Regenerative role of Genistein treatment on Fibrotic and inflammatory Biomarkers Alteration in the Lung of Estrogen deficient Rats

Faeze Daghigh
Islamic Azad University of Tabriz
Masoumeh Majidi Zolbin
Tehran University of Medical Sciences
Pouran Karimi
Tabriz University of Medical Sciences
Alireza Alihemmati
Tabriz University of Medical Sciences
Nasser Ahmadiasl (n.ahmadiasl@gmail.com)
Tabriz University of Medical Sciences

Research Article

Keywords: Genistein, Menopause, Inflammation, Fibrosis, Lung

Posted Date: March 11th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-258892/v1

Abstract

Background

Phytoestrogens are suggested to have estrogenic effects in the pulmonary system and have been revealed with a few adverse side effects. In this study, we tried to investigate the effect of genistein treatment on estrogen deficiency-induced lung injury and demonstrating whether genistein supplementation could replace estrogen hormone in postmenopausal women.

Methods

Forty adult female rats were divided into four groups; sham: rats that underwent surgery without ovariectomy, OVX: rats that underwent ovariectomies, OVX.E: ovariectomized rats with eight weeks period of estrogen treatment (20μg/kg/day), OVX.Gen: ovariectomized rats with eight week period of genistein treatment (1mg/kg/day). At the end of the experiment, lung tissue was removed and inflammatory and fibrotic biomarkers were evaluated with western blotting technique. Hematoxylin-eosin and immunohistochemical staining were used to evaluate histomorphological changes in the lung tissue.

Results

Genistein treatment restored ERK1/2, TGFβ1, MMP2, and IL1β, Bcl-2, and caspase3 expression levels, implying the effectiveness of genistein supplementation in targeting estrogen deficiency symptoms.

Conclusions

Genistein supplementation exerted protective effects against ovariectomy-induced lung injury with reducing inflammation and fibrosis, moreover, it can be recommended as a natural alternative to postmenopausal hormone therapy.

Background

There are few studies on respiratory system health and lung function in postmenopausal women. Estrogen, as a steroid hormone, exerts a vital regulatory role in pulmonary functions [1]. Estrogen hormone improves alveolar size and number and induce alveolar regeneration after their loss in ovariectomized (OVX) mice [2]. Lung alveolar units are estrogen dependent [2], indicating that estrogen deficiency might be responsible in age related pulmonary dysfunction [3]. The role of estrogen in the regulation of inflammatory and fibrotic responses has been established over the past decades [1].

Studies have highlighted the incisive role of IL1β and TGFβ1 in pulmonary fibrosis and inflammation [4], by promoting collagen synthesis in myofibroblast and promotion of fibroblast proliferation [5]. Estrogen

replacement was found to have a profound impact on respiratory function in postmenopausal women with genital prolapse [6].

Recent studies have focused on non-hormonal treatment that exert estrogenic effects in postmenopausal women. Phytoestrogens mimic estrogenic effects in various tissues including respiratory system and considered as a possible alternative to hormone therapies [1]. It has been shown that phytoestrogens can exert protective effects against estrogen deficiency induced lung inflammation and fibrosis [1].

Of note, anti- inflammatory and anti- fibrotic effects of genistein has been suggested in the recent studies [7, 8]. Genistein was found to exert anti-inflammatory effect in LPS-treated macrophages through the attenuation of inflammatory responses and reduction TNFα protein level [9]. More importantly, genistein has been shown to attenuate pulmonary hypertension (PH) induced lung fibrosis. In this study, we aimed to investigate the role of genistein and estrogen treatment on ERK1/2, TGFβ1, MMP2, IL1β, Bcl-2 and caspase3 expression levels as inflammatory, fibrotic biomarkers in the lung of ovariectomized rat model which is a resemble of menopause status.

Methods

Animals

Forty female wistar rats (10 weeks old, weighing 180–220 g**)** were purchased from Experimental Animal Research Center, Faculty of Medicine, Tabriz Medical Science, Tabriz, Iran. Rats were housed under standard condition (12h light: 12 h dark cycle with temperature of 22–24°c) and fed ad libitum. All the animals had free access to water. The study was carried out in compliance with the ARRIVE guidelines. Animals were treated in accordance with Guide for the Care and Use of Laboratory Animals (8th edition, National Academies Press). The study was approved by the ethics committee of Tabriz Medical University. Ethical Committee of Tabriz Medical Science (code number: IR.TBZMED.REC.1396.450).

Experimental design

Forty female rats randomly were assigned into four groups (n = 10 in each group); sham: rats underwent laparotomy without ovariectomy, OVX: rats that underwent bilateral ovariectomy, OVX.E: ovariectomized rats with eight weeks of 17β-estradiol treatment (20 μg/kg/day, s. c.) [10], OVX.Gen: ovariectomized rats with eight weeks of genistein treatment (1mg/kg/day, s. c.) [11].

Two weeks after surgery and recovery, animals in OVX.Gen group received genistein (Sigma-Aldrich, USA) [1 mg/kg in 500 μl of a mixture of PEG-400 (98.75%) and DMSO (1.25%), s. c. daily] and OVX.E group received 17β-estradiol (20μg/kg in 100 ml cottonseed oil, s. c. daily) for 8 weeks. Rats in the sham and OVX groups were administered vehicles. Body weights in all animals were measured weekly.

