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Genistein Supplementation Alters the Expression Levels of miR-155 and miR-181b in the Uterus of Estrogen-Deficient Rats

Genistein Takviyesi Östrojen Eksikliği Olan Sıçanların Uterusunda miR-155 ve miR-181b Ekspresyon Düzeylerini Değiştiriyor

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Abstract

Objective: Estrogen deficiency in menopausehas been associated with several complications like dementia, diabetes, and sexual disturbances. Estrogen modulated microRNA (miR)-155 and miR-181b are involved in various inflammatory responses. Phytoestrogens have been suggested to modulate the expression levels of uterine miRs. The present work evaluated the effect of genistein treatment on the expression levels of miR-155 and miR-181b in the uterus of ovariectomized rats. **Material and Methods:** Thirty female Wistar rats (180-220 g) were randomly divided into 3 groups (n=10 in each group), viz., sham: Rats that underwent laparotomy without ovariectomies, OVX: Rats that underwent bilateral ovariectomy, and OVX.G: Ovariectomized rats with 8 weeks of genistein treatment (1 mg/kg, subcutaneous, daily). Later, the uterus tissue was removed, and the expression levels of miR-155 and miR-181b were evaluated by real-time polymerase chain reaction in the experimental groups. Hematoxylin-eosin staining was used to assess histomorphological changes occurring in the uterus. **Results:** Genistein treatment decreased miR-155 and increased miR-181b expression levels in the uterus of estrogen-deficient rats. Moreover, genistein supplementation ameliorated histological changes in the uterus of ovariectomized rats. **Conclusion:** Genistein supplementation was responsible for partially ameliorating histological changes induced by estrogen deficiency and expression levels of miRNAs in the uterus of ovariectomized rats. Further investigations are warranted to investigate the inclusive effects of genistein treatment in menopausal women.

Keywords: Genistein; miR-155; miR-181b; ovariectomy; inflammation

Özet

Amaç: Menopozdaki östrojen eksikliği; demans, diyabet ve cinsel sorunlar gibi çeşitli komplikasyonlarla ilişkilendirilmiştir. Östrojenin modüle ettiği mikroRNA (miR)-155 ve miR-181b, çeşitli inflamatuar yanıtlarda rol oynar. Fitoöstrojenlerin, uterus miR'lerinin ekspresyon seviyelerini modüle ettiği öne sürülmüştür. Bu çalışmada, genistein tedavisinin overektomize sıçanların uterusundaki miR-155 ve miR-181b ekspresyon seviyeleri üzerine etkisi değerlendirilmiştir. **Gereç ve Yöntemler:** Otuz dişi Wistar sıçanı (180-220 g) rastgele 3 gruba ayrıldı (her grupta n=10); sham: Overektomi yapılmadan laparotomi uygulanan sıçanlar, OVX: Bilateral overektomi yapılan sıçanlar ve OVX.G: Overektomi yapılan ve 8 haftalık genistein tedavisi (1 mg/kg, subkütan, günlük) alan sıçanlar. Daha sonra uterus dokusu çıkarılarak, deney gruplarında miR-155 ve miR-181b ekspresyon seviyeleri gerçek zamanlı polimeraz zincir reaksiyonu ile değerlendirildi. Uterusta meydana gelen histomorfolojik değişiklikleri değerlendirmek için hematoksilen-eozin boyaması kullanıldı. **Bulgular:** Genistein tedavisi, östrojen eksikliği olan sıçanların uterusunda miR-155 ekspresyon seviyelerini azalttı, miR-181b ekspresyon seviyelerini ise artırdı. Dahası genistein takviyesi, overektomize sıçanların uterusundaki histolojik değişiklikleri iyileştirdi. **Sonuç:** Genistein takviyesi, overektomize sıçanların uterusunda östrojen eksikliğinin neden olduğu histolojik değişiklikleri ve miRNA'ların ekspresyon seviyelerini kısmen iyileştirmiştir. Menopozdaki kadınlarda, genistein tedavisinin kapsayıcı etkilerini araştırmak için daha fazla araştırma yapılması gereklidir.

