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Effect of genistein on expression of pancreatic SIRT1, inflammatory cytokines and histological changes in ovariectomized diabetic rat

Hadi Yousefi [1], Alireza Alihemmati [2], Pouran Karimi [3], Mohammad Reza Alipour [1], Parisa Habibi [1], Nasser Ahmadiasl [1*]

[1] Drug Applied Research Center, Tabriz University of Medical Sciences, Tabriz, Iran
[2] Department of Histology & Embryology, Faculty of Medicine, Tabriz University of Medical Sciences, Tabriz, Iran
[3] Neurosciences Research Center, Tabriz University of Medical Sciences, Tabriz, Iran

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ABSTRACT

Objective(s): Genistein is reported to have anti-diabetic and anti-inflammatory functions, in particular, direct effects on β-cell proliferation and insulin secretion. In this study, we investigated the anti-inflammatory effect of genistein on the pancreatic β-cells in ovariectomized diabetic rat.

Materials and Methods: Forty female rats were divided into four groups: sham, bilateral ovariectomy (OVX), OVX.D (OVX+diabetes) and OVX.D.G (OVX.D+genistein). After bilateral ovariectomy, rats in the diabetic groups were fed high-fat diet (HFD), *ad libitum* for 4 weeks, and then a low dose of streptozotocin (STZ) (30 mg/kg) injected intraperitoneally. Genistein (1 mg/kg/day; SC) was administrated for 8 weeks. At the end of 8 weeks, pancreas tissue was removed and used for western blotting and Hematoxylin-Eosin staining.

Results: Treatment with genistein declined inflammation and tissue injury, and this decline was correlated with the expression of SIRT1. OVX and OVX.D significantly increased Nf-κB and IL-1β expression and decreased SIRT1 levels compared to sham group (P<0.05). Significant reduction of Nf-κB and IL-1β, and increasing of SIRT1 were observed during genistein treatment (P<0.05).

Conclusion: Estrogen deficiency alone or with HFD increased pancreatic inflammation. However, subcutaneous administration of gtenistein prevented from these inflammatory changes in the pancreas of a surgery animal model of ovariectomy with or without diabetes. Our results support the potential preventing effect of genistein from pancreatic injury.

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Introduction

Diabetes, which is characterized by a deficit in β-cell mass, is developing to epidemic proportions. However, the mechanisms underlying β-cell destruction are not clear, it has been suggested that cytokines may be involved. In diabetes, cytokines are important mediators in the impaired function and destruction of pancreatic β-cells (1, 2).

Many pieces of evidence have demonstrated that the reduction in ovarian function with menopause or surgical is related with spontaneous rises in pro-inflammatory cytokines. The pro-inflammatory cytokines that have obtained the more attention are interleukin (IL)-1β, and tumor necrosis factor (TNF-α). Estrogen deficiency has also been revealed to augment the responsiveness of cells to these cytokines by up-regulating cytokine receptors (3).

During the inflammatory process, pro-inflammatory cytokines such as IL-1β and TNF-α are secreted by immune cells affecting the pancreas and contribute to β-cell dysfunction and apoptosis (4). Activation of the transcription factor Nf-κB is necessary for cytokine-induced β-cell death (5). SIRT1 belongs to family of histone/protein deacetylases (class III) and may play an important role in the inflammation. SIRT1 is expressed in the endocrine cells of the Langerhans islets. Furthermore, the effect of SIRT1 is inhibition of Nf-κB by deacetylating p65; it can protect β-cells from cytokine (6).

Phytoestrogens, such as genistein, are naturally occurring plant estrogens that are suggested for use in postmenopausal women. These compounds have a chemical structure similar to human estrogen and have the ability to attach the estrogen receptors (7). Studies have demonstrated that phytoestrogen genistein has anti-diabetic effects; it has in particular direct effects on β-cell proliferation, insulin secretion and anti-apoptotic effects (8). Genistein decreases many pro-inflammatory mediators and some pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6. These effects of genistein involved in the protection of human pathological processes (9).

