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Effects of genistein treatment on molecular markers related to fibrosis: addition to histological changes in the lung of ovariectomized diabetic rats

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Objective Postmenopausal women experience physiological changes in several organs related to estrogen deficiency. On the other hand, the role of estrogen, as a sex hormone, in the physiological function and structure of respiratory system has been stablished. In the present study, we hypothesized that the administration of genistein could reverse lung fibrosis disturbed by estrogen deprivation.
Methods Forty female sprague–dawley rats were assigned into four groups: Rats underwent surgery without ovariectomy (sham), rats that underwent ovariectomies (OVX), ovariectomized rats that were fed with high fat diet (OVX.Dia), ovariectomized diabetic rats with genistein administration (OVX.Dia.Gen). Western blotting technique and hematoxylin and eosin were used to assess of protein levels and histological changes in the lung.
Results Transforming growth factor beta-1 and matrix metalloproteinase-2 were found to have differential expression among the groups. Ovariectomy alone and with diabetes increased the levels of growth factor beta-1 and matrix metalloproteinase-2 as compared with the sham ($P < 0.05$). On the other hand, genistein treatment reversed ovariectomy-induced changes in the protein levels as compared with OVX.Dia group ($P < 0.05$).
Conclusion Genistein ameliorated expression levels of fibrotic biomarkers in the lung of ovariectomized diabetic rats. Our data suggest that genistein may be beneficial for menopausal women through modulation of pulmonary fibrotic biomarkers expression levels.
Keywords genistein, menopause, lung, fibrosis, TGF-β1

Introduction

There are few studies done globally on healthy respiratory and lung function in postmenopausal women. Estrogen, as a sex hormone exerts important regulatory role in women's pulmonary function. Several lines of evidence suggest that estrogen deficiency associated with decrease in pulmonary function in elderly women.[1] Likewise, estrogen replacement in ovariectomized (OVX) mice has been shown to reverse lung architecture.[2] More importantly, anti-inflammatory and anti-fibrotic effects of estrogen have been established over the past few decades.[3]

People with diabetes mellitus are suspiciously affected by pulmonary disorders such as edema, inflammation and fibrosis in the lung.[4] Thus, histopathological findings in diabetic subjects have revealed thickening of basal lamina and fibrosis leading to restrictive lung function.[5] The cytokine transforming growth factor beta-1 (TGF-β1) is known as a main pro-fibrotic factor in diabetic statues. Moreover, transient elevation of blood glucose, even in healthy subjects leads to an increase in TGF-β1 urinary levels.[6]

Hormone replacement therapy (HRT) has been proposed to ameliorate insulin sensitivity in postmenopausal diabetic women.[7] However, HRT in menopausal women is accompanied by serious concern as it has been linked with breast cancer and cardiovascular diseases.[8,9] In recent years, there has been a growing interest in researching new procedures with estrogenic effects in menopausal women. Phytoestrogens have been shown to exert similar effects as HRT without unwanted harmful side effects.[10]

Accordingly, genistein, as a phytoestrogen can exert protective effects against metabolic disorders induced by diabetes mellitus.[11] Thus, genistein is shown to be as an effective therapeutic agent in pulmonary fibrosis.[12] In the light of recent data, the present study was designed to evaluate effects of genistein on the lung injury induced by estrogen deficiency in ovariectomized diabetic rats.

Materials and Methods

Animals and groups

Forty female wistar rats were obtained from experimental animal research institute, Tabriz Medical University, Tabriz, Iran. Rats (180–220 g) were kept in cages and acclimated in facility conditions (22–24°C) with a 12-h light, 12-h dark. The study was approved by the ethics committee of Tabriz University of Medical Science (code number: IR.TBZMED.REC.1396.450). Animals were randomly divided into four groups (n = 10 in each group): sham, laparotomy without ovariectomies, OVX, rats that underwent bilateral ovariectomy, ovariectomized rats that were fed with high fat diet (HFD) (OVX.Dia), for 4 weeks and injected with a single injection of STZ (30 mg/kg, intraperitoneally), ovariectomized diabetic rats with genistein administration (OVX.Dia.Gen) (Sigma-Aldrich, USA) (1 mg/kg/day; subcutaneously) for 8 weeks with concurrent HFD feeding.

