Original Article

The Effect of Troxerutin on Apelin-13 and Its Receptor Gene Expression in Ovarian of Pregnant Rats Fed a High-Fat Diet

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Abstract

Background & Objective: Apelin is a newly discovered member of adipocytokines that acts as an endogenous ligand for the G-coupled receptor for the protein orphan (APJ). This study aimed at investigating the beneficial effect of troxerutin (TRO) on apelin-13 and its receptor mRNA expression in the ovary of high-fat diet (HFD) fed rats.

Materials & Methods: 40 three-week old female Wistar rats received control diet (CD) or HFD for 8 weeks. After mating, pregnant animals were divided into 4 subgroups: CD, CD +TRO, HFD, and HFD + TRO. Respective diets continued to the end of lactation. CD +TRO and HFD +TRO groups received troxerutin (150 mg/kg/day, P.O.) during pregnancy. After weaning offspring, animals in the maternal group were sacrificed. Then blood samples and ovarian tissue samples were collected for further evaluation.

Results: The results indicated that HFD significantly increased serum apelin-13 in HFD dams, which was reversed by TRO treatment. Also, analysis showed that ovarian mRNA expression of the apelin receptor markedly down-regulated in the HFD group compared to control groups. It was also revealed that treatment with troxerutin in the HFD group could significantly increase apelin receptor mRNA expression in comparison with the both CD and HFD groups.

Conclusion: Troxerutin treatment in obese pregnant mothers can affect the function of the reproductive system by modulating the serum apelin 13 and the expression of its receptor gene.

Keyword: Apelin-13, APJ receptor, Maternal high-fat diet, Troxerutin

Introduction

Adipokines are bioactive peptides that are secreted by adipose tissue and they are involved in the regulation of glucose and lipid metabolism, energy homeostasis, nutritional behavior, insulin sensitivity and vascular function (1). Apelin known as the ligand for an orphan G-protein coupled receptor, APJ (2, 3). Apelin was first discovered in 1998 by Professor Fujino's research team. Apelin also has a peptide origin and its gene in humans contains 77 amino acids and its receptor shows some similarities in gene sequence with Ag II (angiotensin II) (4, 5). Reports indicate that the most important

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source of secretion of this hormone is white adipose tissue, and for this reason, apelin is known as an effective adipokine in the body (6). Studies have shown that apelin and its receptors are expressed in many human tissues as well as in rodents, including the lungs, cardiovascular system, kidneys, mammary glands, white adipose tissue, central nervous system, gastric mucosa, testes, and uterus (7).

Various forms of apelin have been observed in body tissues. Aplin-13 is the main circulatory form in the bloodstream and its biological activity is higher than that of apelin 17 and 36 (8).The presence of APJ in areas of the brain associated with reproduction, such as the hypothalamus and pituitary gland, suggests a possible role for apelin in controlling the release of LH and FSH from the anterior pituitary. According to a study, intraventricular injection of apelin-13 may play a role in central regulation and reduce testosterone release by inhibiting LH secretion (9).

Control of pituitary hormone release, drinking behavior, stimulation of glucose uptake in insulin-sensitive tissues, retinal angiogenesis, positive inotropy, atherosclerotic plaque formation, Granulosa cells atresia in the process of ovulation and fluid homeostasis are among the other roles of the apelinergic system (8, 10, 11). However, the most important physiological role of apelin in the body is the direct interaction with the hormone insulin. Some studies have shown that, the amount of insulin secretion is the main regulator of apelin gene expression and its release into plasma. In the case of insulin resistance and type II diabetes, the expression of apelin by adipose tissue and its plasma amount increases. On the other hand, reducing its expression from normal levels in adipocytes reduces insulin sensitivity (9).

Research shows that the expression of apelin protein is significantly affected by nutritional status, so that its plasma level decreases sharply during starvation and increases to higher levels with refeeding (2, 12).

Some studies have reported changes in plasma levels of apelin in disorders such as obesity, type II diabetes, polycystic ovary syndrome (PCOS), endometriosis, and hypertension (13). Roche et al. demonstrated that apelin and its receptor play a role in human follicular development and the pathogenesis of PCOS (14) and recent studies have shown that apelin can be targeted in pathological cases.

