Diabetic neovascularization defects in the retina are improved by genistein supplementation in the ovariectomized rat

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Abstract

Genistein seems to have a protective and therapeutic effect on conditions associated with neovascular growth in the retina. This study investigated the angiogenesis, antioxidant, and anti-inflammatory effect of genistein on the retinas in ovariectomized diabetic rats. In this study, 40 female albino Wistar rats were divided into four groups (n = 8 per group): sham, ovariectomized group (OVX), OVX + diabetes (OVX.D), and OVX.D + genistein (OVX.D.G). OVX induced by removal of bilateral ovaries and then high-fat diet (HFD) and a low dose of streptozotocin (STZ) (1 mg/kg; intraperitoneal (IP) injection) was used for diabetes induction (OVX.D) with 8 weeks of genistein treatment (OVX.D.G). At the end of 8 weeks, the retina was removed under anesthesia. The samples were used to measure extracellular signal-regulated kinase (ERK), matrix metalloproteinase 2 (MMP-2), vascular endothelial growth factor (VEGF), and nuclear factor NF-kappa-B (NF-κB) by western blotting and inflammatory factors ELISA and oxidative stress. Measurements of glutathione (GSH) and malondialdehyde (MDA) showed that OVX and especially OVX.D significantly decreased GSH and increased MDA level in the retina, but genistein reversed these effects in OVX.D.G groups. Also, OVX and OVX.D significantly increased VEGF, MMP-2, p-ERK, NF-κB, interleukin-1beta (IL-1β), and tumor necrosis factor alpha (TNFα) expression in the retina of OVX and OVX.D groups in comparison to the sham group (p < 0.05). However, a significant reduction of these proteins was observed in the genistein-treated group (p < 0.05). In conclusion, bilateral ovariectomy and subsequently estrogen deficiency caused the development of inflammation, neovascularization, and then retinopathy in STZ-induced diabetic ovariectomized rats. On the basis of the results, genistein administration may be a practical approach for improving symptoms and complications of ovariectomized diabetic retinopathy.

Keywords Angiogenesis · Genistein · Antioxidant · Ovariectomized rats · Inflammatory

Introduction

Retinal neovascularization is an essential pathologic condition in several ocular diseases, such as proliferative diabetic retinopathy, and leading to the defeat of vision. The abnormal new generated blood vessels can develop into the vitreous of the eye and result in retinal detachment (Wang et al. 2005). Oxygen therapy cessation in premature newborns induces relative retinal hypoxia, and then neovascularization in the retina happens in an attempt to overcome retinal hypoxia. The proliferative retinopathy in diabetes is the leading cause of new blindness in a diabetic population (Echouffo–Tcheugui et al. 2013). Several angiogenic factors, such as vascular endothelial growth factor (VEGF), are associated with the progress of diabetic retinopathy (Noma et al. 2002). VEGF has a prominent role in both standard
and abnormal retinal vascular growth (Zhao et al. 2021). VEGF is distributed in animal and human organs such as the brain, kidney, liver, eye, and other tissues. Under normal conditions, there is a low concentration of VEGF in the retinal cells (Sant et al. 2018). Under physiological conditions, this low concentration of VEGF in the eyes is essential to preserve the integrity of the ocular blood vessels. However, overexpression of this angiogenic factor in ocular tissue will cause neovascularization (Du et al. 2018). VEGF plays a vital role in the pathogenesis of diabetic retinopathy. VEGF is produced by the retinal ganglion cells, pigment epithelium cells, smooth muscle cells, and Müller cells in the human choroids and retinas, and its expression is regulated mainly by tissue oxygen levels (Ishida et al. 2003). Molecular studies have been shown that extracellular signal-regulated kinase (ERK) expression is related to vascular endothelial cell proliferation, thereby contributing to angiogenesis. Activated ERK was detected in accelerated neovascularization during wound healing and ischemia (Elsherif et al. 2014).