Ovariectomy

Ovaries were removed under anesthesia with a mixture of 50 mg/kg ketamine chloride and 5 mg/kg xylazine chloride. In brief, after skin and muscle walls dissection, peritoneal cavity retrieved and then ovaries were removed with minimal skin and soft disruption.[13]

Diabetes induction

Ten days after ovariectomy, diabetic rats continued to feed with HFD (25% protein, 58% fat and 17% carbohydrate) for a 4-week period and injected with a single low-dose of STZ (30 mg/kg) dissolved in a 0.1-mol/L citrate buffer (pH 4.5) for induction of type II diabetes.[13] At the end of diabetes induction period, animals were given access to standard diet for 4 weeks. Blood samples were collected 72 h after STZ injection and at the end of the protocol. Rats with blood glucose levels higher than 200 mg/dl were considered to have diabetes.[14]

Immunoblotting analysis

Snap-frozen lung tissue was homogenized on ice in RIPA buffer containing proteinase inhibitor cocktail (leupeptin, chymostatin, pepstatin, antipain and aprotinin). After storing at 4°C for 20 min, the samples were centrifuged at 12,000 rpm for 10 min at 4°C. The supernatants were separated and stored at −80°C. Proteins were separated by SDS-PAGE and transferred onto polyvinylidene difluoride. Membranes were blocked with 5% (w/v) nonfat dry milk in Tris-buffered saline (TBS) (pH 7.5) for 2 h and incubated with primary antibodies (Anti-TGF-β1 and anti-MMP2, Santa Cruz, USA) at dilution containing 1% (w/v) nonfat dry milk in 0.05% (v/v) TBS plus 0.05% (v/v) Tween 20 (TBST). Samples were washed with TBST three times, then incubated for 1 h in secondary antibody (goat anti-rabbit; Santa Cruz, USA) in antibody dilution buffer. Substrates were detected by chemiluminescence (ECL Western Blotting Detection, Pierce, Rockford, IL, USA).[15] Anti-β-actin antibody has been considered as a loading control. Bands on immunoblots were quantified using ImageJ densitometry software.

Histological evaluation

The lungs were prepared for histopathological evaluation. Briefly, after fixing of lungs in 10% formalin and tissues processing, histological sections (5 μm thickness) were stained with standard hematoxylin–eosin. Light photomicroscope (Olympus BH-2, Tokyo, Japan) was used for evaluating of fibrosis in the lung tissues.

Statistical analysis

Data were expressed as the mean ± SEM. Differences between groups were determined using one-way ANOVA with Tukey's multiple comparison test. *P*-value less than 0.05 was considered as statistically significant.

Results

Blood glucose levels in the studied groups

To evaluate the genistein treatment on blood glucose levels, we measured the changes of blood sugar levels under fasting conditions. OVX.Dia rats exhibited hyperglycemia during an overnight fast as compared with sham group ($P < 0.05$). However, genistein treatment significantly lowered blood glucose levels in comparison to OVX.Dia group ($P < 0.05$, Fig. 1).

Genistein treatment on the levels of TGF-β1 in the lung

The lung levels of TGF-β1 significantly augmented in OVX and OVX.Dia groups when compared with the sham ($P < 0.05$). Genistein treatment significantly reduced TGF-β1 levels in comparison to OVX.Dia group, suggesting that genistein may exert its anti-fibrotic effects by reducing TGF-β1 in the lung of ovariectomized diabetic rats ($P < 0.05$, Fig. 2a and b).

