Genistein preserves the lungs of ovariectomized diabetic rats: addition to apoptotic and inflammatory markers in the lung

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**Objective(s)**: The role of isoflavones in pulmonary structure and function during menopause is not well studied. Moreover, the important role of estrogen in the physiological function of respiratory system has been revealed. Genistein, as an isoflavone, mimics estrogenic in diabetic and ovariectomized rats. Here, we hypothesized that genistein would reverse changes in the protein expression levels related to estrogen deficiency in the lung of ovariectomized diabetic rats.

**Materials and Methods**: Wistar female rats were assigned to four experimental groups (n=10 in each group): sham, rats underwent laparotomy without removing the ovaries; OVX, rats that underwent ovariectomy; OVX D, rats underwent bilateral ovariectomy and were fed a high-fat diet (HFD); O VX GD, ovariectomized diabetic rats with genistein administration (1 mg/kg/day). After ovariectomy, rats continued to feed HFD for a 4-week period. After 4 weeks of HFD feeding, a single dose of 30 mg/kg of streptozotocin was administered in the diabetic group. Genistein was administered for eight weeks. At the end of the experiment, lung tissue was removed and Western blotting technique and hematoxylin–eosin staining were used for evaluation of the lung.

**Results**: Treatment with genistein significantly decreased inflammatory and apoptotic biomarkers in the ovariectomized diabetic rats compared to non-treated animals (P<0.05). Also, genistein exerted a protective effect in the lung architecture.

**Conclusion**: Genistein partly reversed ovariectomy-induced changes in apoptotic and inflammatory biomarkers in the lung. Our data suggest that genistein treatment as a natural replacement therapy may prevent the estrogen deficiency effects in the lung of diabetic menopausal women.

**Introduction**

The effects of diabetes mellitus on the pulmonary system have been studied over the past few decades. Increasing evidence has suggested that diabetes is strongly associated with reduction in pulmonary function (1). Accordingly, the risk of pulmonary disorders increases in patients with diabetes (2). Recently, a meta-analysis research has established that the ratio of forced expiratory volume through the first sec and forced vital capacity decreased in type 1 and type 2 diabetes (3). Diabetes can lead to a marked augmentation in caspase 3 activity, as a final biomarker of apoptosis, in the lung of diabetic rats (4). Also, inflammatory biomarkers including Tumor necrosis factor-alpha (TNF-α) and C-reactive protein were shown to increase in diabetic subjects (5). Increasing evidence in the past decades suggests that gender exerts an impact on the incidence, prevalence, and severity of multiple pulmonary diseases among other factors. Moreover, the important role of sex hormones in respiratory health has been established.

Estrogen deficiency in ovariectomized (OVX) mice is associated with an increase in the number of apoptotic cells in the lung (6). Likewise, estrogen replacement has been shown to improve lung architecture in OVX mice (7). Moreover, the anti-inflammatory effect of estrogen has been demonstrated in the lung tissue (7).

Hormone replacement therapy (HRT) has been shown to improve insulin resistance in postmenopausal diabetic subjects (8). However, HRT raises serious concerns such as breast cancer and vascular complications (9, 10). Some studies have focused on other compounds that exert estrogenic effects in menopausal women. Phytoestrogens have been reported to exert similar effects as HRT without having unwanted side effects (11). Genistein, as a phytoestrogen, exerts a protective effect against metabolic disorders related to diabetes mellitus (12). Accordingly, anti-apoptotic and anti-inflammatory effects of genistein have been suggested in previous research (13). Also, our research team previously has reported the protective effect of genistein in the pancreas of OVX rats (14).
Therefore, in this study, we induced ovariectomy and diabetes in rats and hypothesized that genistein could prevent OVX-induced tissue injury in vital organs such as the lungs by attenuating the proinflammatory cytokine response and apoptotic biomarker generation in animal models of diabetes.

Materials and Methods

Animal care

Forty adult female Wistar rats, aged about 10 weeks with average weight of 180–220 g, were used in the current study. The animals were obtained from the Animal Research Institute, Faculty of Medicine, Tabriz University of Medical Sciences (Tabriz, Iran). This study was approved by the Animal Ethics Committee (code no.: IR.TBZMED.REC.1396.450). All rats were kept at a 22–24 °C temperature range in a 12 h light/dark cycle and maintained in a standard condition with access to water ad libitum and standard pellet diet for a week.

Chemical

Genistein and streptozotocin (Sigma St. Louis, Mo, USA), polyclonal rabbit anti-Bcl-2 (sc-492), anti-IL1β (sc-7884), anti-caspase 3 (sc-7148), anti-ERK (sc-292838), and anti-P-ERK (sc-101760) (Santa Cruz, CA, USA) were used in the experiment.