Ovariectomy

Surgery were performed under the IP injection of anesthesia with (50 mg/kg ketamine chloride and 5 mg/kg xylazine chloride), skin and muscle walls of dorsolateral regions were incised (1.5 cm), and ovaries accurately were removed. At the end of operation, oviducts were located back with a minimal soft tissue disruption [12]. At the end of experiment, the serum level of estrogen was determined in blood samples collected by cardiac puncture under anesthesia. Plasma estrogen level was measured using commercial radioimmunoassay kit [10].

Histological evaluation

At the end of eight weeks experimental period, lungs were removed under anesthesia and used for molecular and histological assessment. Hematoxylin-eosin, immunohistochemistry and western blot technique were used for molecular biomarkers assessment in the lung of study groups.

Immunoblotting analysis

Western blotting was used to evaluate of IL1β, Bcl-2, caspase3, TGFβ1, MMP2 and ERK1/2 phosphorylation expression in the lung. Briefly, snap frozen of lung tissue were homogenized in RIPA lysis buffer containing a proteinase inhibitor cocktail (antipain, pepstatin, leupeptin, chymostatin and aprotinin) on ice and left at 4°C at least 20 min and centrifuged 10 min at 12,000 rpm in 4°C. The supernatants were removed and stored at -80°C. Proteins were separated in SDS-PAGE, and then transferred electrophoretically onto PVDF (polyvinylidene difluoride). Non-specific binding sites were blocked by incubation of membranes for 2 h with 5% (w/v) nonfat dry milk in Tris-buffered saline (pH 7.5).

All the blots were incubated at the room temperature for 2 h with primary antibodies (anti- ERK1/2, P-ERK1/2, TGFβ1, MMP2, caspase3, IL1β and Bcl-2) diluted in antibody buffer comprising of 1% (w/v) nonfat dry milk in 0.05% (v/v) TBST (Tris-Buffered Saline plus 0.05% (v/v) Tween 20). Then washed three times with TBST. Samples incubation was done 1 h with secondary antibody (goat Anti-rabbit; Santa Cruz, USA) in the antibody buffer.

Blots were detected with using chemiluminescence (ECL) detecting kit (Pierce, Rockford, IL). Lung tissues from six animals in each group were obtained for western blotting. Quantification of the resulting bands on immunoblots were quantified by using densitometry of the Image j program.

Immunohistochemical staining

To further confirm our results and their applicability to the human disease, we performed immunohistochemistry (IHC) on paraffin embedded microscopic sections from all experimental groups. Whole lungs were fixed in 10% formalin, lung tissues embedded in paraffin. The sections of 5 μm and 4 μm thick were obtained and subjected to hematoxylin-eosin and immunohistochemical staining, respectively. Briefly, following tissue process [13] xylene and alcohol were used for slides deparaffinization and rehydration. Incubation in 3% hydrogen peroxide for 10 min was done for blocking of endogenous peroxidase activity.

Then, the slides were incubated in citrate buffer for antigen retrieval. Slides were incubated with antibodies (TGFβ1, sc-146, 1:400, Santa Cruz Biotechnology) and collagen I (Dako, Copenhagen, Denmark) at 37°C for one hour in a moist chamber. After washing with TBS, slides left at room temperature and examined for histological changes. The images were collected using a light microscope (Olympus BH-2, Tokyo, Japan).

Semi-quantitative analysis

Lung sections were examined by two independent pathologists and scored by evaluating markers expression in the all studied groups. Collagen fibers and TGFβ1 protein were qualified by optical density with the microscopic examination analysis in 10 high power field (HPF), randomly selected alveolar septa, interstitium and epithelial cells, respectively. The result are reported at the percentage of the area occupied by collagen fibers and TGFβ1 protein [14]. A semi-quantitative analysis was used to compare the studied groups using Allred scoring system [15].

Statistical analysis

All results of this study were described as the mean ± SEM. One- way analysis of variance (ANOVA) with Tukey's multiple comparison post-test used to determination differences between groups. $P < 0.05$ was considered statistically significant.

Results

Body weights and 17β-estrogen levels

As shown in Fig. 1A, at the end of experiment, there was a significant increase in body weight in OVX group in comparison to sham ($P < 0.05$). Body weight was reduced in OVX.E group compared with OVX group. Genistein treatment markedly decreased body weight in OVX.Gen compared to OVX ($P < 0.05$) (Fig. 1A). As shown in Fig. 1B, 17β-estradiol level was significantly reduced in OVX group compared to sham ($P < 0.05$). Estrogen administration enhanced estrogen level in OVX.E group compared to OVX ($P < 0.05$) (Fig. 1B).

Expression level of ERK in lung following genistein and estrogen treatment

Ovariectomy led to an increase in the mean value of ERK1/2 levels in the lung as compared to the sham ($P < 0.05$). Estrogen supplementation reduced ERK1/2 levels in OVX.E group compared to OVX. Also, genistein treatment meaningfully reduced ERK1/2 levels in the OVX.Gen group in comparison to OVX group ($P < 0.05$) (Fig. 1C).