*Corresponding author: Nasser Ahmadiasl. Drug Applied Research Center, Tabriz University of Medical Sciences, Tabriz, Iran. Tel/Fax: +98-4133364664; *email*: n.ahmadiasl@gmail.com

Because of anti-diabetic and anti-inflammatory effects of genistein, in this study, we investigated the effect of genistein treatment on inflammation and SIRT1 level in the pancreatic β-cells in ovariectomized diabetic rat.

Materials and Methods

Chemical

Streptozotocin (STZ) and genistein were purchased from Sigma (St. Louis, Mo, USA). Polyclonal rabbit anti-Nf-κB (ab-16502) was purchased from Abcam (Cambridge, MA) and anti-IL-1β (sc-7884), anti-SIRT1 (sc-15404) and HRP-conjugated goat anti rabbit polyclonal antibody (sc-3837) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

Animal care

Female Wistar rats (weighing 180-220 g, about 10-weeks old) were purchased from Experimental Animal Research Center, Faculty of Medicine, University of Tabriz, Tabriz, Iran. The study was approved by University Ethics Committee. All the rats were kept under controlled conditions (temperature of 22-24 ˚C with 12/12 hr of the light-dark cycle), were allowed free access to water *ad libitum* for 1 week.

Protocol

Forty female rats were randomly divided into four groups (10 per each group): Sham operation (this group underwent only surgery without ovariectomy), bilateral ovariectomy (OVX), OVX that induced diabetes with high fat diet (HFD) and single dose of streptozotocin (STZ) (OVX.D), OVX.D that treated with genistein (OVX.D.G). Ovaries in these groups were removed under anesthesia with ketamine chloride (50 mg/kg) and xylazine chloride (5 mg/kg), with minimum disruption to the surrounding soft tissues (10). Ten days after ovariectomy, rats in the diabetic groups were fed HFD (58% fat, 17% carbohydrate, and 25% protein), *ad libitum* for 4 weeks, and then a low dose of STZ (30 mg/kg) was injected intraperitoneally (IP) in a 0.1 M citrate buffer (pH 4.5) for induction of type II diabetes (11, 12). Glucose level was measured with a glucometer in all rats after induction of diabetes. Animals with blood glucose levels more than 200 mg/dl were selected as diabetic rats (12). After induction of diabetes, the animals in diabetic groups received a standard chow diet for 4 weeks. Genistein (Sigma-Aldrich, USA) (1 mg/kg/day; subcutaneously, SC) was dissolved in DMSO and administrated for 8 weeks, concurrent with the onset of HFD regime. Schematic diagram illustrates experimental protocol in Figure 1. The rats in the control and sham group received the vehicle. At the end of 8 weeks, pancreas tissue was removed under anesthesia and used for histological and molecular evaluations (13). Western blot analysis was performed on homogenates of pancreatic tissue and, Hematoxylin-Eosin staining was used for histopathological assessment.

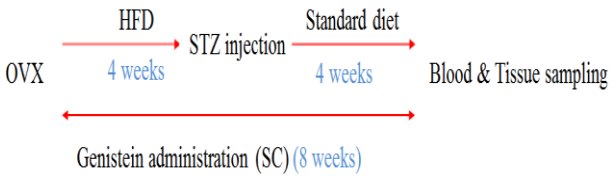

Figure 1. Schematic diagram illustrating experimental protocol for diabetes induction and genistein administration. HFD; high fat diet, STZ; Streptozotocin, OVX; Ovariectomy, SC; Subcutaneous

Plasma glucose, insulin and lipid measurements

At the beginning of the experiment, the fasting blood glucose (FBG) levels in tail vein blood sample were measured using a glucometer (Roche) to ensure that rats were euglycemic. After STZ injection, the levels of blood glucose were measured to assess the onset of hyperglycemia (FBG 200 mg/dl). Plasma insulin level was measured by ELISA (Mecodia, Winston-Salem, NC) in rats fasted for 4 hr. Fasting plasma triglycerides (TG), total cholesterol, HDL-cholesterol (HDL-C) and LDL-cholesterol (LDL-C) were measured by using a commercial diagnostic kit (Randox (UK)) in accordance with the manufacturer's instructions.