Studies have indicated that obesity during pregnancy can increase the risk of a number of diseases in mothers, including gestational diabetes, preeclampsia and gestational hypertension (15, 16). Moreover, congenital malformations are more likely to occur in infants born to these mothers than in infants born to mothers with normal body mass index (BMI) (16, 17).

Troxerutin, or vitamin P4, is a natural flavonoid derivative of rutin, found in tea, coffee, grains, and a variety of fruits and vegetables (18, 19). This substance is easily absorbed from the gastrointestinal tract (20) and has various biological effects such as anti-inflammatory, antioxidant and anti-diabetic properties (21-23). Also, troxerutin has been reported to prevent obesity by boosting the insulin signaling pathway, leading to levels of glucose, fatty acids and blood cholesterol returning to normal (24). Another beneficial effect of troxerutin is that this flavonoid, inhibits oxidative stress by increasing the expression of the adipokine gene and helps maintain lipid homeostasis in the liver of rat fed a high-fat diet (25). Although some of the beneficial properties of troxerutin have been demonstrated in previous studies, we did not find any studies about troxerutin treatment during pregnancy

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on the reproductive system and adipokines such as apelin in high-fat diet fed rats. . Due to the effects of obesity and high-fat diet on the hypothalamic-pituitary-gonadal axis and uterine tissue, and considering the important role of apelin in regulating the physiological and metabolic activities of the body and, on the other hand, the anti-inflammatory and protective role of troxerutin, we aimed to examine the effects of troxerutin administration during pregnancy on serum apelin-13 level and its receptors mRNA expression of in the ovary of HFD fed rats.

Materials & Methods

Animals, diets, and experimental design

40 female Wistar rats, about 3 weeks old and weighing 30 to 40 grams, were purchased from the Laboratory Animal Breeding Center of Tabriz University of Medical Sciences. Animals were kept 3 per cage in standard condition: 24- 25 temperature, 40 to 60% humidity, and 12 hour of light-12 hour of darkness and had free access to water and food. All experimental protocols were performed according to the instructions of care and use of laboratory animals of the ethics committee of Tabriz University of Medical Sciences. (No: IR. TBZMED. REC.1395.534).

Animals were randomly divided into 2 groups before mating (n=20). They were fed control diet (CD) (14.7% lipids, 33.0% protein, 52.2% carbohydrate) or high-fat diet (HFD) (52.0% lipids, 27.1% protein, 20.9% carbohydrate) for 8 weeks which was purchased from Behparvar CO, Tehran, Iran. For mating, female animals in each group were housed with adult male rats overnight. Pregnancy was confirmed by examining vaginal smears for the presence of vaginal plug. Thereupon pregnant rats were randomized to 4 subgroups as follows: CD, CD+TRO, HFD, HFD+TRO (n=10).

Animals of the second and fourth groups received troxerutin (Merck, Germany) 150 mg/kg/day (26, 27) in the form of gavage during pregnancy. HFD diet continued until the end of lactation in the related groups. Animals in CD and HFD groups were gavaged with saline as the vehicle of troxerutin. Apelin serum levels were measured in the pre-pregnancy period and at the end of lactation.

It should be noted that in the pre-pregnancy period, the desired blood was collected from the eye area and capillary tubes to measure the amount of apelin, and for this purpose, the animals were semi-conscious to avoid excessive stress.

Sampling

All animals in maternal group were anesthetized with intraperitoneal injection of ketamine (80 mg/kg) and xylazine (12 mg/kg)(27, 28) at the end of the lactation. Then, the ovaries were removed and rapidly frozen with liquid nitrogen and then kept at -70 °C for molecular examination. Blood samples were centrifuged at 4000 rpm for 15 min, and then serum samples were collected and stored at –70 °C for apelin-13 measurement.

Assessment of apelin serum concentration

The serum level of apelin-13 was measured using rat-specific apelin-13 enzyme linked immuno-absorbent assay (ELISA) kit (EAST BIOPHARMA, China) according to the manufacturer's instructions (27, 29).