On the other hand, one complication in postmenopausal women, especially in its acute and non-physiological form, is visual impairment. Recent studies emphasize the protective effect of estrogen in maintaining visual health in women, especially in menopause. The presence of estrogen receptors in the retina, lens, cornea, and iris is one reason for this claim (Feola et al. 2019; Sengupta et al. 2019). There are different interrelating biochemical pathways that have been anticipated in the progress of retinopathy, specifically amplified expression of growth factors such as vascular endothelial growth factor (VEGF) and insulin-like growth factor 1 (IGF1), stimulation of protein kinase C (PKC), hemodynamic changes, enhanced formation of progressive glycation end products (AGEs), oxidative stress, subclinical inflammation, activation of the renin–angiotensin–aldosterone system (RAAS), augmented polyol pathway, and capillary obstruction. These mechanisms cause vessel abnormalities in the form of cellular capillaries and loss of pericytes as well as ganglion cells, finally resulting in vascular proliferation, glial proliferation, and neural degeneration (Stewart 2010; Jo et al. 2013; Mancini et al. 2013).

Some evidence showed that hormone replacement therapy increases blood flow in a retinal artery and has a protective effect on the retina in postmenopausal women, which indicates the protective role of estrogen in the retina (Deschênes et al. 2010).

The sex hormone estrogen receptors in the retina suggest that estrogen has many functions in this tissue. Additionally, estrogen has potent antioxidation properties, which may cause neuroprotection without including a receptor-mediated process (Azcoitia et al. 2011). In recent years, flavonoids derived from natural plants have become a topic of interest because they have potent antioxidant activity and low toxicity and side effects, which makes them very effective as potential therapeutic agents. Soy isoflavone genistein seems to have a protective and therapeutic effect on conditions associated with neovascular growth in the retina (Ronghua et al. 2018; Dongare et al. 2015). Soybean genistein can also prevent choroid capillary development and corneal neovascularization induced by fibroblastic growth factor (Wang et al. 2005). Also, genistein significantly decreased VEGF protein expression induced by hypoxia in cultured rabbit retinal pigment epithelium cells (Wang et al. 2005; Ronghua et al. 2018).

Therefore, according to the effect of genistein in reducing inflammation, oxidative stress, and angiogenesis, and the role of inflammation, oxidative stress, and angiogenesis in visual impairment caused by diabetes and ovariectomy, in this study we investigated the effect of genistein on the expression of factors involved in inflammation, oxidative stress, and angiogenesis in the retina of diabetic ovariectomized rats.

Materials and methods

Materials

The materials used in this study were ketamine and xylazine (Sigma Aldrich, USA), streptozotocin (Sigma, St. Louis, Mo, USA), pure genistein powder (Sigma Aldrich, USA), first antibodies (ERK, p-ERK, VEGF, matrix metalloproteinase 2 (MMP-2), and β-actin (Santa Cruz, USA; 1:500), anti-p65 NF-κB (Abcam, Germany; 1:1250), secondary antibody (goat anti-rabbit; Santa Cruz, USA; 1:5000), enhanced chemiluminescence (ECL) detection kit (Pierce, Rockford, IL), glutathione (GSH) and malondialdehyde (MDA) kit (Kiazist, Iran), TNFα ELISA kits (Diaclone, France), IL-1β ELISA kit (Abcam, Germany).

Animal care

Forty female Wistar rats (weighing 180–220 g, 6–7 weeks old) were purchased from the Experimental Animal Research Center, Faculty of Medicine, University of Hamadan, Hamadan, Iran. All rats were preserved under controlled conditions (temperature of 22–24 °C with 12/12 h of the light–dark cycle) and received chow diet and water ad libitum. Before the experiments, all rats were given at least 1 week to adjust to their new surroundings. All experimental procedures using rats were conducted by the animal care and use guidelines approved by the institutional ethics committee at Hamadan University of Medical Sciences (Code of Ethics Committee: IR.UMSHA.REC.1397.637) and were performed following the National Institutes of Health Guide for Care and Use of Laboratory Animals.
Protocol