Genistein treatment on the levels of MMP2 in the lung

Ovariectomized with or without diabetes led to a markedly increased in matrix metalloproteinase-2 (MMP2) levels ($P < 0.05$). Genistein treatment decreased MMP2 levels in OVX.Dia.Gen group as compared with OVX.Dia, indicating that decrease of MMP2 might be one of the mechanisms by which genistein exerts its protective effects against ovariectomy induced lung fibrosis ($P < 0.05$, Fig. 3a and b).

Histological changes in the lung tissue

Histological examination of lung in the sham group showed healthy lung tissues with thin interalveolar septa (Fig. 4a, Table 1). Section of lung in OVX and OVX.Dia groups showed thickened interalveolar septa (black arrow) with morphological changes of alveoli (asterisk). Alveolar cells deformities with a denuded alveolar epithelium (green arrow) were also detected in OVX and OVX.Dia groups. Ovariectomy with diabetes were associated with a significant alveolar hemorrhage (blue arrow) and intravascular coagulation (yellow arrow) ($P < 0.05$, Fig. 4b–d, respectively, Table 1). Genistein treatment declined hemorrhage and intravascular coagulation in OVX.Dia.Gen group as compared with OVX.Dia group ($P < 0.05$). Also, relative decline in number of alveolar cells deformities (green arrow) and alveolar morphological changes (asterisk) were observed in OVX.Dia.Gen group (Fig. 4e, Table 1).

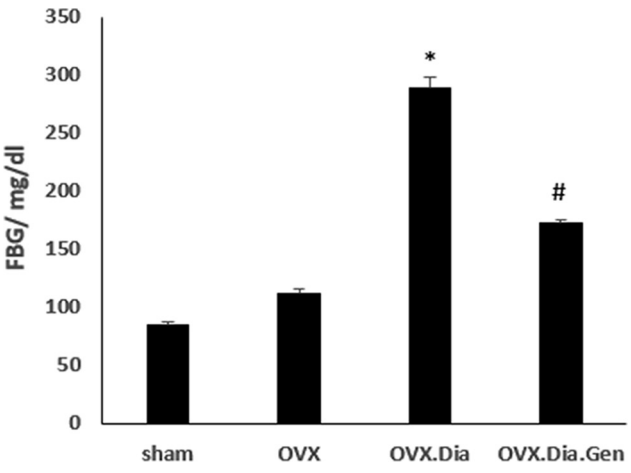

Fig. 1 **Effect of genistein treatment on blood levels of glucose in the studied groups. OVX: ovariectomized, OVX.Dia: ovariectomized diabetic rats, OVX.Dia.Gen: ovariectomized diabetic rats with 8 weeks of genistein treatment. Data are expressed as mean ± SEM. *$P < 0.05$ vs Sham group; #$P < 0.05$ vs OVX.Dia group.**

