with saline solution; 60% + Flu: animals in 60% hyperoxic conditions treated with fluoxetine). Scale bars: 37.5 μm.

Table 2. Expression of Vascular Endothelial Growth Factor (VEGF) and Metalloproteinases 2 (MMP2) and 12 (MMP12) in the four experimental groups.

	21% + Saline	21% + Flu	60% + Saline	60% + Flu	
VEGF/GAPDH					
Mean ± SD	100±8.97	101.9±17.30	71.71±9.577	73.19±16.16	21% + Saline and 21% + Flu
[Median]	[98.75]	[100.0]	[72.97]	[75.20]	vs
(95% CI)	(93.61-106.4)	(90.30-113.6)	(64.86-78.57)	(65.15-81.23)	60% + Saline and 60%+Flu (P<0.001)
MMP2/GAPDH					
Mean ± SD	100.0±22.55	104.2±16.11	90.60±22.24	82.97±23.34	
[Median]	[95.44]	[108.2]	[94.03]	[82.65]	P>0.05
(95% CI)	(83.90-116.2)	(93.33-115.0)	(74.69-106.5)	(71.36-94.58)	
MMP2 activity					
Mean ± SD	100.0±28.05	117.9±18.51	59.42±27.31	94.07±24.08	60% + Saline vs
[Median]	[99.05]	[112.9]	[57.34]	[95.40]	21%+Saline, 60%+Flu (P<0.01)
(95% CI)	(79.95-120.1)	(105.5-130.4)	(39.88-78.96)	(82.09-106.0)	and 21%+Flu (P<0.001)
MMP12/GAPDH					
Mean ± SD	100.0±23.93	184.7±49.48	105.1±11.01	141.0±25.85	21% + Saline and 60% + Saline vs 21%+Flu (P<0.001)
[Median]	[102.3]	[178.0]	[103.8]	[136.5]	60% + Flu vs 21%+Flu; 21% + Saline (P<0.01)
(95% CI)	(82.92-117.2)	(151.5-217.9)	(97.24-113.0)	(128.2-153.9)	and 60% + Saline (P<0.05)