Rats were randomized into two groups, including sham and OVX groups. OVX was re-randomized into three new groups: OVX only, OVX + diabetic (OVX.D), OVX + D + genistein (OVX.D.G) (n=8 per each group). The ovariotomy induction was performed under anesthesia (ketamine 50 mg/kg and xylazine 5 mg/kg: intraperitoneal (IP) injection). Then the ovaries were bilaterally removed from a small incision of the ventral abdominal midline area, and after ovariotomy, the abdominal wall was sutured finally. After removal of the ovaries, rats were housed under good conditions to recover. In the sham surgery, animals were anesthetized with ketamine hydrochloride (50 mg/kg) and xylazine (5 mg/kg). The ventral abdominal midline wall was opened similarly to the OVX rats. Then the abdominal wall was sutured (Habibi et al. 2016). After surgical recovery, diabetes in animals was induced with high-fat diet (HFD) ad libitum for 4 weeks. The HFD component included protein (25%), carbohydrate (17%), and fat (58%). Then a single dose streptozotocin (STZ) (30 mg/kg; IP injection), dissolved in 10 mM sodium citrate, pH 4.5, with 0.9% NaCl, was injected into rats in the diabetic groups at 4 weeks after beginning the HFD regime. In the sham surgery, rats were injected with 0.4 ml of sodium citrate buffer, pH 4.5. Fasting blood glucose levels were measured with a glucometer after 6 h of fasting. Rats in the diabetic groups were confirmed as a diabetic model when tail vein blood glucose became greater than 200 mg/dl (Habibi et al. 2020). Genistein was dissolved in dimethyl sulfoxide (DMSO) and subcutaneously injected for 8 weeks (1 mg/kg/day) (Habibi et al. 2017; Kuzu et al. 2007), concurrent with the onset of the HFD regime. At the end of 8 weeks, the retina tissues were removed under anesthesia and used for molecular evaluations. Western blotting protocol and ELISA analysis were performed on retinas tissue homogenates.

Western blotting

The western blotting protocol was performed on homogenates of retinal tissue. In this method, snap-frozen retinal tissue was homogenized in radioimmunoprecipitation assay buffer (RIPA buffer). This buffer was supplemented with a protease inhibitor cocktail containing leupeptin, pepstatin, chymostatin, aprotinin, and antipain (5 μg/ml each). The homogenized tissues were rotated for 20 min and then centrifuged at 12,000×g for 10 min at 4 °C. After centrifuging, the supernatant was collected and stored at −80 °C. The proteins were detached by sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (10 μg supernatant loaded per each well) and then electrophoretically transferred onto polyvinylidene difluoride (PVDF) membranes. Nonspecific binding was blocked by 2-h incubation of the membranes in 5% (w/v) nonfat dry milk in Tris-buffered saline (pH 7.5). The polyvinylidene fluoride or PVDF membranes were then incubated for 2 h at room temperature (at 4 °C) with primary antibodies (ERK, p-ERK, VEGF, MMP-2, and β-actin: Santa Cruz, USA (1:500); anti-p65 NF-κB: Abcam, Germany (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), and washed three times with TBS-T, then incubated for 1 h with a secondary antibody (goat anti-rabbit; Santa Cruz, USA; 1:5000) in the antibody buffer. The PVDF membranes were developed for visualization using ECL detection kit (Pierce, Rockford, IL). Anti-β-actin was used as a loading control. Band intensities on the PVDF membranes were quantified by densitometry using ImageJ software.

Enzyme-linked immunosorbent assay (ELISA)

Freshly isolated retina tissues were homogenized with PBS and centrifuged at 11,100×g for 20 min at 4 °C, and then the supernatants were isolated. The retina extracts were used for ELISA analysis. The levels of IL-1β and TNFα were measured using ELISA kits according to the manufacturers’ protocol.

Oxidative stress

The accumulation of TBARS (thiobarbituric acid reactive substances) in the retina homogenates expressed as MDA level was used for lipid peroxidation. MDA (nmol/mg protein) was evaluated at 532 nm (UV/visible U-200L spectrophotometer, Spectra Max 190, Molecular Devices, USA).

In the present study, a GSH kit (Kiazist, Iran) was used to measure the GSH level. The mechanism of this kit is based on the binding of Ellman’s reagent (DNTB) to the regenerated thiol groups and the formation of a colored complex. The level of free thiol groups in each sample was calculated using the standard curve and reported as nmol/mg protein after dividing by the protein concentration.

Statistical analysis

All data were analyzed with SPSS software version 20.0 and described as the mean ± SEM, and differences between all groups were evaluated by one-way analysis of variance (ANOVA) with Tukey’s multiple comparison post-test. A value of p<0.05 was considered statistically significant.
Results

Effect of genistein on oxidative stress levels in the retina

At 8 weeks after STZ administration and ovariectomy surgery, MDA and GSH levels were measured in the retina. Measurements of MDA (Fig. 1a) and GSH (Fig. 1b) showed that OVX and OVX.D significantly decrease GSH and increase MDA level in the retina, but genistein reversed these effects in OVX.D.G groups (Fig. 1, p < 0.05).