Total RNA extraction and real-time PCR

Real-time reverse transcription polymerase chain reaction (RT-qPCR) was performed to determine the expression level of the target genes. According to the manufacturer's instructions, total RNA was extracted from the ovarian tissue samples using the RNX-Plus solution kit (Cinagen Co. Iran). Purity of the RNA was measured using a NanoDrop 1000 Spectrophotometer (Thermo Scientific, USA). cDNA synthesis was performed using the PrimerScript RT Master Mix Perfect

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Real-Time Kit (Takara Bio Inc.). Real-time PCR was done using SYBR Green PCR Master Mix (Takara Bio, Shiga, Japan) in a total volume of 25 µl on a real-time PCR instrument (RotorGene 3000). PCR primer sequences for APJ receptor and β-actin (housekeeping) were as follows:

APJ (Forward 5′-CCTGGCTTGATGCAGTTGGA-3′, Reverse 5′- TCTGGCCTGAGACATGCAGAG-3′; β-actin (Forward 5′TACAGCTTCACCACCACAGC-3′, Reverse 5′ ATGCCACAGGATTCCATACC-3′). The relative quantity of mRNA for each gene was calculated in relation to its threshold cycle (Ct) compared to the Ct of the internal control gene (β-actin). Relative expression of the target gene was calculated using the 2-ΔΔCt method as follows:

2-[(Ct APJ gene – Ct β-actin) experimental - (Ct APJ gene -Ct β-actin) Control] (30).

Statistical analysis

Data were expressed as mean ± SEM (standard error of the mean). Statistical analysis was performed by SPSS 16. Paired sample t- test was used to compare apelin 13 serum concentration before pregnancy and the end of lactation period. One-way ANOVA followed by Tukey post-hoc test was used for analysis of apelin 13 serum concentration and its mRNA expression of APJ receptor between related group. It was considered significant if $P<0.05$.

Results

Effect of HFD and TRO treatment on serum apelin-13 level

Serum apelin-13 concentration in the HFD group significantly increased in comparison with the CD group ($P<0.05$, Chart1). Conversely, troxerutin treatments during pregnancy significantly ($P<0.05$, Chart1) decreased serum apelin-13 levels in the HFD+TRO group as compared to the HFD group. Also, there was no significant difference between apelin-13 serum levels before and after pregnancy.

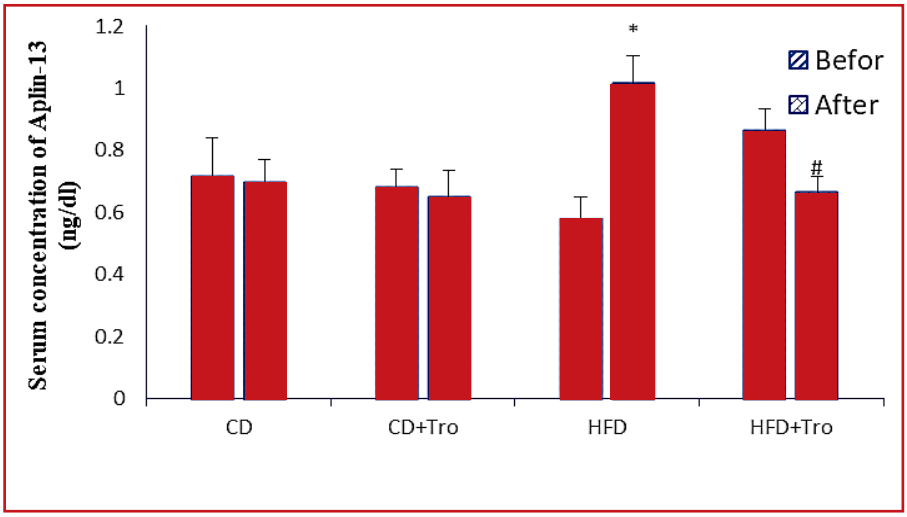

Chart 1. Effect of high fat diet and troxerutin treatment during pregnancy on serum apelin-13 concentration. Paired sample t-test was used to compare serum apelin-13 level before and after pregnancy. One-way ANOVA followed by Tukey's post hoc test; *P<0.05 vs CD group, #P<0.05 vs HFD group. Data are expressed as Mean±SEM (n=8)
[CD: control diet, CD+TRO: control diet +Troxerutin, HFD: High-fat diet; HFD+TRO: High-fat diet + Troxerutin]