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

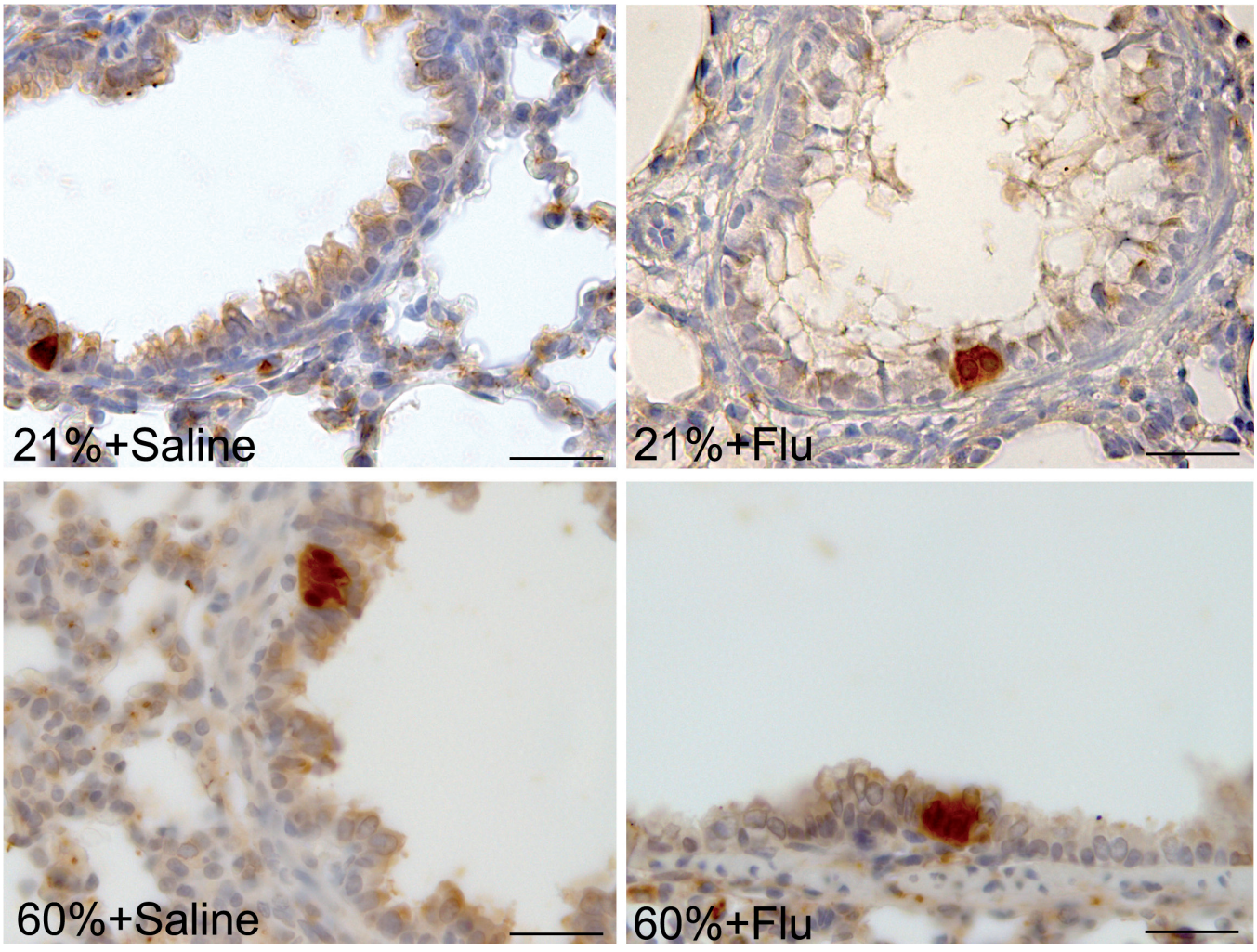

Fig. 4. Representative lung sections stained immunohistochemically for PGP9.5 showing higher numbers of pulmonary neuroendocrine cells (PNEC) in hyperoxia-exposed animals irrespective of fluoxetine treatment. (21% + Saline: control animals housed in room air and treated with saline solution; 21% + Flu: animals housed in room-air and treated with fluoxetine; 60% + Saline: animals in 60% hyperoxic conditions treated with saline solution; 60% + Flu: animals in 60% hyperoxic conditions treated with fluoxetine). Scale bars: 23.8 μm.

levels in the rats under normoxic conditions than in those exposed to hyperoxia.

Discussion

Hyperoxia exposure has been used in other studies mainly to develop experimental animal models of BPD in prematurely born infants, because hyperoxia disrupts postnatal alveolar and microvascular development (e.g., Dauger et al., 2003; Balasubramaniam et al., 2007). The present study confirmed that alveolarisation is impaired by exposure to hyperoxia, resulting in fewer, larger-diameter alveoli with reduced septation and patchy areas of parenchymal thickening. Morphometric analysis also showed a lower alveolar density in the groups exposed to hyperoxia; the absence of any significant differences in the RAC was probably due to the heterogeneity of the histopathological changes involved, as reported previously (Yi et al., 2004). However, fluoxetine treatment did not significantly modify alveolar density in either normoxic or hyperoxic groups, revealing neither a therapeutic nor an exacerbating effect of fluoxetine on the changes in alveolarisation caused by exposure to hyperoxia.

Apart from the above changes, exposure to hyperoxia was found to increase the thickness of the muscle layers of the bronchi and of arteries 50- to 100-μm in diameter (but not of smaller arteries), with consequent right ventricular hypertrophy. The above findings are consistent with published reports, showing that exposure to hyperoxia may cause airway smooth muscle hypertrophy and/or airway hyper-reactivity (e.g., Shenberger et al., 1997; Belik et al., 2003) and thickening of the medial muscle layer of the intra-acinar and pre-acinar arteries, with pulmonary hypertension (Jones et al., 1984; Koppel et al., 1994).