Experimental design

Adult female Wistar rats were assigned to four experimental groups: sham (n=10), these animals underwent surgery without ovariectomy and received the vehicle (DMSO 100 µl/day); OVX (n=10), rats underwent bilateral ovariectomy; OVX.D (n=10), diabetes was induced in OVX rats with HFD; OVX.D.G (n=10), in this group OVX diabetic rats were treated with genistein. Ovariectomy was performed under anesthesia with xylazine chloride (15 mg/kg, IM) and covered with sterile gauze. Each rat was injected with gentamicin (60 mg/kg, IM) and covered with sterile gauze. After 30 minutes, the incisions on both sides of the back were sutured with sterile sutures (15). Ten days after surgery, rats received an HFD consisting of 17% carbohydrate, 58% fat, and 25% protein in the diabetic group for four weeks. After 4 weeks of HFD, a single dose of 35 mg/kg of streptozotocin was dissolved freshly in citrate buffer (0.1 M, pH 4.5) and injected intraperitoneally for inducing diabetes type II (16, 17). Blood sugar level was checked with a glucometer, and rats were confirmed diabetic when blood glucose levels exceeded 200 mg/dl (17). After 4 weeks on HFD, animals received a normal diet for a period of four weeks. Genistein dissolved in DMSO was administered daily for up to eight weeks (1 mg/kg; subcutaneously), concurrent with the onset of diabetes induction (18). After 8 weeks, lung tissue was removed under anesthesia and prepared for molecular and histological analysis.

Plasma glucose measurements

Measurement of fasting blood glucose (FBG) was done at the beginning of the experiment by using a glucometer (Roche) in order to ensure rats were euglycemic. After injection of streptozotocin, the blood glucose concentration was checked and the levels higher than 200 mg/dl were considered as hyperglycemia.

Immunoblotting analysis

Evaluation of ERK1/2 phosphorylation, Bcl-2, IL1β, and caspase 3 was performed by using the Western blotting technique in the lung (19). Briefly, all samples after snap freezing were homogenized in RIPA lysis buffer comprising proteinase inhibitor cocktail (aprotinin, pepstatin, antipain, leupeptin, and chymostatin) on ice and left 20 min at 4 °C. The samples were then centrifuged at 4 degrees centigrade at 12,000 rpm for 10 min, then supernatant was collected and kept at -80 °C. The proteins were separated in SDS-PAGE. After separation, proteins were transferred electrophoretically onto polyvinylidene difluoride (PVDF). Incubation of membranes was done with 5% w/v nonfat dry milk in Tris-Buffered Saline (pH 7.5) for 2 hr in order to block non-specific binding sites. Blots were located at the room temperature for 2 hr with anti- Bcl-2, anti- P-ERK1/2, anti- caspase 3 , anti-ERK1/2, and anti-IL1β (primary antibody) in antibody dilution buffer comprising 1% w/v nonfat dry milk in 0.05% v/v Tris-Buffered Saline with 0.05% v/v Tween 20. Then samples were washed with TBST 3 times, incubation was prepared for 1 hr with goat Anti-Rabbit (secondary antibody) in an antibody dilution buffer. Chemiluminescence (ECL) detection kit (Pierce, Rockford, IL) was used for detection of blotting substrate. The density of the bonds on immunoblots was quantified using the ImageJ software.

Histological evaluation

At the end of the experiment, lung tissue was fixed in 10% formaldehyde after isolation, sectioned with 5 µm thicknesses, stained with hematoxylin and eosin (H&E), and examined using light microscopy.
Menopause, genistein, lung

Figure 1. ERK1/2 proteins hyperphosphorylation with genistein treatment in the lungs of experimental groups. ERK immunoblotting among different studied groups. (a) Quantitation of immunoblotting of P-ERK expression levels against ERK. (b) OVX: ovariectomized group, OVX.D: ovariectomized diabetic group, OVX.D.G: ovariectomized diabetic with genistein treatment. Data expressed as mean±SEM. *P<0.05 versus sham group; # P<0.05 versus OVX.D group

Data analysis and statistics
Data are presented as the mean±SEM. One way ANOVA with post hoc Tukey’s test was used for statistical analysis of all data. P-values<0.05 was considered as statistically significant.

Results
Genistein treatment on ERK1/2 level in the lung
As shown in Figure 1, the levels of ERK1/2 increased significantly in the OVX rats compared with the sham group and this increase was higher in the OVX diabetic rats (P<0.05). Treatment with genistein markedly decreased ERK levels in the genistein treatment group as compared with OVX and OVX.D rats (P<0.05).

Genistein treatment on caspase 3 and Bcl-2 levels in the lungs
When compared with the sham group, the level of activated caspase 3 was markedly elevated in the OVX group and this increase was more in the OVX.D rats (P<0.05) (Figure 2). Also, the Bcl-2 protein was significantly reduced in the ovariectomized and ovariectomized diabetic groups as compared with the sham (P<0.05). Genistein treatment significantly increased Bcl-2 and decreased caspase 3 expression levels as compared with ovariectomized diabetic groups (P<0.05).

Effect of genistein on IL1β level in the lungs
As shown in Figure 3, ovariectomy with diabetes noticeably increased the expression level of IL1β as compared with the sham (P<0.05). Genistein treatment significantly reduced IL1β expression level when compared with the ovariectomized diabetic group (P<0.05).