Analysis of protein expression of Bcl-2, ILβ1 and caspase3 in the studied groups

The expression levels of caspase3 and IL1β significantly increased in OVX group compared with sham. Our study's findings demonstrated that ovariectomy decreased Bcl-2 levels as compared to the sham ($P < 0.05$). Genistein supplementation and estrogen treatment significantly reversed the protein levels in comparison to the ovariectomized group ($P < 0.05$) (Fig. 1D).

Genistein treatment on protein levels of TGFβ1 and MMP2 in the lung of study groups

The proteins of TGFβ1 and MMP2 were found significantly up regulated in the OVX group as compared to the sham ($P < 0.05$). Estrogen supplementation decreased the expression levels of TGFβ1 and MMP2 in OVX.E group compared with OVX group ($P < 0.05$). Genistein treatment markedly reduced the protein levels in the OVX.Gen group when compared with OVX ($P < 0.05$) (Fig. 1E).

Histological and immunohistochemical results

H&E results in the lung tissue

Histological evaluations of lung sections revealed normal lung architecture with thin inter alveolar septa in the sham group (Fig. 2A). In OVX group, perivascular edema along with the blood vessels was observed. Histologic evaluation in the lung of OVX group revealed thickened inter alveoli septa with morphological change in the alveoli. Also, ovariectomy induced macrophage and leukocytes infiltration in the lung. Alveolar deformity was also observed in this group ($P < 0.05$) (Fig. 2B).

In OVX.E group, relatively thin inter alveolar septa was detected. Estrogen supplementation reduced perivascular edema and inflammatory cell infiltration in OVX.E group compared to OVX. Estrogen administration alleviated alveolar deformities in OVX.E group compare to OVX group ($P < 0.05$) (Fig. 2C).

In the OVX.Gen group, decrease of inflammatory cell infiltration and perivascular edema were observed ($P < 0.05$). Alveoli with relatively thin inter alveolar septa were detected in OVX.Gen group compared to OVX ($P < 0.05$). Genistein with reduction of morphological changes improved alveolar deformity (Fig. 2D), (Table 1).

Table 1
Histological evaluation in the lung of experimental groups (H&E)

Group	Leukocyte infiltration	Macrophage infiltration	Perivascular edema	Interstitial thickening	Alveolar architectural alteration	Alveolar epithelium deformity
sham	0.60 ± 0.05	0.70 ± 0.24	0.0 ± 0	0.20 ± 0.20	0.40 ± 0.24	0.20 ± 0.20
OVX	1.80 ± 0.08*	2.60 ± 0.10 *	2.40 ± 0.37*	2.90 ± 0.24*	2.80 ± 0.20*	2.80 ± 0.20*
OVX.E	1.36 ± 0.08**	1.7 ± 0.28**	1 ± 0.21**	1.20 ± 0.31**	2.40 ± 0.40	2.30 ± 0.19
OVX.Gen	1.06 ± 0.08**	2.05 ± 0.20	1.10 ± 0.27**	1.30 ± 0.22**	2.68 ± 0.32	2.60 ± 0.05

A minimum of 5 fields for each lung tissue slide were assigned for the tissue changes (n = 10 in each group). Data are presented as the means ± SEM.* P < 0.05 versus sham group; ** P < 0.05 versus OVX group

Immunohistochemical results in the lung tissue

Histomorphological evaluation of lung samples revealed normal tissue architecture in the sham group (Fi 3a, e). The histomorphology study results confirmed a higher expression of cytoplasmic protein of TGFβ1 and collagen I in the lung of ovariectomized group. Additionally, replacement of smooth muscle with collagen fibers and higher infiltration of inflammatory factors was detected in OVX group compared to the sham (Fig. 3b, f). Estrogen and genistein treatment groups demonstrated significantly decrease of lung tissue fibrotic change and the complication associated with fibrosis in the experimental groups (Fig. 3c, d, g and h), (Table 2).

Table 2
Imonohistochemical evaluation of the lung tissue in the experimental groups

Group	sham	OVX	OVX.E	OVX.Gen
TGFβ1	74.65 ± 0.42	98.16 ± 2.02 *	87.19 ± 1.50	84.66 ± 2.08 **
Collagen I	75.22 ± 0.50	110.64 ± 4.65 *	87.93 ± 1.65 **	84.19 ± 2.11 **

Examination analysis of TGFβ1 and collagen I fibers in 10 high power field (HPF). Data are presented as the means ± SEM.* P < 0.05 versus sham group; ** P < 0.05 versus OVX group

Discussion

To the best of our knowledge, this project is the first report describing the effect of genistein treatment in the lung of ovariectomized rats with focus on its recovering role in the fibrosis and getting over the inflammation. These findings are novel and have implications in either understanding the pathogenesis of menopause and its treatment. In current study, genistein treatment significantly ameliorated estrogen deficiency induced alteration in the expression of TGFβ1, IL1β, MMP2, ERK, caspase3 and Bcl-2 in the studied groups.