Effect of genistein on IL-1β and TNFα level in the retina

After sampling, the IL-1β and TNFα protein levels were measured by ELISA. Measurements of IL-1β and TNFα level (Fig. 2a and b) with ELISA showed overexpression of these proteins in OVX and especially OVX.D in the compared sham group. However, a significant reduction of IL-1β and TNFα was observed in genistein treated group (OVX.D.G).

Effect of genistein on VEGF, MMP-2, NF-κB, and p-ERK levels in the retina

At 8 weeks after STZ administration and ovariectomy surgery, VEGF, p-ERK, MMP-2, and NF-κB protein levels were measured by western blotting. Densitometric measurements of western blots (Fig. 3a) with ImageJ software showed that OVX and OVX.D cause an increase in VEGF protein levels (p < 0.05), but a significant reduction of VEGF was observed in the genistein-treated group. Genistein significantly decreased VEGF protein levels in this group (Fig. 3a, p < 0.05). Besides, OVX.D causes an increase in ERK phosphorylation, NF-κB, and MMP-2 protein levels compared to the sham (Fig. 3b–d, p < 0.05); genistein significantly decreased ERK phosphorylation, NF-κB, and MMP-2 protein level. Genistein in OVX.D.G significantly decreased these proteins compared to the OVX.D group (Fig. 3b–d, p < 0.05).
Diabetic neovascularization defects in the retina are improved by genistein supplementation...

Discussion

This study evaluated the effects of genistein on the ERK signaling pathway and neovascularization factors in retinal tissue of ovariectomized diabetic rats. We showed that the ERK phosphorylation, VEGF, MMP-2, and NF-κB protein levels and inflammatory factors were significantly higher in the OVX.D group than in the sham group at 4 weeks after OVX surgery and STZ administration. Genistein reversed these effects in OVX.D.G groups in comparison to OVX.D groups. Also, glutathione decreased and MDA increased in OVX.D, but genistein reversed these effects in OVX.D.G groups. Studies suggest that the combination of HFD-fed and low-dose STZ-treated rat serves as an alternative animal model for type 2 diabetes simulating the human syndrome (Guo et al. 2018). In these models, an HFD for a few weeks and a low-dose STZ injection are used simultaneously. It has been demonstrated that the HFD significantly increases body weight, basal plasma glucose (PGL), insulin (PI), triglycerides (PTG), and total cholesterol (PTC) levels. Moreover, the HFD decreases the glucose disappearance rate on the insulin glucose tolerance test (IGTT). Hyperinsulinemia...
together with reduced glucose disappearance rate suggests that the feeding of HFD induces insulin resistance in rats. Also after 2 weeks of HFD use, intraperitoneal injection of a low dose of STZ (35 mg/kg) increased hyperglycemia without diminishing insulin. In addition, the levels of PTG and PTC increase further after STZ treatment in HFD-fed rats. Thus, these fat-fed/STZ-treated rats simulate natural disease progression and metabolic characteristics typical of individuals at increased risk of developing type 2 diabetes because of insulin resistance and obesity (Guo et al. 2018).

A causal association between diabetic abnormal high glucose level and the progress of retinal vascular dysfunction has been recognized, such that glucose regulation in patients with diabetes decreases the development of the disease (Control and Group 1993). Therefore, hyperglycemia has been associated with the activation of more critical signaling pathways, including nitrative and oxidative stress, which contribute to retinal neovascularization and diabetic retinopathies (Huang and Sheibani 2008). Hypoxia/reoxygenation conditions in diabetes or any pathological conditions are the most important causes of ERK, MMP-2, and VEGF expression, which are implicated in various neovascularization (Zhang and Tao 2017). Also, Kamalden et al. showed that ischemia causes a marked increase in ganglion cell mRNAs of caspase 3 and caspase 8 in the optic nerve. Changes in these mRNAs by ischemia were significantly reduced by genistein (Kamalden et al. 2011). It has been shown that the expression of IL-1β, ERK, and caspase 3 increases in the lungs of the OVX.D group; genistein decreased them in lung tissue of ovariectomized diabetic rats (Daghigh et al. 2017). These results indicate the role of IL-1β, ERK, and caspase 3 in tissue damage, while genistein improves these effects. One of the present study findings was an increase of MMP-2 in the OVX and ovariectomized diabetic group, which was decreased by genistein. Similar to this study, it has been shown that MMP proteins decrease in retinal pigment epithelium choroid complex when treated with soy isoflavone genistein after choroidal neovascularization induction (Kinoshita et al. 2014).