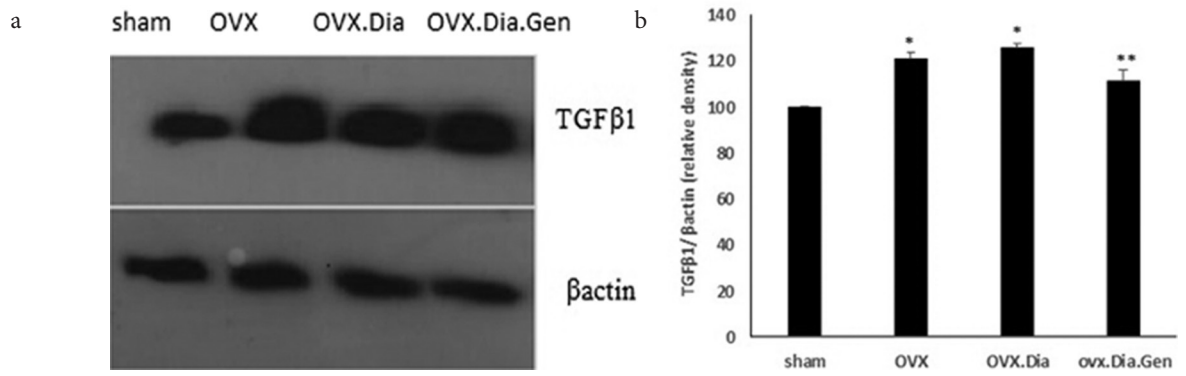

Fig. 2 **Effect of genistein treatment on TGF-β1 expression levels in the lung of studied groups. Immunoblotting of TGF-β1 among different studied groups (a), immunoblotting quantitation of TGF-β1 against expression of βactin in lung (b), OVX: ovariectomized group, OVX.Dia: ovariectomized diabetic rats, OVX.Dia.Gen: ovariectomized diabetic rats with genistein treatment. Data are expressed as mean ± SEM. *$P < 0.05$ vs sham group; **$P < 0.05$ vs OVX.Dia groups.**

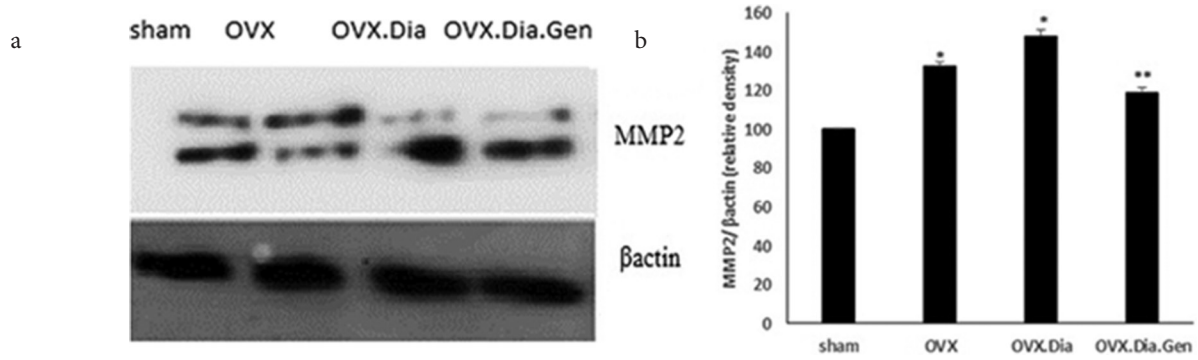

Fig. 3 **Effect of genistein treatment on MMP2 expression levels in the lung of studied groups. Immunoblotting of MMP2 within different groups (a), immunoblotting quantitation of MMP2 against expression level of βactin in the lungs (b), OVX: ovariectomized group, OVX.Dia: ovariectomized diabetic rats, OVX.Dia.Gen: ovariectomized diabetic rats with genistein treatment. Data are expressed as mean ± SEM. *$P < 0.05$ vs sham group; **$P < 0.05$ vs OVX.Dia groups.**

