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

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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 estrogeneric effects in postmenopausal women. Phytoestrogens mimic estrogeneric 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-estradiol 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 ± 0.22**      | 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 (Fig. 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
Immunohistochemical 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 | 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.

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Not Applicable.

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