Loss of anti-fibrotic, anti- inflammatory and anti- apoptotic effects of estrogen beginning with menopause is theorized to be responsible for occurrence of pulmonary disease. Thus given that, ovariectomy can cause inflammation and fibrosis [1]. Estrogen deficiency resulted in an increase in TGFβ1 gene transcription in oophorectomized female rabbits [16]. Of note, increase in TGFβ1 mRNA has been shown in fibrotic human lungs [17].

The important role of estrogen in matrix remodeling has been established in the recent years. Previous studies have demonstrated that estrogen replacement can reduce collagen deposition in the lung of OVX rats [1]. Accordingly, estrogen continues to provide a protective effect against cardiac fibrosis [18]. However, there are some contradictory reports indicating that, estrogen can enhance fibrogenesis in a model of fibrotic lung disease [19].

Activation of MAPK/ Erk pathway exerts an important role in fibrosis through regulating the matrix synthesis and/or fibroblast into myofibroblast trans- differentiation [20]. Recent studies have also revealed a new molecular mechanism involved in the MAPK/ Erk pathway by TGFβ1. Thus, activation of ERK1/2 and MAPK pathway by TGFβ1 has been proposed in dermal fibroblast cells [21]. There is increasing evidence that epithelial mesenchymal transition (EMT) has a major role in the progression of pulmonary fibrosis [22]. Also, it has been shown that activation of the Ras/ Erk MAPK signaling pathway might be responsible for EMT induced by TGFβ1 [23].

Several researches suggest that aging associated with a morphological change in the lung alveolar cells [24]. Repetitive alveolar injury resulting in pulmonary fibrosis has been noted to have serious histological abnormality such as denuded basal lamina [25]. In accordance with the last studies, we found significant morphological alterations in alveolar cells characteristic by the alveolar epithelium deformity in the lung of ovariectomized rats.

More studies have highlighted the role of IL1β, as an inflammatory marker, in the progression of lung fibrosis [26]. Evidence will be presented indicating the potential role of IL1β in lung injury and inflammation [27]. Of note, fibroblasts with a long exposure to IL1β enhance MMPs levels in the lung [28]. MMPs are believed to be involved in several pulmonary diseases. Accordingly, MMP2 expression was found up-regulated in pulmonary fibrosis [29]. Besides the important role of MMPs in extracellular matrix degradation, these molecules are involved in activation of latent TGFβ1 protein [30]. Glassberg et al. have reported a significant increase in MMP2 activity and collagen production in the lung of old female mice [29]. Moreover, Dogru and colleagues have found a dramatic change in lung structure in ovariectomized rats characterized by marked histological abnormality as well as mononuclear cells infiltration, edema,

fibrosis and hemorrhage [31]. Moving forward with the previous researches, we found a marked increase in IL1β and MMP2 expression levels as well as perivascular edema and increase of inflammatory cells infiltration in the lung of estrogen-deficient rats.

In recent years, the potential link between inflammation and apoptosis is being actively investigated. There are strong evidence confirming that relation between pulmonary fibrosis and apoptosis. Recent studies have found that TGFβ1 increases expression of apoptotic mediators including Fas-L and caspase3 in the mouse lung [32]. Accordingly, exposure to hyperoxia has also been shown to lead to an increase in TGFβ1 induced apoptosis in the alveolar type II cells [33].

Anti- inflammatory and anti- apoptotic effects of estrogen has been shown in the last decades [1]. It has been suggested that inflammatory and apoptotic biomarkers increase in the lung of post-menopausal animal model [29, 34]. Estrogen replacement therapy (ERT) has been demonstrated to reverse architectural changes in OVX lung [2]. Of note, estrogen treatment has been shown to exert protective effects against pulmonary apoptosis and inflammation in estrogen deficiency animal model [29].

Recent research has shown that estrogen supplementation decreases apoptotic and inflammatory markers including ERK, MMP2 and caspase3 in estrogen- deficient mice [29]. Accordingly, in the present study, estrogen administration remarkably inhibited OVX induced increase in the expression levels of TGFβ1, IL1β, MMP2, ERK, caspase3 in the lung tissue. Moreover, estrogen administration markedly reversed OVX induced histologic inflammation and fibrosis in the lung of estrogen- treated rats.

In the present study, we also aimed to evaluate whether genistein treatment could affect lung fibrosis and inflammation. For the first time this report is describing the effect of genistein treatment on TGFβ1, MMP2, IL1β, Bcl-2, caspase3 and ERK1/2 in the lung of surgical model of menopause. In the present study, genistein administration remarkably down regulated the expression levels of TGFβ1, IL1β, MMP2, ERK1/2 and caspase3 and meaningfully upregulated the expression of Bcl-2 in the lung of estrogen-deficient rats.

Recent finding strongly supports the idea that genistein can reverse perivascular and interstitial lung fibrosis [11]. Genistein has also been reported to exert an anti-inflammatory effect in OVX mice through reducing IL1β serum levels [35]. Of note, genistein is able to suppress IL1β induced MMP2 expression in fibroblast-like synoviocytes of rheumatoid arthritis [36]. Anti- fibrotic effect of genistein has been suggested in the previous studies.

