

The expression of HoxB5 and SPC in neonatal rat lung after exposure to fluoxetine

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Objective: Approximately 10% of pregnant women suffer from pregnancy-associated depression. Fluoxetine, as a selective serotonin reuptake inhibitor, is being employed as a therapy for depressive disorders. The present study aimed to determine the effects of fluoxetine on neonatal lung development.

Methods: Thirty pregnant Wistar rats (weighing 200–250 g) were treated daily with 7 mg/kg fluoxetine from gestation day 0 to gestation day 21, via gavage. The control group received a similar volume of distilled water only. Following delivery, the newborns and their lungs were immediately weighed in both of the groups. The right lung was fixed for histological assessments while the left lung was used for evaluation of the expression of SPC and HoxB5 by the real-time polymerase chain reaction method.

Results: Results have indicated that even though the body weight and the number of neonatal rats in both groups were the same, the lung weight of neonates exposed to fluoxetine was significantly different compared to the control group ($P < 0.05$). Expression of both genes was increased, nonetheless, only elevation of HoxB5 was significant ($P < 0.05$). Histological studies demonstrated that lung tissue in the fluoxetine treatment group morphologically appears to be similar to the pseudoglandular phase, whereas the control group lungs experienced more development.

Conclusion: According to the upregulated expression of HoxB5 concerning histological findings, results of the present study showed that fluoxetine can influence lung growth and may in turn lead to delay in lung development. So establishment of studies to identify the effects of antidepressant drugs during pregnancy is deserved.

Keywords: fluoxetine, rat, lung, HoxB5, SPC

Introduction

Approximately 350 million people are suffering from depression as a frequent disorder worldwide. This disorder affects several aspects of life, including an individual's relationship, capacity to work, and financial status and can lead to self-harm and even suicide. The prevalence of depression in females is two-fold higher than in males. Women are at increased risk of depression during periods of hormone changes in various states such as during maturity, pregnancy, and menopause.¹

Evidences revealed that depression is a risk factor for both preterm birth and low birth weight, and hence it is important to cure gestational depression.² A number of studies have suggested that selective serotonin reuptake inhibitors (SSRIs) are the first-line treatment drugs for depression therapy.³ Fluoxetine is an SSRI medication that is known as an important antidepressant drug for gestational depression which has been used during the last 2 decades.⁴ Fluoxetine is able to cross the placenta and therefore induces some side effects on fetal development, including risk of persistent

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pulmonary hypertension of the newborn (PPHN). PPHN has been reported to occur in one to two per 1,000 live-born infants and is associated with some important diseases and mortalities in newborns. Epidemiological studies have reported positive statistical correlation between PPHN and fluoxetine consumption during pregnancy.⁵

Several studies have demonstrated that fluoxetine affects both the level of pulmonary hypertension^{6,7} and alters the thickness of the pulmonary arterial wall.^{8,9} Although many studies have examined the effects of fluoxetine on exposed neonates, there exists no studies to evaluate the evolutionary changes in bronchial tree and alveolar cells. Concerning the most recent studies, it is essential to evaluate the effect of fluoxetine on lung tissue and evolution of the bronchial tree. The expression of the *HoxB5* gene is reported to commence at the earliest stages of lung development, peak at the end of the pseudoglandular stage, and finally diminish when branching morphogenesis is quite completed. After the birth, *HoxB5* is expressed by type I, while *SPC* genes are expressed in type II alveolar epithelial cells.^{10–13} The *SPC* gene provides a direction for the building of one of the four surfactant proteins and mutations in the *SPC* gene that will lead to chronic interstitial lung diseases in both infants and adults.^{14,15} Therefore, the present study aimed to evaluate the effects of fluoxetine on the processes of lung development via measuring the expression of *SPC* and *HoxB5* levels in neonatal rat lung.

Materials and methods

Animals

All animal protocols were carried out in accordance with the guidelines for the care and use of laboratory animals at the Rafsanjan University of Medical Sciences and rules and regulations of the European Communities Council Directive, November 24, 1986 (86/609/EEC). Thirty female Wistar rats weighing 200–250 g were kept and used under controlled conditions at 22°C ± 2°C and a constant 12 hours light/12 hours dark cycle. Rats had free access to food and water. Every three female rats were placed in contact with an adult male rat for mating. After 24 hours, vaginal smears were evaluated in female rats. The day of sperm detection in

the vaginal smear was considered day 0 of pregnancy (gestation day 0).¹⁶ Then, the female rats were randomly separated into treatment and control groups. The treatment group was initially treated by gastric gavage with fluoxetine at 10 mg/kg once per day¹⁷ from days 0 to 21 of gestation. Because rats could not tolerate this dose, the dose was reduced and the maximum dose for this study was 7 mg/kg. The control rats received a similar volume of distilled water. Immediately after delivery, the weights of pups were recorded and lungs were removed surgically. Then, lung weights were recorded, next, the left lung was fixed in TRizol reagent for real-time polymerase chain reaction (PCR) and the right lung was fixed in formalin for histological analysis.