Anahtar kelimeler: Genistein; miR-155; miR-181b; overektomi; inflamasyon

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Introduction

The physiological role of sex hormoneson the female reproductive system has been well established. Estrogen deprivation in menopause causes multiple complications such as dementia, osteoporosis, diabetes, and sexual disturbances (1). Hormone replacement therapy is an effective alternativeto combat menopause-related complications (2,3). However, replacement therapy with synthetic estrogen drugs has been accompanied by a higher incidence of endometrial and breast cancers (4,5).
Phytoestrogensmimic estrogenic effects (6) and have side effects like gastrointestinal, gynecological, and neurological disorders (7). They can potentially act as a natural substitution for estrogen therapy in menopause (8).
MicroRNAs (miRs) are endogenous non-coding RNAs and regulate post-transcriptional gene expression (9). MiRs exert a crucial role in multiple aspects of cell biology such as development, differentiation, immune responses, inflammation, and apoptosis (9). Unmodulated expression levels of miRs are associated with coronary artery disease, cancer, endometriosis, and other disorders (10-13). Estrogen has been found to regulate the expression levels of various uterine miRs (14). Accordingly, the administration of antagonistic estrogen hormone receptors such as antagonist ICI 182 and 780 (ICI, fulvestrant, Faslodex, United Kingdom) blocked the expression levels of some miRs (14).
miR-155 and miR-181b are known as the significant estrogen modulated miRs in the uterus (15). The expression levels of miR-155 and miR-181b were unmodulated during inflammatory responses (16,17). miR-155 plays a significant role in oncogenic transformation using redox generation (18). Nassar et al. have reported higher levels of miR-155 in postmenopausal women having breast cancer, as compared to premenopausal women (19). Accordingly, miR-181b has been changed in early-stage breast cancer in older women (20).
Phytoestrogen genistein has shown protective effects against inflammatory diseases in estrogen-deficient animal models (21). In addition, genistein has also been reported to exert anti-proliferative effects against breast cancer cells through the attenuation of miR-155 (22). In this study, we investigated the effect of genistein supplementation on the expression levels of miR-155 and miR-181b in the uterus of ovariectomized (OVX) rats.

Material and Methods

Animal Subjects

Ten weeks old 30 female Wistar rats, weighing 180-220 g, were used in this study. Rats were purchased from the Animal Research Center, Faculty of Medicine, Tabriz University of Medical Sciences, Tabriz, Iran. All rats were kept in standard conditions (22-24°C temperature, 12 h light-dark cycles) with ad libitum, pellet diet, and free access to drinking water. All the procedures were as per animal rights, following the Helsinki Declaration Principles. All animals were subjected to humane treatment in accordance with the Guide for the Care and Use of Laboratory Animals (www.nap.edu/catalog/ 5140.html) and an Ethics Committee of Experimental Animals approval report was obtained. Procedures performed on the animal were performed without pain, suffering and discomfort. The Ethics Committee of Tabriz University of Medical Sciences also approved this study (Date: 2/9/2018, Code number: IR.TBZMED.REC.1396.1299).

Experimental Model

Animals were assigned into 3 groups with ten rats in each. In the sham group, rats underwent laparotomy without ovariectomy and were administered dimethyl sulfoxide (DMSO) (100 µL per day), in OVX, rats had bilateral ovariectomy; and in OVX.G, there were ovariectomized rats with genistein therapy (1 mg/kg, subcutaneous, daily) (Sigma-Aldridge, USA). Ovariectomy surgery was done under general anesthesia using a mixture of ketamine chloride and xylazine chloride (50 mg/kg, respectively). The operation site was shaved and then cleaned with 70% alcohol and povidone-iodine. After incision of both sides of the spine, ovaries were ultimately exposed and then removed. Finally, oviducts were placed with minimum injury to the adjacent tissues. Then, the muscle layers and the exposed

skin were sutured using a sterile suture (23). Two weeks after surgery, genistein (1 mg/kg; subcutaneously) was dissolved in DMSO and administered continuously for up to 8 weeks (24). At the end of the operation, the uterus was removed under general anesthesia. Finally, the tissue was frozen in liquid nitrogen and maintained at -70 °C for further molecular and histological analysis.

Real-Time Quantitative Polymerase Chain Reaction

Total RNA comprising miRNA and mRNA was extracted from the uterus using RNX-Plus solution kit (Fermentase, Cinagen Co. Iran) and miR-amp kit (Parsgenome Co. Iran). NanoDrop 1000 (Thermo Scientific, Waltham, and Mass The United States.) evaluated the purity and quantity of RNA. The expression levels of miR-155 and miR-181b were quantitatively assessed by real-time polymerase chain reaction (PCR). The number of PCR products was normalized to miR-191a for miRs samples (internal control). Synthesis of cDNA in miRs models was performed according to the miR-amp kit (Parsgenome Co. Iran). Real-time quantification was undergone by detecting the fluorescence intensity after binding the SYBR Green (Master Mix,Thermo Scientific, USA). dye to a double-stranded DNA at the end of every amplification cycle. The thermal cycler included one preliminary denaturation of 5 min (at 94°C), then 40 cycles at 94°C (20 sec), annealing at 60°C (20 sec), and extension at 72°C for 30 sec. The actual amount of miRs for target genes was calculated based on the threshold cycle (Ct) compared tothe Ct of the housekeeping gene (miR-191a). The quantification was done by the 2-ΔΔ Ct method (25).