In the neovascularization process, angiogenic factors increased endothelial cells’ basement membrane degradation, migration, and proliferation, causing new capillary tube formation (Hanahan and Folkman 1996). The endothelial cell’s basement membrane is disrupted by proteolytic enzymes such as MMPs (Vu and Werb 2000). MMP-2 and MMP-9 play an essential role in the hydrolyzation of endothelial cells’ basement membrane components (Kinoshita et al. 2014; Hiraoaka et al. 1998). Also, our results showed that the NF-κB expression markedly increased in the ovariectomized diabetic group compared with in the sham control groups. This shows that MMP-2 was upregulated via the increased activity of the NF-κB. This result is consistent with the previous investigations, which showed that the NF-κB activation increased proliferation, angiogenesis, and apoptosis in endometriosis (Kunikata et al. 2019; Calibasi-Kocal et al. 2019). It has been shown that NF-κB acts via regulation of the MMPs expression in the adhesion and invasion of endometriosis cells on the peritoneal surface (Lee et al. 2010; Gottschal et al. 2002). A previous study has shown that MMP-2 is expressed in the endometrial graft in the peritoneum (Ueda et al. 2002).

Previous studies have shown that hyperglycemia can increase mitochondrial reactive oxygen species (ROS) and stimulates many signaling pathways causing tissue injury (Wu et al. 2014). Increasing retinal ROS stabilizes hypoxia-inducible factor-1α and causes the upregulation of VEGF and other angiogenic factors (Masuda et al. 2017). On the other hand, Weng et al. showed that genistein treatment can enhance the integrity of the retinal vessels perhaps by diminishing iNOS and increases morphological changes of the retina of STZ-induced diabetic mellitus which indicates the protective role of genistein in the retina (Weng et al. 2019).

In the present study, we demonstrated VEGF overexpression in the ovariectomized diabetic group. Similar to our investigation, in previous studies, it was shown that VEGF expression increases in the retina of STZ-induced diabetic rats. These results suggest that diabetes induces neovascularization through increasing VEGF expression in the retinas. Our results showed that genistein decreases the VEGF levels in the OVX.D.G group.

On the other hand, estrogen insufficiency by menopause or ovariectomy causes inflammation and oxidative stress by increasing the TNFα and MDA levels, respectively (Daghigh et al. 2017), which can cause eye complications in these patients. MDA is a main byproduct of polyunsaturated fatty acid peroxidation and increased levels of MDA have been reported in eyes of patients with age-related macular degeneration. MDA induces autophagy dysfunction and VEGF secretion in the retinal pigment epithelium (Ye et al. 2016). In this study, we showed that oxidative factors such as MDA increased and antioxidants decreased in OVX.D. However, genistein reversed these effects in OVX.D.G groups; reducing oxidative stress may lead to a decrease in neovascularization in the retina by reducing VEGF and inflammatory factors. Moreover, blockade of ERK signaling pathway decreases VEGF-induced proliferation of endothelial cell in a placental artery (Liao et al. 2009). MMP-2 is another factor involved in angiogenesis. The processing of angiogenesis is related to vascular basement membrane degradation, which is necessary for migration of endothelial cells and proliferation (Song et al. 2015).

VEGF expression is increased by hypoxia and hyperglycemia, two major causes of retinal neovascularization. Besides, VEGF is regulated by the ERK signaling cascade (Hashimoto et al. 2006). In the present study, we showed that ERK phosphorylation, VEGF, MMP-2, and NF-κB protein
levels were significantly higher in the OVX.D group and can induce retinal neovascularization and injuries. Nevertheless, genistein reversed these effects in the retina of OVX.D.G.

Conclusion

Oestrogen deficiency induced by bilateral ovariectomy caused retinopathy and neovascularization in STZ-induced diabetic rats. On the basis of the results, genistein may be a practical approach for improving symptoms and complications of ovariectomized diabetic retinopathy.

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Availability of data and materials

The data are available for any scientific use with kind permission.

Declarations

Conflict of Interest

The authors declare that they have no competing interests.

Ethics Approval and Consent to Participate

All experimental procedures using rats were conducted by the animal care and use guidelines approved by the institutional ethics committee at Hamadan University of Medical Sciences (Code of Ethics Committee: IR.UMSHA.REC.1397.637) and were performed following the National Institutes of Health Guide for Care and Use of Laboratory Animals.

Consent for Publication

Not applicable.

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