Real-time PCR

Total RNA was extracted from the lung tissue using TRizol reagent according to the manufacturer's protocol. Extracted RNA was purified by isolation kits and used as a template for reverse transcription in cDNA synthesis. The cDNA synthesis kit was used for cDNA synthesis using both oligo (dT) and random hexamer primers. Real-time PCR was performed on all lung samples. This reaction was undertaken for *HoxB5*, *SPC*, and β -actin (housekeeping gene) genes in triplicate. Designed primers for each gene were controlled thermodynamically, then, they were evaluated in the BLAST database to verify the absence of nonspecific binding to other regions of the genome. The sequences of primers used are described in Table 1.

Real-time PCR was performed using 10 μ L of SYBR green master mix, 3 μ L of cDNA, 5 μ L of distilled water, 2 μ L of each primer, either forward or reverse primer. The following program was programmed on a Bio-Rad CFX96 system (Bio-Rad Laboratories Inc., Hercules, CA, USA) for PCR amplification. There was one cycle of 95°C for 10 minutes to activate the hot start Taq polymerase enzyme and DNA denaturation, 40 cycles of 95°C for 15 seconds, and 40 cycles of 58°C for 1 minute. Drawing of separation curve was performed using the following cycle: 95°C for 15 seconds, 60°C for 30 seconds, and 95°C for 15 seconds, and the temperature was reduced from 95°C to 60°C at 0.03°C per second in this step. The relative quantification

Table 1 The sequence of primers used for real-time polymerase chain reaction in the study

Gene	Forward primer	Reverse primer
<i>SPC</i>	CCTTGAGATGAGCATCGGAG	AGAAGGTAGCGATGGTGTCT
<i>HoxB5</i>	CTCCTTCTCGGGCGTTATC	CGGTTGACGCTGAGATCCAT
<i>β-Actin</i>	GATATCGCTGCGCTCGTCG	CCCATACCCACCATCACACC

of PCR products was determined using the $2^{-\Delta Ct}$ formula.¹⁸ The melting curves, quantitative analyses, and dissociation stages of the data were performed using the CFX manager software.

Histological and morphological analyses

The right lung samples which had already been fixed in formalin were embedded in paraffin, then the tissues were sectioned at 8 μ m and were stained with hematoxylin and eosin. The samples were studied under a light microscope.

Results

Body and lung weight

Our results showed that the mean body weight of newborns of the fluoxetine-exposed group (5.75 ± 0.05 g) was not significantly reduced compared to the control group (5.90 ± 0.09 g) (Figure 1). We observed that fluoxetine-exposed newborns' lung weight was significantly reduced compared to control newborns' lung weight (0.11 ± 0.006 g and 0.14 ± 0.004 g, respectively) ($P=0.001$) (Figure 2).

Number of newborns

There were no statistically significant differences between the average number of live-born infants in the fluoxetine treatment group and the control group (average: 7 ± 1.5 and 8.25 ± 1.6 , respectively) (Figure 3).

HoxB5 and SPC gene expression

The mRNA of *HoxB5* was expressed in rat lungs. When a comparison was made between the fluoxetine treatment group and the control group, a significant difference was observed. The expression of the *HoxB5* gene in the fluoxetine treatment

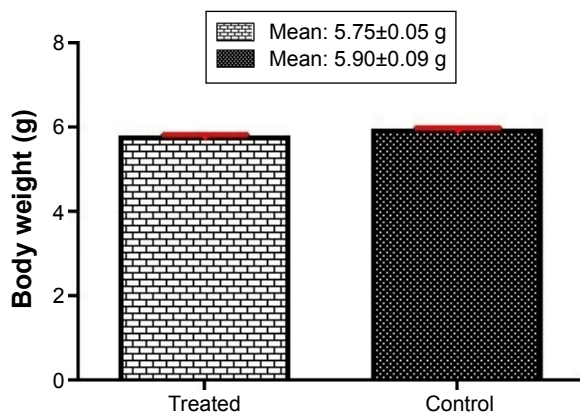


Figure 1 Body weight in studied groups.