Immunoblotting analysis

Western blot analysis was performed on homogenates of pancreatic tissue. Briefly, snap-frozen pancreatic tissue was homogenized on ice in RIPA buffer. This buffer supplemented with a protease inhibitor cocktail containing leupeptin, pepstatin, chymostatin, aprotinin and antipain (5 µg/ml each), rotated for 20 min at 4 ˚C, and centrifuged at 12,000×g for 10 min at 4 ˚C. The supernatant was collected and stored at −80 ˚C. The amount of protein in the supernatant was quantified using the Bradford assay with commercial reagents (Bio-Rad Laboratories, CA, USA) and spectrophotometric (Jenway 6305 spectro-photometer, Bibby Scientific Ltd, UK) measurements. Proteins were separated by SDS-PAGE (10 µg protein loaded per each well) and electrophoret -ically transferred onto PVDF membranes. Nonspecific binding was blocked by 2 hr incubation of the membranes in 5% (w/v) nonfat dry milk in Tris-buffered saline (pH 7.5). Blots were then incubated for 2 hr at room temperature (or overnight at 4 ˚C) with primary antibodies (Anti- IL-1β, SIRT1 and β-actin: Santa Cruz, USA (1:500); Anti-p65 Nf-κB: Abcam (1:1250)) diluted in the antibody buffer containing 1% (w/v) nonfat dry milk in TBS-T (0.05% (v/v) Tween-20 in Tris-buffered saline), then washed 3 times with TBS-T, and finally incubated for 1 hr with a secondary antibody (Goat anti-Rabbit; Santa Cruz, USA (1:5000)) in the antibody buffer. Blots were developed for visualization using enhanced chemiluminescence (ECL) detection kit (Pierce, Rockford, IL). Anti-β-actin was used as a loading control. Band intensities on the immunoblots were quantified by densitometry using the Image j software.

Histological evaluation

The pancreas tissues were fixed in 10% of formalin solution in continuing dehydration in ascending grades of Ethanol (Merck, Germany), cleared and embedded in xylol and paraffin, respectively. Sections of 5 µm were taken, stained with Hematoxylin-Eosin (H-E), and investigated under the light microscope (Olympus BH-2, Tokyo, Japan) in a blinded manner by a pathologist. A minimum of five fields for each pancreas slide were examined and assigned for severity of tissue changes (n=7 for each group). Pancreas tissue was examined for tissue inflammation and injury in islets of Langerhans.

Statistical analysis

All values were described as the mean±SEM. Differences between groups were determined by one-way analysis of variance (ANOVA) with Tukey's multiple comparison post-test using SPSS program version 16.0. A value of $P<0.05$ was considered statistically significant.

Results

Body weight changes in studied groups

Body weight was measured weekly. We observed a significant increase in body weight in the OVX animal compared to sham ($P<0.05$). However, ovariectomized diabetic animals showed significant reduction of the body weight in comparison with sham and OVX groups ($P<0.05$). Genistein treatment markedly increased body weight in OVX.D.G compared to OVX.D group ($P<0.05$), but it was less than OVX animals (Figure 2).

Blood glucose and insulin levels

To ascertain the effects of genistein on metabolic function, we measured FBG and serum insulin. OVX.D rats exhibited hyperglycemia and hyperinsulinemia during an overnight fast (Figure 3). Genistein improved glucose homeostasis in OVX.D.G rats to near normogly - cemia (Figure 3). Genistein replacement significantly lowered serum glucose and insulin contents (Figure 3a & b). In OVX.D.G rats, genistein decreased the FBG and serum insulin levels compared to OVX.D animals ($P<0.05$) (Figure 3).