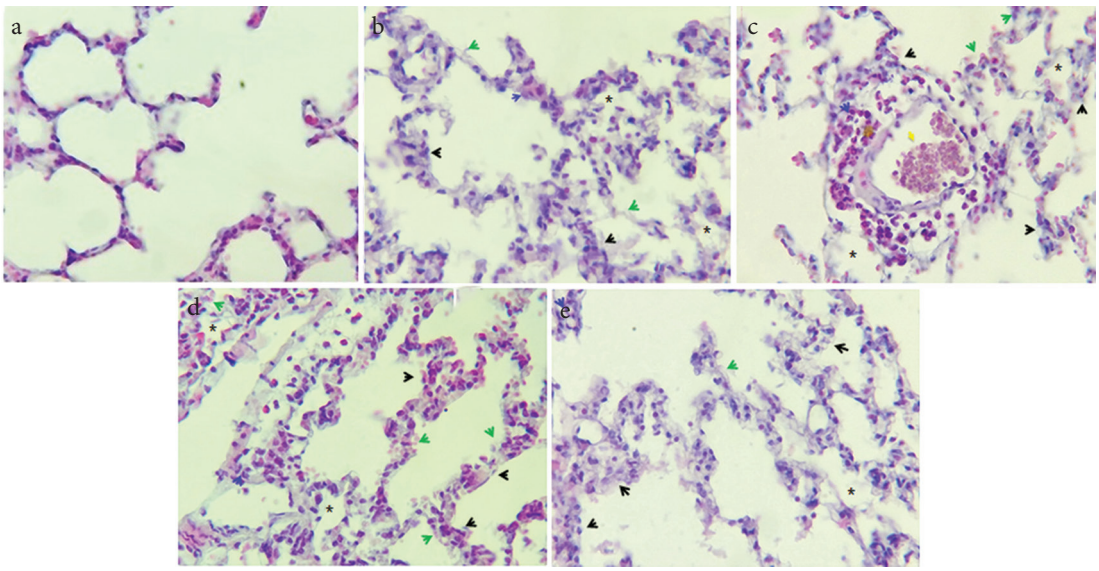

Fig. 4 **Histological micrograph in the lung of different studied groups. Sham (4a): normal lung with thin inter alveolar septa, OVX (4b): hemorrhage (blue arrow), thickened inter alveolar septa (black arrow), morphological change of alveoli (asterisk), and alveolar deformity (green arrow) observed in OVX group, OVX.Dia (4c and 4d): severe hemorrhage (blue arrow) in OVX.Dia group and thickening of interalveolar septa (black arrows) and intravascular coagulation (yellow arrow). OVX.Dia.Gen (4e) relative thin inter alveolar septa (black arrow) and decrease of hemorrhage (blue arrow), alveolar denudation (green arrow) and intravascular coagulation (yellow arrow) observed in OVX.Dia.Gen compared with OVX.Dia group.**

Table 1. **Histological changes in the lung of sham, ovariectomized, ovariectomized diabetic and genistein treatment groups (H&E)**

Group	Interstitial hemorrhage	Interstitial thickening	Alveolar epithelium deformity	Intravascular coagulation	Alveolar architectural alteration
Sham	0	0.20 ± 0.20	0.20 ± 0.20	0	0.40 ± 0.24
OVX	1.20 ± 0.20*	2.60 ± 0.24*	2.80 ± 0.20*	2.80 ± 0.20*	2.80 ± 0.20*
OVX.Dia	3.80 ± 0.20*	4 ± 0.24*	3.60 ± 0.24*	3.80 ± 0.20*	3.80 ± 0.20*
OVX.Dia.Gen	3 ± 0.50**	2.80 ± 0.20**	3 ± 0.001	2.80 ± 0.20*	3 ± 0.001

A minimum of five fields for each slide were evaluated for lung tissue changes ($n = 10$ in each group). Data are expressed mean ± SEM. *$P < 0.05$ vs sham group. **$P < 0.05$ vs OVX.Dia group.

Genistein treatment in OVX.Dia.Gen group revealed a moderate thickened interalveolar septa as compared with OVX.Dia group ($P < 0.05$, Fig. 4e, Table 1).

Discussion

Decrease in ovarian function with surgical or physiological menopause is associated with an increase in TGF-β1 expression levels in several organs.[16] Similarly, higher blood glucose concentration has been found to stimulate urinary TGF-β1 levels in healthy individuals.[6] There appears to be a strong association between the deregulation of TGF-β signaling and various fibrotic diseases. Aberrant activation and proliferation of fibroblasts or myofibroblast contribute to an increased collagen deposition in the fibrotic lung. There is also evidence that TGF-β1 might induce fibroblast to myofibroblast transdifferentiation. Interestingly, the cytokine TGF-β1 has been identified locally at the sites of fibrosis in fibroblast-to-myofibroblast trans-differentiation.[17]

Previous studies demonstrated a strong induction of coagulation factor XII (FXII) expression by TGF-β1. In particular, TGF-β1 expected to assume a major role in increasing the expression of coagulation FXII in the lung fibroblast cells.[18] In keeping with previous reports, we observed fibrosis with markedly increased in TGF-β1 expression levels and a marked activation of blood coagulation detecting in the lung of OVX.Dia group as compared with the sham.