Histological Evaluation

At the end of the operation, the uterus was removed during general anesthesia and then evaluated for histologic changes. Processing of tissues (fixing of the uterus in 10% formalin) and histological sections (5 µm thickness) was stained with hematoxylin-eosin. Finally, a light photomicroscope (Olympus BH-2, Japan) was used to evaluate inflammatory responses in uterus tissue.

Statistical Methods

All results were reported as mean±standard error of the mean or median and range. Kruskal-Wallis test and Dunn's post hoc test were used in comparing the experimental groups. A p value of <0.05 was considered statistically significant.

Results

Genistein Treatment on miR-155 and miR-181b Expressions in Uterus Tissue

There was a significant difference in miR-155 expression levels in the OVX group compared to sham ($p<0.05$). Ovariectomy significantly increased miR-155 expression levels in the uterus compared to the sham group [median expression value: 2.47 (fold) in OVX vs. 1 (fold) in the sham group]. Genistein treatment partially reduced miR-155 expressions in the OVX.G group in comparison to the OVX group [median expression value: 1.83 (fold) in OVX.G vs. 2.47 (fold) in OVX group] (Figure 1, Table 1, Table 2).

The current study showed that ovariectomy significantly decreased miR-181b expressions in the uterus tissue compared to the sham group ($p<0.05$) [median expression value: 0.33 (fold) in OVX vs. 1 (fold) in the sham group]. Treatment with genistein partially increased miR-181b expressions in the OVX.G group compared to the OVX group [median expression value: 0.55 (fold) in OVX.G vs. 0.33 (fold) in OVX] (Figure 1, Table 1, Table 2).

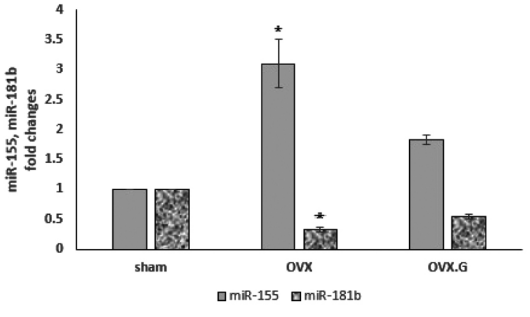

Figure 1. Expression levels of miR-155 and miR-181b in the uterus of studied groups (fold change).
Data are expressed as mean±standard error of the mean. *p<0.05 vs. sham group. OVX: Ovariectomized group; OVX.G: Ovariectomized rats with genistein treatment.

Table 1. The primer sequences for genes.

Genes	Target sequense[a]
rno-miR-155-5p	UUAAUGCUAAUUGUGAUAGGGGU
rno-miR-181b-5p	AACAUUCAUUGCUGUCGGUGGGU
rno-miR-191a-5p	CAACGGAAUCCCAAAAGCAGCUG

[a]Sequences were derived from miR Base (www.mirbase.org).

Histological Results

Histological evaluation of endometrium in the uterus of studied groups

Histological evaluation of endometrial samples revealed typical tissue architecture in the sham group (Figure 2A). Endometrium in the OVX group revealed hyperemia along with dilated blood vessels in the uterine tissue (Figure 2B).

Estrogen deficiency significantly increased the area of blood vessels in OVX compared to sham (median value: 27,100 μm^2 in OVX vs. 7,000 μm^2 in sham) ($p<0.05$). Genistein treatment partially alleviated histological changes in the OVX.G group. In OVX.G, stroma was relatively less edematous in comparison to the OVX group. Genistein supplementation reduced blood vessel area (median value: 12,600 μm^2 in OVX.G vs. 27,100 μm^2 in OVX) and inflammatory cell infiltration in OVX.G group compared to ovariectomized group (median value: 129 cellsin OVX.G vs. 155 cells in OVX) (Figure 2C, Table 3).

Discussion

The present study describes the effect of genistein treatment on the uterus of estrogen-deficient rats. Genistein treatment ameliorated histological changes and miRs expression levels in the estrogen deficiency-induced uterus of ovariectomized rats. Moreover, ovariectomy increased miR-155 and decreased miR-181b expression levels in uterus tissue compared to the sham group.