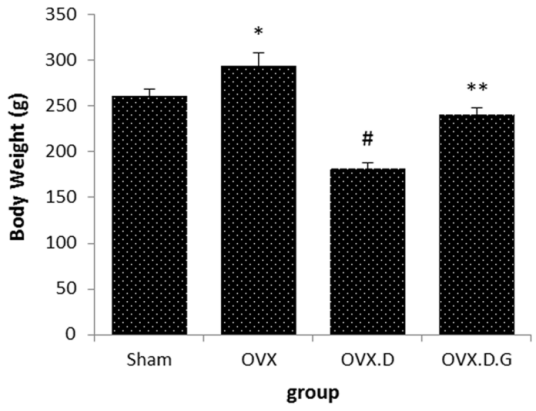

Figure 2. Final body weight (BW) evaluation in different groups. OVX: ovariectomized, D: diabetic and .G: genistein-treated groups respectively. Data are expressed as mean±SEM; * $P<0.05$ vs. Sham group; # $P<0.05$ vs. Sham and OVX group; ** $P<0.05$ vs. OVX and OVX.D

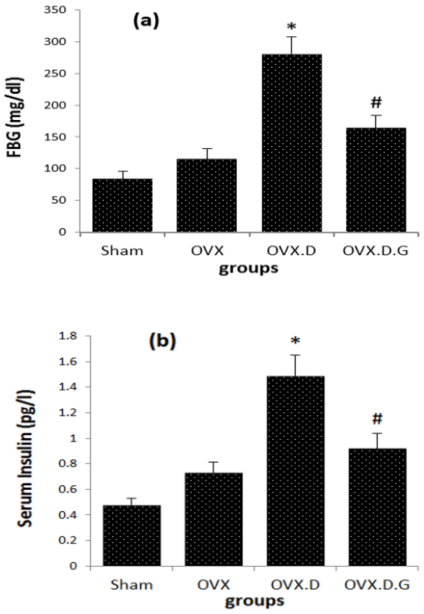

Figure 3. Blood levels of glucose (a) and insulin (b) in different groups. OVX: ovariectomized, D: diabetic and .G: genistein-treated groups respectively. Data are expressed as mean±SEM. * $P<0.05$ vs. Sham group; # $P<0.05$ vs. OVX.D group

Biochemical analysis

The plasma lipid profiles are presented in Figure 4. Total-cholesterol (t-Chol), Triglyceride (TG) and low-density lipoprotein cholesterol (LDL-C) levels were significantly higher in the OVX and OVX.D groups than the OVX.D.G and Sham operation groups ($P<0.05$). High-density lipoproteins cholesterol (HDL-C) in the OVX.D.G and sham operation groups were markedly higher than the OVX.D and OVX group ($P<0.05$).

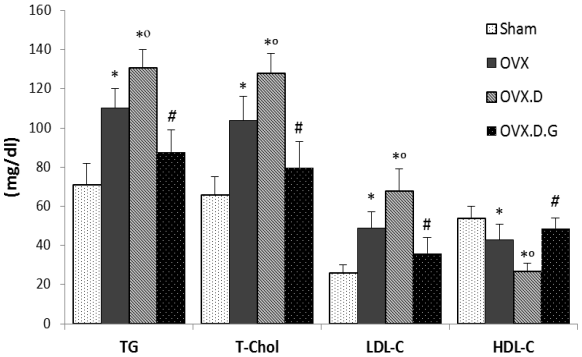

Figure 4. Plasma lipid profiles levels in different groups. TG; Triglyceride. T-Chol; total cholesterol. LDL-C; low density lipoprotein cholesterol. HDL-C; High density lipoprotein cholesterol. OVX: ovariectomized, D: diabetic and .G: genistein-treated groups, respectively. Data are expressed as mean±SEM. *$P<0.05$ vs. Sham group; #$P<0.05$ vs. OVX.D & OVX groups; °$P<0.05$ vs. OVX and sham operation groups