Genistein treatment on histological changes in the lung
Histological study of the sham group revealed lung tissue without macrophage and leucocytes infiltration and perivascular edema (Figure 4A). In the OVX group, cellular infiltration and perivascular edema were obviously observed (Figure 4B). Diabetes and ovariectomy exerted severe perivascular edema and severe macrophage and leucocyte infiltration (Figure 4C). Treatment with genistein was associated with obviously decreased in cellular infiltration. Perivascular edema was prominently diminished in the genistein treatment group (Figure 4D).
Overview of the document

The document discusses the effects of diabetes mellitus on cellular function in different types of tissues. It highlights the role of estrogen in reducing inflammation and apoptosis in the lungs of O VX (ovariectomized) mice. The study shows that estrogen deprivation increases IL1β protein expression and caspase 3 activity, while genistein treatment reduces these effects.

**Discussion**

Diabetes mellitus alters cellular function in different types of tissues; it causes unsuitable effects called diabetic complications. Inflammation and apoptosis are two important cellular processes that are affected by diabetes. On the other hand, reduction in ovarian function has been shown to induce inflammation and apoptosis in the lungs of OVX mice. In this work, we studied the protective role of genistein in the lungs of OVX diabetic rats.

The Ras/Raf/ERK pathway has a key role in lung injury induced by hyperoxia. It has also been shown that inhibition of Raf-1 kinase protects retinal endothelial cells against increased apoptosis under hyperglycemic conditions. The effect of estrogen on apoptosis in the lung cells is not well known; however, some studies have revealed that estrogen may exert a protective role in the development of pulmonary apoptosis in the OVX mice. Accordingly, estrogen deficiency in old female mice has been associated with increased levels of IL1β and caspase 3 activity in the renal epithelial cells. Zhuang and colleagues showed that ERK activity can induce apoptosis through enhancing in caspase 3 activity in the renal epithelial cells. Additionally, ERK/MEK activity has been found to be associated with down-regulation of the Bcl-2 protein, as an anti-apoptotic marker, in osteoblasts.

In our study, caspase 3 was elevated and Bcl-2 declined in OVX diabetic rats as compared with the sham. Estrogen deprivation also induced an increase in Caspase 3 as a final biomarker of apoptosis in the lung of OVX mice. Moreover, elevated levels of caspase3 activity have been found in the lung of diabetic rats. Zhuang and colleagues showed that ERK activity can induce apoptosis through enhancing in caspase 3 activity in the renal epithelial cells. Additionally, ERK/MEK activity has been found to be associated with down-regulation of the Bcl-2 protein, as an anti-apoptotic marker, in osteoblasts.

On the other hand, investigators have suggested that IL1β, an inflammatory mediator, has a key role in lung inflammation. Accordingly, neutralization of IL1β is associated with decreased silica-induced inflammation by inhibiting other inflammatory mediators in the lung. The pathogenesis of diabetes in different tissues is associated with chronic inflammation. Moreover, increased levels of IL1β have been found in the retina of diabetic rats. Genistein, a naturally occurring isoflavonic phytoestrogen, can bind to estrogen receptors; it has been proposed as a natural replacement to estrogen. It has also been shown to have beneficial effects on the prevention of metabolic disorder related to diabetes.
Similarly, the anti-inflammatory effect of genistein has been proposed in diabetic mice. Genistein has been shown to reduce inflammatory markers including NF-kB in diabetes-induced renal damage (29). Also, another study reported that genistein reduces P-ERK production in diabetic nephropathy in mice (30). Consistent with the previous studies, we observed significantly decreased inflammatory and apoptotic markers including IL1β and ERK in genistein-treated rats compared to the OVX.D group. We hypothesized that OVX diabetic rats would be protected against developing lung inflammation and apoptosis with genistein treatment. Genistein has also attracted much attention for its anti-apoptotic properties in diabetes. It is now well known that high glucose concentration triggers endothelial cell apoptosis, and genistein treatment decreases cellular apoptosis by the Bcl-2 dependent pathway (31).

Results of previous studies by our group revealed that genistein exerts a strong protective effect against inflammatory and apoptotic markers in the pancreas of OVX diabetic rats (14). In our study, genistein significantly decreased caspase 3 and increased Bcl-2 expression levels in the genistein treatment group compared with the OVX.D group. In this study improving the effect of genistein on pulmonary apoptosis and inflammation could result in protection from lung damage in OVX diabetic rats. Although the beneficial effects of genistein on pulmonary function is not well known, our results encourage further works in this area.

Conclusion

Our study revealed increased levels of IL1β, ERK, and caspase 3, and also decreased Bcl-2 expression level in the OVX.D group compared to the sham. Estrogen deficiency alone or with diabetes increased inflammatory and apoptotic biomarkers in the lungs. However, administration of genistein prevented these apoptotic and inflammatory changes in the lungs of OVX diabetic rats. The results of this work support the beneficial effects of genistein for prevention of lung injury induced by estrogen deficiency in OVX diabetic subjects.

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Conflict of interest

There are no competing interests in the present work.

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