The anti-inflammatory effect of estrogen has been studied in recent years. A decrease in ovarian function with surgical or natural menopause is associated with elevated pro-inflammatory cytokine levels

Table 2. miR-155 and miR-181-b in the experimental groups.

Groups	miR-155						miR-181-b					
	Mean	Median (fold)	Range (Minimum, maximum)	SD	SE	Significant	Mean	Median (fold)	Range (Minimum, maximum)	SD	SE	Significant
sham	1	1	0.00	0.0	0		1	1	0	0	0	
OVX	3.1	2.47	5.22 (1.05, 6.27)	1.66	0.4	<0.0001*	0.34	0.33	0.43 (0.14, 0.57)	0.14	0.037	<0.0001*
OVX.G	1.85	1.83	1.05 (1.26, 2.31)	0.27	0.07	0.12	0.54	0.55	0.47 (0.29, 0.76)	0.13	0.036	0.12

*$p<0.05$ considered statistically significant; Median, mean expression values, range, SE and SD, in the experimental groups; sham; OVX: Ovariectomized rat; OVX.G: Genistein treated ovarectomized rat; SD: Standard deviation; SE: Standard error.

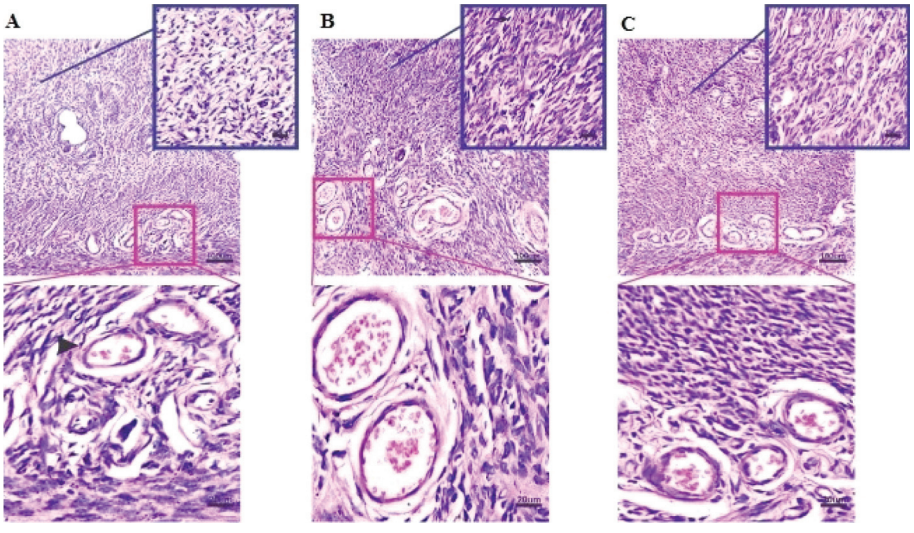

Figure 2. Histological observation of endometrium (Hematoxylin and eosin staining). Sham: standard endometrium architecture (A), OVX: Thick-walled blood vessels, hyperemia along with dilated blood in the endometrium (10x, 40x) (B), OVX.G: Relatively less edematous stroma, reduced vascular diameter, and somewhat reduced inflammatory cell infiltration (10x, 40x) (C), sham, OVX: Ovariectomized group; OVX.G: Ovariectomized rats with genistein treatment (10x, 40x).

(26). In addition, aging in female mice was accompanied by vascular and structural changes (27). Accordingly, aging has been shown to have a positive correlation with vascular inflammatory markers (27).

Previous studies have documented that estrogen deprivation could induce a potential increase in the diameter of the uterine blood vessels and blood vessel area (28). More importantly, estrogen administration in ovariectomized animals has been significantly associated with cellular and structural changes in the uterus similar to those observed in estrus or pregnancy (29).

Many inflammatory and immune cells were responsive to estrogen. In addition, postmenopausal women had higher levels of monocyte chemo attractant protein than premenopausal women (30). Notably, endothelial dysfunction and vascular inflammation are common complications of estrogen deficiency in menopausal women (31).

The significant role of miR-155 in the immune system and inflammatory responses has been established in recent studies (16,32). miR-155 is also involved in the production and secretion of inflammatory cytokines (33). It is also reported that miR-155 is involved in the atherosclerotic and inflammatory processes (34). In addition, growing evidence has demonstrated that miR-155 is involved in endometrial inflammatory responses (33).

There have been a few studies regarding the role of miR-181b in inflammatory disorders. In recent years, miR-181b has been suggested as a valuable target for treating vascular inflammatory disease. Overexpression of miR-181b is indicated to inhibit tumor necrosis factor-alpha-induced adhesion molecules, including ICAM-1 and E-selectin, promoting leukocyte adhesion to the endothelial cells (35). Moreover, administration of the drug methotrexate, a miR-181b activator, is associated with a significant decrease in vascular inflammation (17).