Yuan et al. have reported the effectiveness of genistein treatment in hyperglycemia induced kidney fibrosis by down- regulating of TGFβ1 [37]. It has been reported that genistein protects the hippocampal neurons against apoptosis in OVX rats. Also, long term intervention with genistein leads to a significant decrease in neural apoptosis with upregulation of Bcl-2 in OVX rats [38].

Caspase3 is found to exert a key role in the execution phase of apoptosis [39]. In the present study, we showed that genistein administration markedly ameliorated OVX induced lung injury by decreasing of

caspase3 and enhancement of Bcl-2 expression levels, as apoptotic markers, in genistein- treated rats. There has been confirmed many other identified molecules which are involved in cell apoptosis. This finding is simply suggestion for protective effects of genistein and estrogen treatment against OVX induced apoptosis in the lung tissue. Thus, it needs to more evaluation for other apoptotic markers in estrogen- deficient lung.

Conclusions

Our data demonstrated that genistein supplementation and estrogen treatment could protect against OVX induced lung injury. Genistein, as a non- hormonal treatment may exert protective effect against lung injury in estrogen deficient rats. However, further preclinical and clinical research studies are needed to establish genistein, as a novel agent, for protective treating of lung injury in postmenopausal women.

Abbreviations

OVX
ovariectomized
Bcl-2
B-cell lymphoma 2
ERK
The extracellular-signal-regulated kinase
IL1β
Interleukin 1 beta
MMP2
Matrix Metallopeptidase 2
TGFβ1
Transforming growth factor beta 1

Declarations

Ethics approval and consent to participate

The study was approved by the ethics committee of Tabriz Medical University. All the methods used in the present study were prepared in accordance with the relevant guidelines, Ethical Committee of Tabriz Medical Science, (the code number is IR.TBZMED.REC.1396.450).

Consent for publication

All the authors have given their consents for this publication.

Availability of data and materials

The data used during the present study is available for all the readers from the corresponding authors on reasonable request.

Competing interests

The authors declare that they have no competing interests.

Funding: This study was supported by Tuberculosis and lung disease research center, Tabriz University of medical sciences, Tabriz, Iran.

Acknowledgements

Not Applicable.

References

1. Elfattah, L.I.A., A comparative study between the effects of dietary soya and estrogen replacement therapy on the lung of ovariectomized albino rats: histological and immunohistochemical study. Egyptian journal of histology, 2012. **35**(1): p. 34–42.
2. Massaro, D. and G.D. Massaro, Estrogen regulates pulmonary alveolar formation, loss, and regeneration in mice. American Journal of Physiology-Lung Cellular and Molecular Physiology, 2004. **287**(6): p. L1154-L1159.
3. Carlson, C.L., et al., Hormone replacement therapy is associated with higher FEV1 in elderly women. American journal of respiratory and critical care medicine, 2001. **163**(2): p. 423–428.
4. Gasse, P., et al., IL-1R1/MyD88 signaling and the inflammasome are essential in pulmonary inflammation and fibrosis in mice. The Journal of clinical investigation, 2007. **117**(12): p. 3786–3799.
5. Dukovich, M., et al., Stimulation of fibroblast proliferation and prostaglandin production by purified recombinant murine interleukin 1. Clinical immunology and immunopathology, 1986. **38**(3): p. 381–389.
6. Stipic, I., et al., Estrogen replacement therapy improves pulmonary function in postmenopausal women with genital prolapse. Rejuvenation research, 2012. **15**(6): p. 596–600.
7. Alipour, M.R., et al., Effects of genistein treatment on molecular markers related to fibrosis: addition to histological changes in the lung of ovariectomized diabetic rats. Journal of Contemporary Medical Sciences, 2018. **4**(3).
8. Daghigh, F., et al., Genistein preserves the lungs of ovariectomized diabetic rats: addition to apoptotic and inflammatory markers in the lung. Iranian journal of basic medical sciences, 2017. **20**(12): p. 1312.
9. Ji, G., et al., Genistein suppresses LPS-induced inflammatory response through inhibiting NF-κB following AMP kinase activation in RAW 264.7 macrophages. PLoS One, 2012. **7**(12): p. e53101.