Effects of fluoxetine on vascular and bronchial musculature

Studies indicate different effects, depending on the lung's developmental stage (adulthood or foetal period). In adult rats, experimental findings indicate that SSRIs prevent hypoxia (Marcos et al., 2003) or monocrotaline induced pulmonary hypertension (e.g., Guignabert et al., 2005; Zhai et al., 2009; Li et al., 2011), although treatment with SSRIs does not demonstrate any statistically significant improvement in the mortality rate of adult patients with pulmonary artery hypertension (Kawut et al., 2006). Conversely, an association has been reported between maternal SSRI therapy after the 20th week of gestation and pulmonary hypertension in the newborn (Chambers et al., 2006). It has also been reported that fluoxetine treatment in rats during gestation results in foetal pulmonary hypertension, with a higher ratio of the right ventricle to the left ventricle plus septum, increased medial thickness of pulmonary arteries 50- to 200-μm in diameter, and decreased arterial oxygen saturation on the first postnatal day. Fluoxetine can also induce pulmonary artery smooth muscle contraction in foetal, but not in adult animals. *In vitro* exposure to low concentrations of fluoxetine also significantly increase cell proliferation in the pulmonary artery smooth muscle cells of foetal but not adult rats (Fornaro et al., 2007).

The present work is the first to consider the effects of fluoxetine treatment in the early postnatal period in rats exposed to normoxia or hyperoxia. The drug revealed no therapeutic effect, but showed a muscularisation effect on the larger arteries of normoxic rats and caused a further significant increase in the thickness of the muscle layer in hyperoxic conditions, clearly exacerbating the hyperoxia-induced arterial muscularisation effect. In this sense, hyperoxia-exposed fluoxetine-treated rats also showed a further significant increase in right ventricular hypertrophy than hyperoxia-exposed saline-treated ones. Thus, the effects of fluoxetine in the early postnatal period seem to be more similar to those during gestation than to the therapeutic effects seen in adult rats, confirming the relevance of the developmental stage of lung vessels.

In the present work, fluoxetine was also found to further increase the muscle layer areas of the bronchi less than 150 μm in diameter in hyperoxia-exposed animals, worsening hyperoxia-induced histopathological changes. Further studies will be needed to verify the effect of fluoxetine on neonatal rats after its administration to nursing maternal rats, as the human infants are obviously not directly exposed to fluoxetine except through maternal intake.

Effects of fluoxetine treatment on PNEC

To investigate the possible pathogenic mechanisms behind the above-described effects of fluoxetine on the muscle layers of the bronchi and arteries, we considered the PNEC population and VEGF and MMP expression. There are many reports in the literature of increased PNEC populations in BPD and in experimental models of this condition. Increased numbers of bombesin-like peptide-, calcitonin- and serotonin-immunoreactive PNECs have been reported in infants dying of BPD (e.g., Johnson et al., 1985), and high bombesin-like peptide levels have been demonstrated in early postnatal urine from infants at higher risk of BPD (Cullen et al., 2002). Shenberger et al. (1997) also reported that exposing 21-day-old rats to 2 weeks of >95% hyperoxia prompted an increase in the numbers of PNECs. Our study is the first to confirm this increase in neonatal rats exposed to moderate hyperoxia in the first two weeks after birth.

We also hypothesised that fluoxetine might modify PNEC populations, since they are known to be the main source of serotonin in the lung. However, the drug produced no statistically significant changes in them, in either normoxia or hyperoxia. Further studies will be needed to reveal fluoxetine-induced changes in the

synthesis and/or release of PNEC mediators.

Effects of fluoxetine treatment on VEGF expression

Prolonged hyperoxia exposure during the newborn period has been reported to reduce lung VEGF expression in many experimental animals (e.g., Remesal et al., 2009; Hosford and Olson, 2003), and lower VEGF levels have also been reported in the lungs of infants dying of BPD (Lassus et al., 1999; Bhatt et al., 2001). Lower VEGF expression in the lung was also seen in our hyperoxia-exposed groups, confirming the literature data.