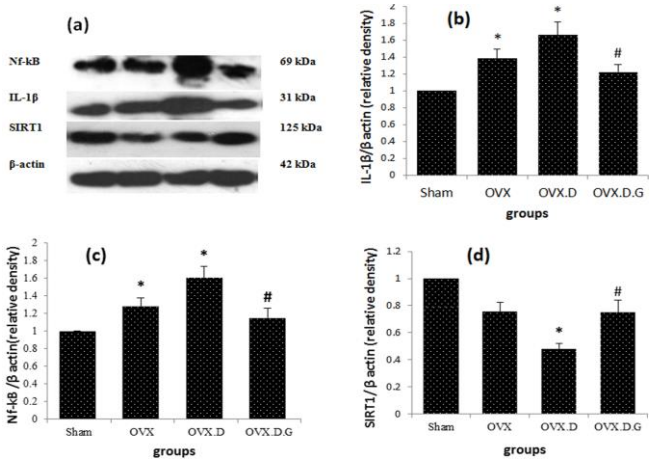

Figure 5. Immunoblotting of Nf-κB, SIRT1 and IL-1β among different groups (a). Quantitation of immunoblotting of IL-1β (b), Nf-κB (c) and SIRT1 (d) against expression of β-actin in pancreas. Data are shown as the means±SEM of 5–6 individual experiments. **P*<0.05 vs. Sham group; #*P*<0.05 vs. OVX.D groups

Effect of genistein on Nf-κB and IL-1β levels in pancreas

OVX with or without diabetes caused an increase in Nf-κB and IL-1β expression (Figure 5 b & c, *P*< 0.05)). Treatment with genistein, significantly inhibited Nf-κB and IL-1β protein levels in these groups (Figure 5, *P*<0.05), suggesting that inactivation of the Nf-κB and reduction of IL-1β might be one of the mechanisms by which genistein protects pancreatic β-cells from cytokine toxicity.

Effect of genistein on SIRT1 levels in pancreas

The present study showed that ovariectomy with diabetes in rats led to a significant reduction in the mean value of pancreatic SIRT1 levels (Figure. 5d, *P*< 0.05), and genistein treatment significantly increased SIRT1 protein levels. Treatment with genistein significantly stimulated SIRT1 protein levels in OVX.D.G group than OVX.D group (Figure 5d, *P*<0.05).

Effect of genistein on histological changes in pancreas

Histological examination of pancreatic tissue in the control group showed the normal range and size of the islets of Langerhans (Figure 6a and Table 1). Structure of the islets of Langerhans, with relative atrophic size, is evident in OVX group. Few inflammatory cells were observed around the islet and sinusoid, which indicates the low degree of inflammation compared to the ovariectomized diabetic group (Figure 6b and Table 1). Islet atrophic and irregular size and leukocyte infiltration was evident in the pancreas of OVX.D group. In addition, sinusoids between islets cells, vessels congestion and dilation, necrotic changes in the Langerhans cells, cytoplasmic acidophilic degeneration and amyloid depositions were observed in this group (Figure 6c and Table 1). Treatment with genistein in OVX.D.G group showed few inflammatory cells around the islet and sinusoid, indicating the low degree of inflammation to the OVX.D group. Sinusoidal dilation was observed in restricted areas with lesser congestion. Amyloid deposition was rarely found in some focal areas in pancreas of OVX.D.G group (Figure 6d and Table 1).