10. Squadrito, F., et al., Genistein supplementation and estrogen replacement therapy improve endothelial dysfunction induced by ovariectomy in rats. Cardiovascular Research, 2000. **45**(2): p. 454–462.
11. Matori, H., et al., Genistein, a soy phytoestrogen, reverses severe pulmonary hypertension and prevents right heart failure in rats. Hypertension, 2012: p. HYPERTENSIONAHA. 112.191445.
12. Irigoyen, M.-C., et al., Exercise training improves baroreflex sensitivity associated with oxidative stress reduction in ovariectomized rats. Hypertension, 2005. **46**(4): p. 998–1003.
13. Churg, A., An inflation procedure for open lung biopsies. The American journal of surgical pathology, 1983. **7**(1): p. 69–72.
14. Rozin, G., et al., Collagen and elastic system in the remodelling process of major types of idiopathic interstitial pneumonias (IIP). Histopathology, 2005. **46**(4): p. 413–421.
15. Harvey, J.M., et al., Estrogen receptor status by immunohistochemistry is superior to the ligand-binding assay for predicting response to adjuvant endocrine therapy in breast cancer. Journal of clinical oncology, 1999. **17**(5): p. 1474–1481.
16. Abramov, Y., et al., Transforming growth factor beta1 gene expression during vaginal wound healing in a rabbit menopause model. BJOG: An International Journal of Obstetrics & Gynaecology, 2013. **120**(2): p. 251–256.
17. Coker, R., et al., Localisation of transforming growth factor β1 and β3 mRNA transcripts in normal and fibrotic human lung. Thorax, 2001. **56**(7): p. 549–556.
18. Neugarten, J., et al., Estradiol suppresses mesangial cell type I collagen synthesis via activation of the MAP kinase cascade. American Journal of Physiology-Renal Physiology, 1999. **277**(6): p. F875-F881.
19. Gharaee-Kermani, M., et al., Gender-based differences in bleomycin-induced pulmonary fibrosis. The American journal of pathology, 2005. **166**(6): p. 1593–1606.
20. Caraci, F., et al., TGF-β1 targets the GSK-3β/β-catenin pathway via ERK activation in the transition of human lung fibroblasts into myofibroblasts. Pharmacological research, 2008. **57**(4): p. 274–282.
21. Samuel, G.H., et al., Autocrine transforming growth factor β signaling regulates extracellular signal-regulated kinase 1/2 phosphorylation via modulation of protein phosphatase 2A expression in scleroderma fibroblasts. Fibrogenesis & tissue repair, 2010. **3**(1): p. 25.
22. Willis, B.C. and Z. Borok, TGF-β-induced EMT: mechanisms and implications for fibrotic lung disease. American Journal of Physiology-Lung Cellular and Molecular Physiology, 2007. **293**(3): p. L525-L534.
23. Grände, M., et al., Transforming growth factor-β and epidermal growth factor synergistically stimulate epithelial to mesenchymal transition (EMT) through a MEK-dependent mechanism in primary cultured pig thyrocytes. Journal of Cell Science, 2002. **115**(22): p. 4227–4236.
24. Yamamoto, Y., et al., Morphology of aging lung in F344/N rat: alveolar size, connective tissue, and smooth muscle cell markers. The Anatomical Record, 2003. **272**(2): p. 538–547.

25. Geiser, T., Idiopathic pulmonary fibrosis-a disorder of alveolar wound repair? Swiss medical weekly, 2003. **133**(29–30): p. 405–411.
26. Wilson, M.S., et al., Bleomycin and IL-1β–mediated pulmonary fibrosis is IL-17A dependent. Journal of Experimental Medicine, 2010. **207**(3): p. 535–552.
27. Kolb, M., et al., Transient expression of IL-1β induces acute lung injury and chronic repair leading to pulmonary fibrosis. The Journal of clinical investigation, 2001. **107**(12): p. 1529–1536.
28. Moon, D.-O., et al., Curcumin attenuates inflammatory response in IL-1β-induced human synovial fibroblasts and collagen-induced arthritis in mouse model. International immunopharmacology, 2010. **10**(5): p. 605–610.
29. Glassberg, M.K., et al., 17β-estradiol replacement reverses age-related lung disease in estrogen-deficient C57BL/6J mice. Endocrinology, 2014. **155**(2): p. 441–448.
30. Wang, M., et al., Matrix Metalloproteinase 2 Activation of Transforming Growth Factor-β1 (TGF-β1) and TGF-β1–Type II Receptor Signaling Within the Aged Arterial Wall. Arteriosclerosis, thrombosis, and vascular biology, 2006. **26**(7): p. 1503–1509.
31. Dogru, Y.Z., et al., Effects of diabetes mellitus and postmenopausal period on the lungs of rats. African Journal of Pharmacy and Pharmacology, 2012. **6**(27): p. 1989–2010.
32. Li, Z., et al., A potential role of the JNK pathway in hyperoxia-induced cell death, myofibroblast transdifferentiation and TGF-β1-mediated injury in the developing murine lung. BMC cell biology, 2011. **12**(1): p. 54.
33. Alejandre-Alcázar, M.A., et al., Hyperoxia modulates TGF-β/BMP signaling in a mouse model of bronchopulmonary dysplasia. American Journal of Physiology-Lung Cellular and Molecular Physiology, 2007. **292**(2): p. L537-L549.
34. Daghigh, F., et al., Fibrotic and apoptotic markers alteration in ovariectomised rats: addition of swimming training preserves lung architecture. Archives of physiology and biochemistry, 2017: p. 1–6.
35. Li, B. and S. Yu, Genistein prevents bone resorption diseases by inhibiting bone resorption and stimulating bone formation. Biological and Pharmaceutical Bulletin, 2003. **26**(6): p. 780–786.
36. Zhang, Y., et al., Genistein inhibit cytokines or growth factor-induced proliferation and transformation phenotype in fibroblast-like synoviocytes of rheumatoid arthritis. Inflammation, 2012. **35**(1): p. 377–387.
37. Yuan, W.-J., F.-Y. Jia, and J.-Z. Meng, Effects of genistein on secretion of extracellular matrix components and transforming growth factor beta in high-glucose-cultured rat mesangial cells. Journal of Artificial Organs, 2009. **12**(4): p. 242.
38. Peng, Y., et al., Effects of genistein on neuronal apoptosis, and expression of Bcl-2 and Bax proteins in the hippocampus of ovariectomized rats. Neural regeneration research, 2012. **7**(36): p. 2874.
39. Kaufmann, S.H., et al., Apoptosis-associated caspase acti*vation assays*. Methods, 2008. **44**(3): p. 262–272.