VEGF is known to increase the proliferation of smooth muscle cells (e.g., Li et al., 2009), so it seemed worth looking for possible further changes in its expression in fluoxetine-treated rats as potential mechanisms behind the drug's muscularisation effect. Some authors have reported increased VEGF expression in nervous tissues and cells exposed to fluoxetine (Warner-Schmidt and Duman, 2007; Greene et al., 2009; Allaman et al., 2011), but there are no such data on the lung. In our model, fluoxetine exposure did not affect VEGF expression in either normoxia-exposed or hyperoxia-exposed neonatal rats, suggesting that the arterial and bronchial muscle layer thickening seen in fluoxetine-treated animals may not be VEGF-dependent (although changes in VEGF expression at earlier time points cannot be excluded). It must also be recalled that the effects of fluoxetine on VEGF expression are probably tissue- and cell-specific, since fluoxetine has been demonstrated to reduce VEGF secretion in ameloblast-like cells LS8 (Riksen et al., 2010).

Effects of fluoxetine treatment on MMPs

Changes in MMP expression and activation have been reported in many human lung diseases and in experimental animal models of BPD. Data on lung MMP-2 expression and activity in response to hyperoxia are somewhat controversial and probably depend on the experimental model involved. Some authors found no changes in MMP-2 mRNA or pro-enzyme content, but they did see reduced active protein levels and MMP-2 activity in neonatal rats exposed to >95% O_2 (Hosford et al., 2004). Lower total MMP-2 levels in tracheal aspirates from premature infants have also been associated with the onset of BPD (Danan et al., 2002). In our experimental model of hyperoxia exposure, although there were no significant changes in MMP-2 expression, there was a reduction in MMP-2 activity. The differences in MMP-2 responses to hyperoxia may be mainly attributable to the levels and timing of hyperoxia exposure, partly in relation to postnatal age. For instance, MMP-2 activity in newborn rats increased after two days of >90% hyperoxia, then dropped to below control values after 4, 6 and 8 days (Buckley and Warburton, 2002). The reduced MMP-2 activity in our experimental model may be the result of the fairly long period of exposure to moderate hyperoxia.

As regards MMP-12, few studies are available in the literature on its expression in experimental models of BPD: it was found to be higher in premature rats exposed to 100% oxygen during the first two weeks after birth (Wagenaar et al., 2004), but no statistically significant changes emerged in the present study, possibly owing to the limited degree of hyperoxia exposure.

Serotonin has been reported to stimulate the expression of some MMPs (Nocito et al., 2008) and recent studies have also examined the possible role of SSRIs on MMP expression. For instance, fluoxetine treatment has been reported to raise pro-MMP-2 and active MMP-2 plasma levels, respectively, in young and old mice transplanted with melanoma (Kubera et al., 2009). In our study, fluoxetine increased MMP-2 activity in hyperoxia-exposed animals and increased MMP-12 expression in both normoxic and hyperoxic groups. That MMPs are involved in producing the changes seen in the bronchial and arterial walls as a result of fluoxetine treatment is intriguing, because these metalloproteinases may induce smooth muscle cell proliferation and migration (Johnson and Knox, 1999; Dwivedi et al., 2009; Nishihara-Fujihara et al., 2010; Wang et al., 2011). However, increased MMP expression and activity may also be the consequence (not the cause) of muscularisation, because the same bronchial and vascular smooth muscle cells express MMP-2 and MMP-12. Further *in vitro* studies or analyses at earlier time points will be needed to specifically address potential relationships between MMP expression/activity and fluoxetine-related changes in arterial and bronchial muscle layers.

In conclusion, our study shows that fluoxetine may exacerbate BPD-related bronchial and arterial muscularisation. It may be hypothesised that the upregulation of MMP-2 activity and MMP-12 expression plays a pathogenic role in the thickening of these muscle layers, although further functional studies will be necessary.

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Accepted June 27, 2012