Effect of HFD and TRO treatment on ovarian mRNA expression of APJ receptor

The results of real-time PCR showed that ovarian mRNA expression of APJ, apelin-13 receptor, was markedly down-regulated in the HFD group in comparison with the CD group (P<0.05, Chart 2). While treatment with troxerutin in the group that received a high-fat diet, led to a significant increase in the expression of the desired gene as compared to the CD group (p<0/001, Chart 2).

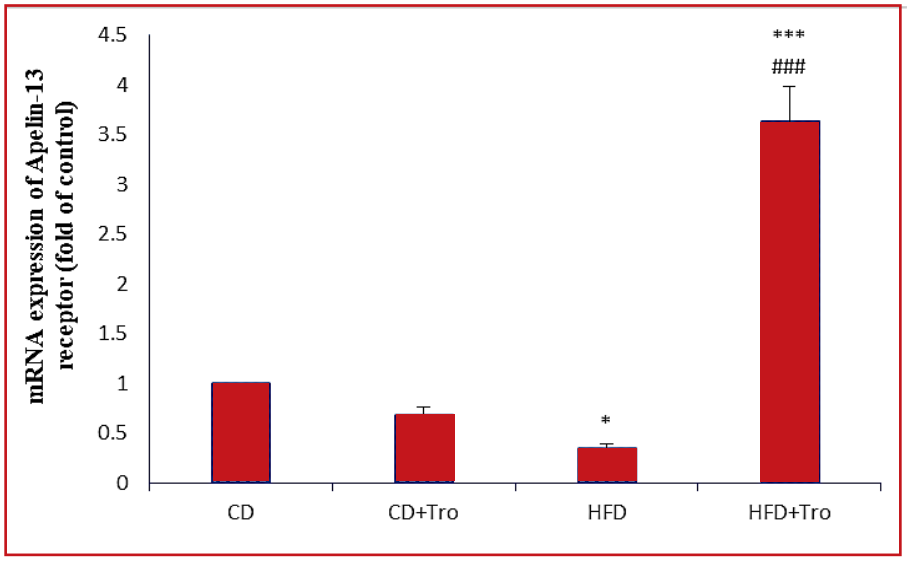

Chart 2. Relative expression of apelin receptor (APJ) in the ovaries of HFD fed dams. One-way ANOVA followed by Tukey's post hoc test; *P<0.05 vs CD group, ***P<0.001 vs CD group, ###P<0.001 vs HFD group. Data are expressed as Mean±SEM (n=6). [CD: control diet, CD+TRO: control diet +Troxerutin, HFD: High-fat diet; HFD+TRO: High-fat diet + Troxerutin]

Moreover, the administration of troxerutin during gestation significantly (P<0.001) up-regulated mRNA expression of the APJ receptor in the HFD+ TRO group in comparison with the HFD group.

Discussion

In the present study, it was demonstrated that maternal HFD led to significant changes in the serum levels of apelin-13 and its receptor mRNA expression, APJ, in the ovary tissue. However, troxerutin treatment during pregnancy decreased serum apelin-13 and upregulated apelin-13 receptor mRNA expression in the ovarian tissue of the HFD fed rats.

Previous studies revealed that HFD feeding for 15 days is related to elevated adipose tissue, decreased adiponectins secretion from the adipose tissue, and reproductive disorders both in the male and female rats (31, 32). The results of a study demonstrated that high fat diet (HFD) during pregnancy led to a metabolic syndrome-like phenomenon through epigenetic modifications of the adipocytokines genes that can persist for multiple generations (33). In another study, it was found that the use of high-fat diet in female rats from infancy to puberty can cause obesity through increasing the weight and body adipose tissue, also the use of high-fat diet leads to increased levels of triglycerides, total cholesterol and LDL (34). Moreover, in vivo studies demonstrated that expression of adipocytokines including apelin is noticeably regulated by nutritional

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status, suppressed by fasting and restored by refeeding, as well as insulin (2, 35).