Figures

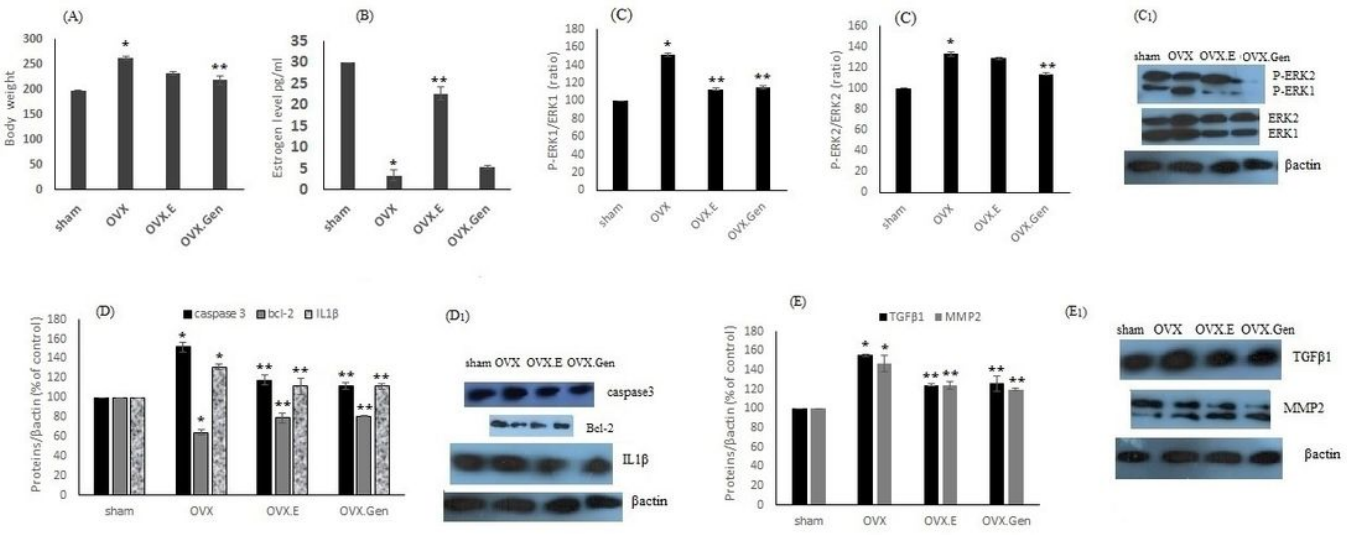

Figure 1

Final body weight evaluation in the groups (A), Plasma 17β-estradiol levels in the study groups (B), Immunoblotting of P-ERK1/2 and ERK1/2 among different groups in the lung (C), quantitation of immunoblotting of P-ERK1/2 against ERK1/2 among different groups (C1), Immunoblotting of Bcl-2, IL1β and caspase3 among different groups (D), Immunoblotting quantitation of Bcl-2, IL1β and caspase3 against expression level of βactin in the lung (D1), immunoblotting of TGFβ1 and MMP2 among different groups in the lung (E), Immunoblotting quantitation of TGFβ1 and MMP2 proteins against the expression level of βactin in the lung (E1). OVX: ovariectomized group, OVX.E: ovariectomized rats with 8 weeks of estrogen treatment, OVX.Gen: ovariectomized rats with 8 weeks of genistein treatment. Data are expressed as mean ± SEM. * $P < 0.05$ vs sham group; ** $P < 0.05$ vs OVX group