Notes: Newborns' body weights were not different in fluoxetine treatment group (mean: 5.75 ± 0.05 g) and control group (mean: 5.90 ± 0.09 g).

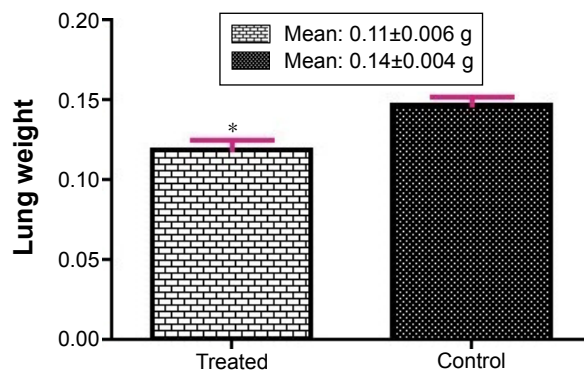


Figure 2 The lung weights.

Notes: Fluoxetine-exposed newborns' mean lung weights (0.11 ± 0.006 g) compared with the control newborns' mean lung weights (0.14 ± 0.004 g) were significantly reduced ($*P=0.001$).

group was significantly increased compared with the control group ($P=0.0276$) (Figure 4).

The mRNA of the SPC gene was expressed in type II alveolar cells. Although SPC was increased in the fluoxetine treatment group compared to the control group, this was nonsignificant ($P=0.1226$) (Figure 5).

Results of histological analyses

Histology survey showed that the amount of mesenchymal tissue in the fluoxetine treatment group was more than in the control group which was elevated relative to the developmental time. The alveolar cells were formed in the control group and the walls between the alveolar cells were thinner than the fluoxetine treatment group (Figure 6).

Discussion

Therapeutic drugs is a path to overcome depression during pregnancy. Approximately 2%–3% of pregnant women

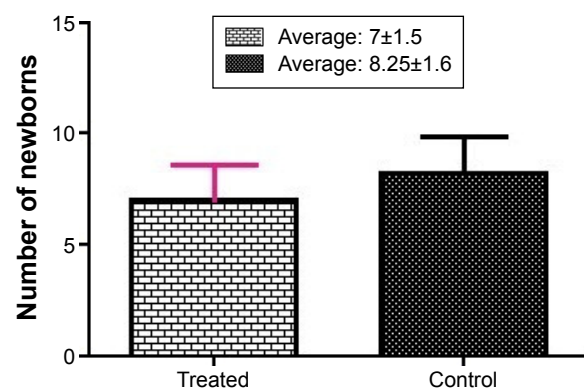


Figure 3 The number of newborns.

Notes: There was no statistically significant difference between average number of live-born infants in the fluoxetine treatment group (7 ± 1.5) and control group (8.25 ± 1.6).

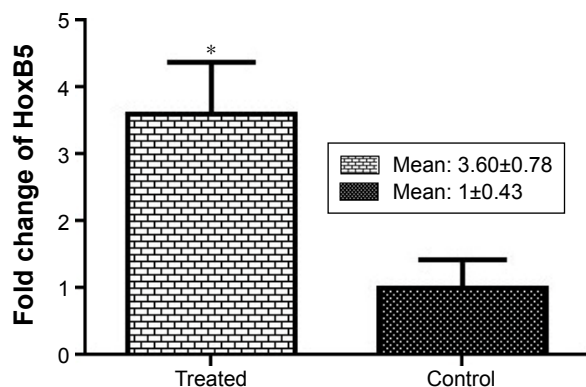


Figure 4 Fold changes of *HoxB5* expression.

Note: The expression of *HoxB5* in the fluoxetine treatment group compared with the control group showed a statistically significant increase (* $P=0.0276$).

with depression receive SSRI drugs (such as fluoxetine).¹⁹ Fluoxetine increases the serotonin level, and an acute increase in serotonin in turn reduces uterine blood flow and leads to preterm birth and reduced fetal growth.³ The time of exposure to fluoxetine is important as Morrison et al demonstrated that first-trimester exposure to fluoxetine caused preterm birth, fetal growth restriction, and increased rates of miscarriage,³ while a fetus exposed to fluoxetine during the third trimester will require respiratory support and hospitalization after birth.²⁰

In the present study, the impact of maternal fluoxetine treatment was evaluated on the development of fetal rat lungs and the results showed the effect of fluoxetine on the fetal rat. In this study the number of live-born infants in the fluoxetine treatment group was lower than the control group, however it was nonsignificant, but the lung weights of fluoxetine-exposed newborns compared to the control newborns' lung weights were significantly reduced ($P<0.05$). These weight changes and mortality were reported in other studies.