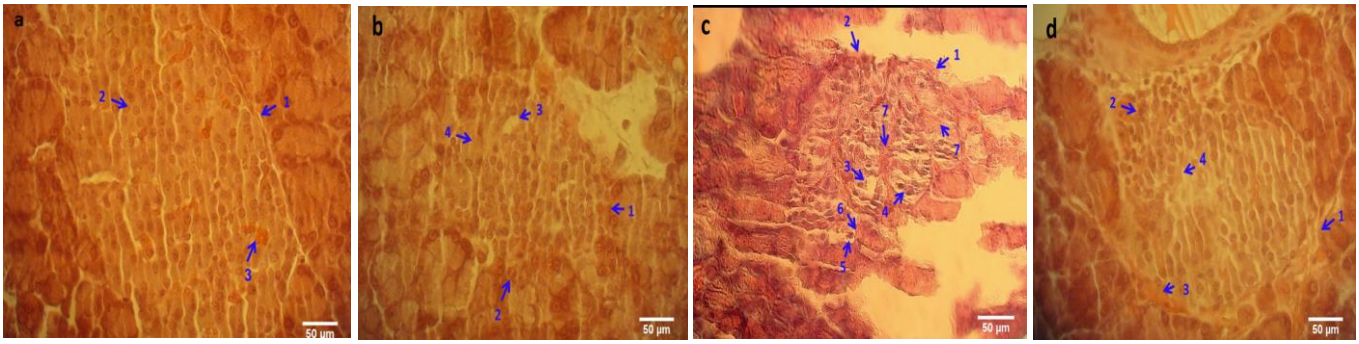

Figure 6. Representative images of histological changes in pancreatic islets stained with Hematoxilin-Eosin in the studied groups: **sham** (a) normal size of the islets (1), endocrine cells nuclei (2), sinusoids containing erythrocytes (3). OVX (b) relative atrophy size (1), inflammatory cells (2), Sinusoidal dilation (3), rarely amyloid deposition (4). OVX.D (c) islet irregular size (1), leukocyte infiltration (2), vessels congestion (3), necrotic changes (4), vacuolar degeneration (5), acidophilic cytoplasm (6), islet amyloid depositions (7). OVX.D.G (d) relatively atrophied size (1), a few inflammatory cells (2), sinusoidal congestion (3), rarely amyloid deposition (4). (Magnification of all images×40)

Table 1. Histological changes / field in the pancreas of studied group (H-E)

Groups	Infiltration and inflammatory cells	Islet cells necrosis	Amyloidosis	Sinusoidal dilatation and congestion
sham	0.3±0.11	0.1±0.1	0.0	0.2±0.1
OVX	0.6±0.21	0.5±0.20	0.7±0.31	1±0.31
OVX.D	4.6±0.51*	4.6±0.44*	4.4±0.67*	4.40±0.47*
OVX.D.G	3±0.27#	2.8±0.22#	2.6±0.29#	2.40±0.30#

A minimum of 5 fields for each pancreas slide were examined and assigned for severity of tissue changes (n=7 for each group). Data are shown as the means ± SEM of 7 individual experiments.* P< 0.05 vs. sham group; # P<0.05 vs. OVX.D group

Discussion

The points of the present study could be summarized as follow that genistein decreased plasma FBG, decreased Nf-κB and IL-1β proteins and downregulated SIRT1 protein. This evidence suggests that the beneficial effects of genistein in pancreatic tissue protection may be partially mediated by inhibition of inflammation and increased anti-inflammatory factor.

Loss of the anti-inflammatory effect of estrogen beginning with menopause is theorized to be responsible for the increasing occurrence of many disorders such as diabetes and its risks (14-16). Some studies have revealed an inflammatory process in the pancreas of patients with type 2 diabetes characterized by the presence of immune cells, cytokines, and β-cell apoptosis (17). Reduction in ovarian function with menopause or surgical procedure has been shown to increase pro-inflammatory cytokines such as IL-1β and TNF-α (3). So, given that both diabetes and ovariectomy can cause inflammation in the pancreas, in this study we investigated the protective effect of genistein on the pancreas of ovariectomized diabetic rat.

We measured body weight weekly in all groups. The increase in final body weight was observed in the OVX animal compared to sham group. While ovariectomized diabetic animals showed significant reduction of body weight compared to sham and OVX groups, genistein treatment prevented body weight loss in OVX.D.G compared to OVX.D group, but it was below than OVX animals. Previous reports have shown that dietary genistein decreases food intake and body weight in ovariectomized mice (18). On the other hand, experimental diabetes induced by STZ leads severe loss of body weights. Dehydration and catabolism of fats, as well as proteins with increased lipid profile and catabolic reaction, are major causes of these signs, but genistein administration increases body weight in genistein treated diabetic rats (19, 20). Genistein directly increases β-cell mass, and serum insulin; in addition, genistein improves glucose tolerance in diabetic animals in a dose-dependent manner (8). Therefore, in our study, it seems that genistein improved glucose metabolism and the signs of diabetes as well as indirectly improved body weight in OVX.D.G.