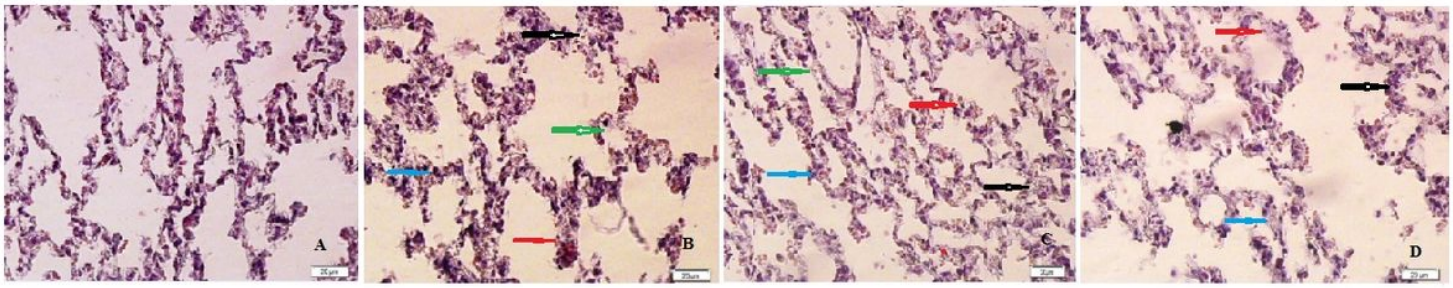

Figure 2

sham: normal lung architecture with thin interalveolar septa (A), OVX: thickened interalveolar septa with morphological change (red arrow), leukocyte and macrophage infiltration (black and blue arrow), respectively. Alveolar deformity with a morphological change in the alveoli (green arrow) (B), OVX.E: relatively thin interalveolar septa (blue arrow), and reduced inflammatory cell infiltration (red and black) (C), Alveolar deformity with a morphological change (green arrow), OVX.Gen group relatively thin interalveolar septa (black arrow) and reduced cell infiltration (blue and red) (D).

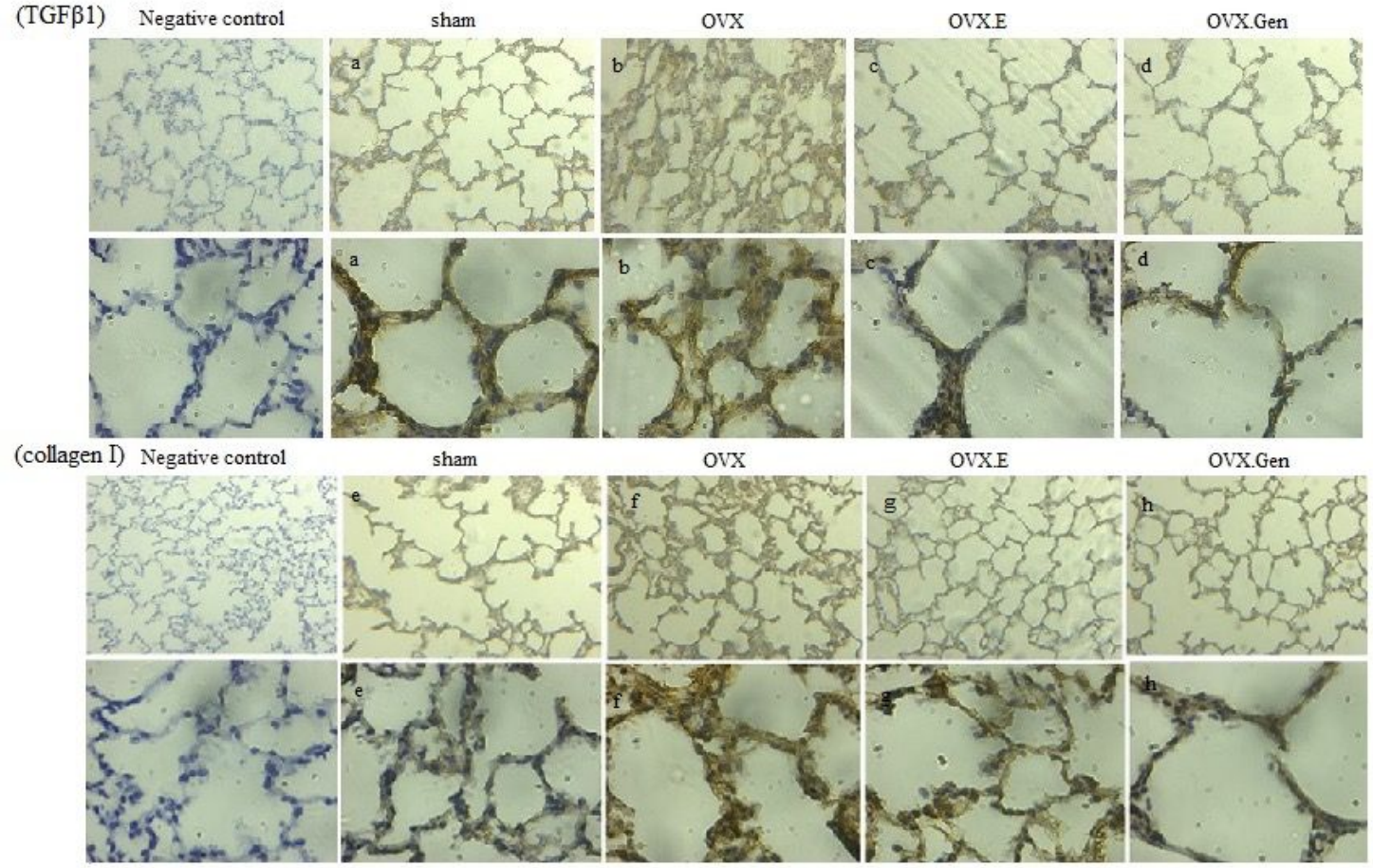

Figure 3

The immunohistochemical staining of TGFβ1 and collagen I in the lung sections in four experimental groups. TGFβ1 immunohistochemical staining in the study groups: sham (a), OVX (b), OVX.E (c), OVX.Gen (d), Collagen I immunohistochemical staining in experimental groups, sham (e), OVX (f), OVX.E (g), OVX.Gen (h).