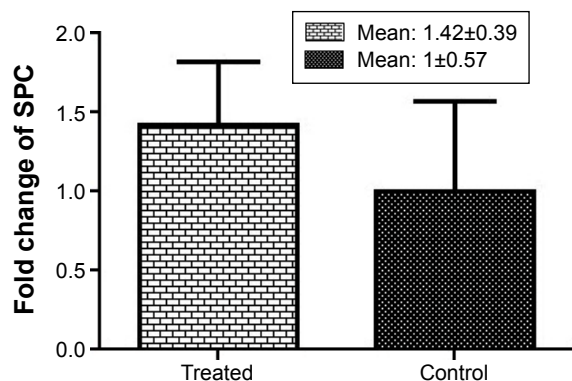


Figure 5 Fold changes of *SPC* expression.

Note: Expression of *SPC* in gene in the fluoxetine treatment group compared with control group showed an increase but this increase was nonsignificant ($P=0.1226$).

Vorhees et al demonstrated that exposure to fluoxetine led to reduction of size in pregnant rats and increased mortality, although its mechanism is yet to be cleared.²¹ Morrison et al reported that the cause of this reduction in weight and the number of the newborns are due to the reduction of uterine blood flow after exposure to fluoxetine.²²

The most important and novel result of this study is the effect of fluoxetine on expression of *HoxB5* and *SPC* genes.

Analysis of the real-time PCR showed the expression of both genes was increased and this increase was significant for the *HoxB5* gene ($P=0.0276$). *HoxB5* is a homeobox-containing gene that plays an important role in the patterning of airway branches.^{11,23,24} Strong expression of this gene is found in lung mesenchyme during branching morphogenesis, and its expression falls in the sacular stage¹¹ and after birth it is expressed only in alveolar type I cells. Regarding, increased or decreased expression of this gene can serve as an indicator to show a problem in lung development. Overexpression of this gene could be indicative of a delay in lung development; however, a reduction in expression of this gene can imply a reduction in the number of alveolar type I cells. According to the histological analysis results of this study, it was found that lung development is delayed in the fluoxetine treatment group compared with the control group. With regard to the expected evolutionary time in this study, the mesenchymal tissue in the lungs of the fluoxetine treatment group is more than the control group and the lung tissue morphology is similar to pseudoglandular phase whereas, mesenchymal tissue has decreased in the control group and more alveolar cells have been established.

We observed in this study that the expression of *SPC* was increased, however this increase was not significant. Thus, according to the alveolar type II cells, cells differentiate into type I, and relative overexpression of the *SPC* gene could be due to the lack of differentiation of alveolar type II cells toward type I and delay in development.

In 2007, Wang et al investigated the effect of alcohol on murine fetal lung and reported that alcohol delays the lung development and explained that the cause of overexpression of *HoxB5* is delay in lung development.²⁵

In Xu et al's study which evaluated the expression of *HoxB5* and its role in neonatal rats with chronic lung disease, the cause of overexpression of *SPC* is related to the lack of differentiation of alveolar type II cells toward type I.¹³

A number of studies also indicated the effects of fluoxetine on tissues and cells.