Hormones have a significant influence on the expressions of miRs. Therefore, estrogen hormone has been observed to regulate miRs expression levels in the mouse uterus (36). miR-181b and miR-155, expressed in the uterus, are the miRs mainly influenced by estrogen (14). In the present study, we found a significant increase in miR-155 and a meaningful decrease in miR-181b expression levels in the uterus of ovariectomized rats.

During the last decades, improvement in the treatment and prevention of chronic diseases has increased the survival and life expectancy of menopausal women. However, with the increasing age of the population,

finding safer strategies to prevent chronic disease-related complications is of great importance. Therefore, compounds that mimic the beneficial effects of estrogen without inducing cell proliferation could prevent complications in estrogen deficiency status (37).

Phytoestrogens have similar characteristics to estrogen hormones due to phenolic rings that bind to estrogen receptors (38,39). However, there are conflicting reports about the mechanisms of phytoestrogen actions in the postmenopausal period. For example, Zhang et al. have reported identical transcriptional responses in the uterus of estrogen and genistein treated mice (40). However, a study indicated partial overlaps between the expressions elicited by genistein and estrogen hormone in the pathological stages (41).

Genistein, as a phytoestrogen, was shown to reduce the inflammatory process through decreasing miR-155 expression and inhibiting the nuclear factor kappa B pathway in human umbilical vein endothelial cells (42). More pertinently, genistein was found to have anticancer effects during the downregulation of miR-155 (22).

Upregulation of miR-181b was associated with reduced edema and inflammatory cell infiltration during pancreatitis (43). It has been suggested that treatment with phytoestrogens could significantly decrease inflammatory biomarkers in estrogen-deficient rats (21). Of note, genistein has been found to exert anti-inflammatory effects on estrogen-deficient animals (21,44). Moreover, anti-inflammatory effects of genistein have also been proposed in lipopolysaccharide-induced inflammation in human endometrial cells (45).

Recently, Liu et al. have reported that Panax notoginseng saponins, extracted from Chinese ginseng, could reduce pancreatic inflammation by increasing miR-181b signaling (43). In our study, genistein treatment partially decreased miR-155 and increased miR-181b expression levels in the OVX.G group compared to OVX. Furthermore, genistein exerted protective effects against estrogen-deficiency-induced inflammation in the uterus of ovariectomized rats. Thus, the consumption of natural supplements could effectively improve menopause-related complications in older women. However, more experimental and clinical studies are needed to evaluate the protective effects of phytoestrogens in postmenopausal women significantly.

Conclusion

In the current study, genistein treatment partially ameliorated estrogen deficiency-induced histological changes in the uterus of ovariectomized rats. The expression levels of miR-155 decreased, and the levels of miR-181b increased in the uterus of genistein-treated rats. This study supports the beneficial effects of genistein treatment against uterine inflammation due to estrogen deficiency in menopausal women.

Table 3. Accumulation of inflammatory cells blood vessels area (µm²).

Groups	Mean	Median (n)	Range (Minimum, maximum)	SD	SE	Significant	Mean	Median (µm²)	Range (Minimum, maximum)	SD	SE	Significant
sham	100	100	22 (89-111)	0.0	0.00		7,200	7,000	4,100 (5,900-10,000)	0.00	0.00	
OVX	155.1	155	31 (138-169)	4	0.05	0.5	27,700	26,500	12,000 (20,000-32,000)	6	5	0.01*
OVX.G	127	129	20 (148-168)	4	0.02	0.4	12,100	12,000	6,600 (8,990-15,590)	6	0.44	0.06

*p<0.05 considered statistically significant; Median, mean expression values, range, SE and SD, in the experimental groups; sham; OVX: Ovariectomized rats; OVX.G: Genistein treated ovarectomized rats; SD: Standard deviation; SE: Standard error.

Source of Finance

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Conflict of Interest

No conflicts of interest between the authors and / or family members of the scientific and medical committee members or members of the potential conflicts of interest, counseling, expertise, working conditions, share holding and similar situations in any firm.

Authorship Contributions

Idea/Concept: Naser Ahmadiasl; Design: Alireza Alihemmati, Naser Ahmadiasl; Control/Supervision: Faeze Daghigh, Arezou Hamzehzadeh; Analysis and/or Interpretation: Naser Ahmadiasl; Literature Review: Faeze Daghigh; Writing the Article: Faeze Daghigh; Critical Review: Faeze Daghigh.

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