In addition, a high-fat diet has been shown to stop the cell cycle and apoptosis of granulosa cells during the period of follicular growth and development. On the other, cell cycle inhibitors p27 and p21 are significantly increased in the ovaries of mice fed HFD compared to mice fed a normal diet (31) Therefore, a high-fat diet can affect various aspects of ovarian function such as proliferation, apoptosis and steroidogenic ovarian function.

High levels of fatty acids can reduce the cells' sensitivity to insulin and cause hyperinsulinemia, which in turn increases apelin secretion adipose tissue (35, 36). Furthermore, in a study conducted by Attane et al., chronic administration of apelin in obese and insulin-resistant mice increased the oxidation of fatty acids in muscle by activating AMPK (37).

Findings of the current study demonstrated that apelin-13 concentration in the HFD fed rats increased in comparison with the CD group.

In a study by Zhang et al, it was found that feeding HFD increased fasting blood sugar and serum insulin levels in mice (38). Thus, increased serum apelin13 levels in rats fed a high-fat diet can be justified by this mechanism.

On the other hand, there is a direct correlation between apelin secretion and BMI, obesity and fat percentage (9, 39). Furthermore, due to high body fat and insulin resistance in obese people, the amount of apelin secretion in these people is higher than people with normal weight (40, 41). A study reported that insulin sensitivity decreases in soleus muscle of mice with apelin deficiency and apelin injection in these mice increased muscle glucose uptake, Akt phosphorylation, and improved insulin sensitivity (42).

In agreement with our findings, previous studies suggest that TRO has a restorative effect on the protein expression of adipokine in the liver of HFD-treated mice (43).

Newly, a study also indicated that TRO prevented the secretion of inflammatory cytokines and reduced adiponectin levels in HFD-received mice. In addition, administration of TRO declines serum insulin levels in HFD-treated mice (38).

Also, the insulin signaling pathway can be improved with troxerutin, thereby restoring blood glucose, fatty acids and cholesterol levels to normal (24).

Therefore, it is possible that TRO can reduce the level of maternal apelin through this mechanism.

Moreover, our data showed that ovarian mRNA expression of APJ, down-regulated in the HFD fed rats. This reduced receptor gene expression may result in increased apelin 13 serum levels via feedback inhibition.

Apelin as an adipokine has an important property in the regulation of fluid homeostasis, immune response, angiogenesis, oxidative stress and glucose metabolism (44). Apelin and APJ are considered as a factor controlling reproduction in both sexes. Also, apelin is associated with different pathologies like polycystic ovarian syndrome (PCOS), infertility, endometriosis, and obesity (45). Therefore apelin can be targeted in pathological cases.

Adipose tissue proliferation in obesity is associated with decreased blood flow and tissue hypoxia, and increases the production of inflammatory factors, including interleukin-6, interleukin-1β, and TNF-α (46).

Our research team recently indicated that TRO treatment during pregnancy reduced IL-6 and TNF-α concentration in maternal serum as well as in the serum and

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of their male offspring (47). Also, in another study conducted by our team showed that troxerutin can improve maternal HFD induced spatial memory impairments of the offspring probably via modulation of serum and hippocampal apelin levels (29).

Several studies suggest that troxerutin can have useful effects on metabolic syndrome complications by improving lipid profiles and antioxidant status (48, 49). Therefore, it seems that troxerutin treatment during pregnancy can affect metabolic status in reproductive system by modulation of serum apelin and its receptor gene expression but further studies are needed to elucidate the exact mechanism of TRO action in this regard.

Conclusion

Overall, the results of the current study showed increased serum apelin and decrease in apelin/APJ mRNA expression of ovarian tissue in HFD fed group, and these changes were restored by troxerutin treatment.

Eventually, to better understand how troxerutin affects the reproductive system, we suggest doing more studies with regard to the evaluation of molecular pathways and intracellular signaling of troxerutin.

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Conflicts of Interests

There is no conflict of interest.

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