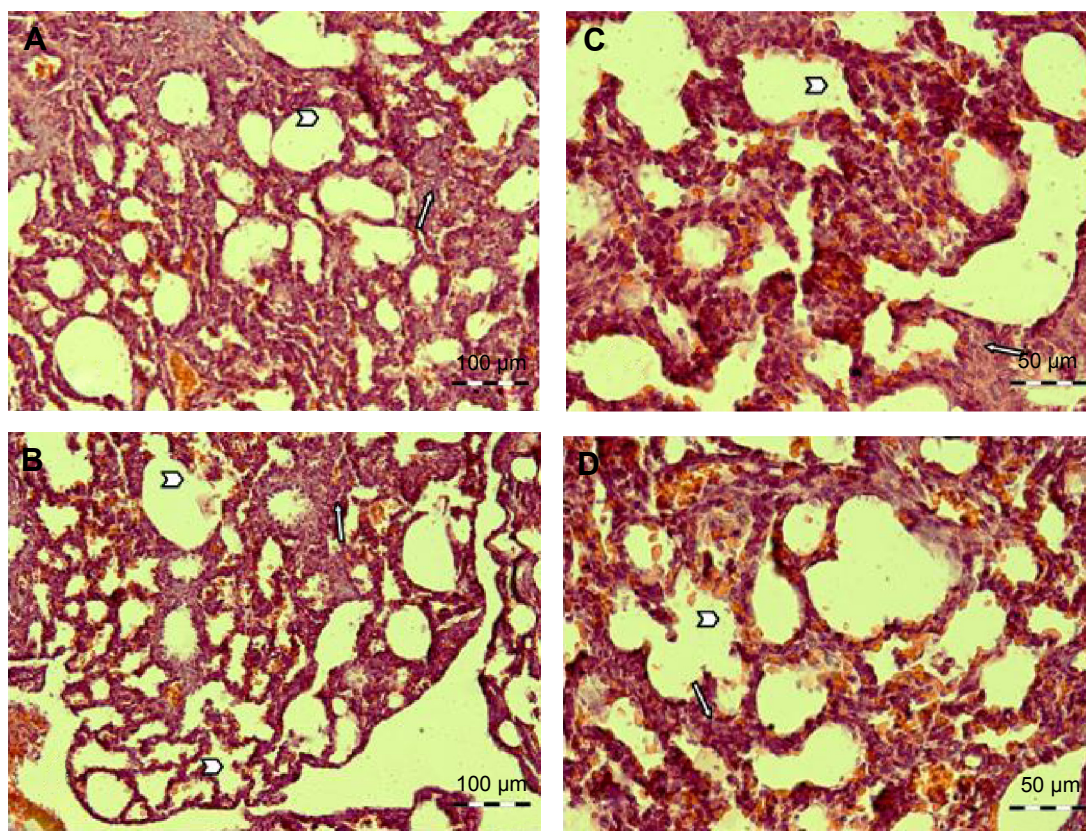


Figure 6 A representative picture of hematoxylin and eosin staining of rat lungs.

Notes: (A, B) Lung of the fluoxetine treatment group. The amount of mesenchymal tissue (arrow) is more than the control group. (C, D) Lung of the control group. The number of alveolar cells (arrowhead) are more than the fluoxetine treatment group and the walls between the alveolar cells are thin. Arrow: mesenchymal tissue. Arrowhead: alveolar cell. The magnification of (A, B) $\times 20$ (scale bar: 100 μm). The magnification of (C, D) $\times 40$ (scale bar: 50 μm).

Swerts et al reported the effects of fluoxetine on nerve cells' development. They stated that the effect of fluoxetine on the elevated levels of serotonin led to the reduction in uterine blood flow which in turn can have an impact on the development of nerve cells.²⁶

More recently in 2013, Kiryanova et al investigated the effects of fluoxetine on heart muscle cells and reported that exposure to fluoxetine during the neonatal period will lead to abnormality in cardiac growth and ventricular septal defects.²⁷

Therefore, in agreement with aforementioned studies, our findings may also confirm fluoxetine's effects on development throughout fetal life. Some studies exist that suggest that SSRIs have an impact on pulmonary hypertension,^{5,28} and they have also reported that the highest concentration of fluoxetine accumulates within lungs after injection of fluoxetine in animals^{29,30} which can lead to PPHN.³¹ Even though the main cause of PPHN is not clear, one of the concerns could possibly be the increase in serotonin levels which results in increased pulmonary and smooth muscle cells' vasoconstriction.³²

Conclusion

In the present study, we demonstrated that the exposure to fluoxetine during pregnancy caused increased expression of *HoxB5* and *SPC* in neonatal rats. The expression of the *HoxB5* gene began at the earliest stages of lung development, peaked at the end of the pseudoglandular stage, and then its expression was moderated in the saccular stage.³³ Regarding histological analysis, it appears that the overexpression of this gene in the fluoxetine treatment group is probably due to the delay in lung development.

According to the other studies,^{5,28} one of the reasons for the delay in lung development can be the increase of serotonin levels in this tissue. On the other hand, pulmonary vascular and the bronchial tree development are equivalent. Therefore, maybe the effect of fluoxetine on vessels development^{9,34,35} can affect the development of the bronchial tree, although it's not clear.

Considering the results of the present study and other studies, it is important to be more scrutinizing before taking fluoxetine during pregnancy, so, it is essential to study the effects of antidepressant drugs during pregnancy.

Acknowledgment

The authors of this article would like to thank Rafsanjan University of Medical Sciences as well as Dr Hasanshahi for editing the manuscript.

Disclosure

The authors report no conflicts of interest in this work.

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