Findings of this study showed that OVX and OVX.D cause a marked increase in Nf-κB and IL-1β expression, and reduction in SIRT1 protein in pancreas compared to sham group. SIRT1, a class of III histone/protein deacetylase, interferes with the Nf-κB signaling pathway and thereby has an anti-inflammatory function (6, 21). Nf-κB has a central role in cytokine-mediated pancreatic β-cell damage, and SIRT1 acts as a preventive biomarker in cytokine-induced pancreatic β-cell damage (6).

Our results showed that genistein has a protective effect against cytokine with an expression of SIRT1 in the pancreas of ovariectomized diabetic rat. The increase of cytokine in pancreas was associated with reduced SIRT1 protein levels. Genistein is capable of protecting islets by stimulating SIRT1 protein and protecting from cytokine.

These results suggest the hypothesis that genistein has an anti-inflammatory role against cytokine in pancreatic β-cells by increasing SIRT1 and reducing IL-1β and Nf-κB protein level in ovariectomized rat with or without diabetes. We first showed that SIRT1 levels were decreased and cytokines increased significantly by OVX and OVX.D in pancreas compared to the sham group. Several cytokines regulate inflammatory responses in the pancreas by modulating the Nf-κB signaling pathway. One of these cytokines is IL-1β that has been implicated in early events of β-cell damage (22). In addition, pro-inflammatory cytokines released from pancreatic-infiltrating macrophages can inhibit glucose-stimulated insulin production and secretion (23). Suppression of IL-1β production or inhibition of its interaction with receptors significantly inhibits IL-1β –mediated injurious effects on β-cells (22). These findings are consistent with the histological results of the present study. Treatment with genistein markedly lowered inflammatory cells around the islet and sinusoid, indicating the low degree of inflammation compared to ovariectomized diabetic group. Consistent with our results, marked vacuolated cytoplasm and pyknotic nuclei has been previously demonstrated in most of the endocrine cells of STZ induced diabetic animal. Genistein treatment improved the morpho-logical changes in the islets of diabetic rat (19). Because

Nf-κB is a molecular target for cytokine toxicity and deacetylation by SIRT1, we investigated the changes of Nf-κB in the genistein-treated ovariectomized rat with or without diabetes. Compared with OVX.D group, expression of SIRT1 was significantly increased in the genistein-treated group. In one study, SIRT1 expression led to inhibition of Nf-κB pathway, and its overexpression decreased transcriptional activities and attenuated cytokine-induced β-cell damage (6).

Our results showed that genistein markedly increased SIRT1 protein, and decreased IL-1β and Nf-κB proteins levels compared to OVX and OVX.D groups. Study on endothelium has shown that genistein inhibits Nf-κB activation with downregulation of interleukin-6 (IL-6) and TNF-α production and expression. These findings suggest that genistein could inhibit inflammation and ameliorate endothelial dysfunction (24). Hirasaka, *et al* found that daidzein and genistein induce the SIRT1 expression and phosphorylation of AMP kinase, which is identified to stimulate SIRT1 expression, while there was no direct effect on SIRT1 activation (25). Genistein at physiological concentrations decreases an excessive production of many pro-inflammatory mediators such as inducible nitric oxide synthases (iNOS) and some pro-inflammatory cytokines (IL-1β, TNF- α, and IL-6), caused by MAPK and Nf-κB signaling pathways (9). The molecular mechanism of genistein inhibition on expression of iNOS gene seems to involve the inhibition of Nf-κB activation.

The cytokine caused an increase in Nf-κB binding activity, and IκBα degradation in cytosol compared to unstimulated cells; treatment with genistein abolished all of these parameters (9, 26). Anti-inflammatory properties of genistein involved in the protection against harmful consequences of pathological processes related to several disorders (9).

Conclusion

Estrogen deficiency alone or with HFD increased pancreas inflammatory markers such as Nf-κB, and IL-1β and decreased SIRT1 as an anti-inflammatory marker. However, subcutaneous administration of genistein prevented from these inflammatory changes in the pancreas of a surgery animal model of ovariectomy with or without diabetes. The results of this study support the potential preventing effect of genistein from pancreatic injury after menopause.

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Conflict of interest

The authors declare that there is no conflict of interest in this study.

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