Matrix metalloproteinase exert essential roles in functioning of several tissues during aging. Thus, aging female mice were characterized by enhanced MMP2 activity, that it was ameliorated by estrogen therapy in the lung.[19] Interestingly, in a previous study we found an increase in MMP2 expression level in the lung of OVX rats.[20] Clinical studies have revealed a close relationship between MMP2 expression and pulmonary fibrosis. It is noted that, MMP2 involved in TGF-β1 induced epithelial mesenchymal transition (EMT), indicating that production of MMP2 in the lung alveolar cells may exert a major role in the development of pulmonary fibrosis.[21] Additionally, excess activation of MMPs has been shown to destroy extracellular matrix and induce further inflammation in the lung.[22] On the other hand, lung epithelial cells that undergo EMT show enhanced expression of fibrogenesis markers like MMP2 protein levels.[21]

There appears to be a strong link between dysregulation of MMP/TIMP system and diabetes status. Thus, blood levels of MMP2 has been shown to significantly increase in type 2 diabetes.[23] Yusuf et al.[4] have found a dramatic change in lung structure in diabetic rats characterized by infiltration of mononuclear cells, edema, hemorrhage, and fibrosis. Similarly, ovariectomized diabetic lung is associated with a marked histological abnormalities.[4] In the present study, to the best of our knowledge we demonstrated that MMP2 is significantly increased in the lung of ovariectomized diabetic rats. It is also well known that, fibrotic pulmonary remodeling associated with repetitive minor injuries of alveolar epithelial cells lining with a denuded basal lamina.[24] Our results confirmed previous studies indicating markedly increased in MMP2 expression with severe fibrosis in the lung. We also found significant morphological change in alveolar cells in OVX and OVX.Dia groups reflected by denudation of basal lamina.

It has become apparent that, phytoestrogens exert beneficial effects on diabetic complications.[11] Genistein, as a phytoestrogen plays a protective role in the pulmonary fibrosis. As previously reported, genistein could exert a protective effects against interstitial and perivascular lung fibrosis induced by pulmonary hypertension.[12] Genistein has also been shown to block TGF-β-mediated activation of MMP2 in prostate epithelial cells.[25] Activation of the Ras/Erk MAPK pathway has been implicated in EMT induced by TGF-β1.[26] In other study, we showed that genistein can decrease ERK expression levels in the lung of ovariectomized diabetic rats.[22] In the current study, TGF-β1, and MMP2 significantly decreased in the genistein treatment group as compared with the ovariectomized diabetic rats.

There are few researches regarding the role of phytoestrogens on the blood coagulation activities. It is known that genistein increases some coagulation related genes in OVX rats.[27] However, in another study high doses administration of isoflavone (up to 300 mg/day) did not change coagulation parameters in the postmenopausal women.[28] In the present study, genistein administration partially decreased intensity of intravascular coagulation, but there was no significant change between genistein treatment and ovariectomized diabetic groups.

Our results are supported by recent studies that highlighted the important role of genistein supplementation in ovariectomy induced lung injury. Although the beneficial effects of genistein on respiratory system is not well known. Therefore, our results encourage further studies in ovariectomized diabetic states.

Conclusion

Estrogen deficiency alone or with HFD increased fibrotic biomarkers such as TGF-β1 and MMP2 in the lung of ovariectomized diabetic rats. Genistein treatment ameliorated

proteins expression and histological changes in the lung of ovariectomized diabetes rats.

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Conflict of Interest

None.

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