Effect of Testosterone Enanthate Modeling of Polycystic Ovary on Liver Irs-2 mRNA Expression in Rats: A Brief Report

Seyyed Amir Yasin Ahmadi1, Amin Hasanvand4, Mandana Beigi Boroujeni2, Afshin Hasanvand5, Naser Pajouh3, Seyed Behnamedin Jameie6, Asghar Aaliehpour2, Reza Nekouian7

1 Student Research Committee, Iran University of Medical Sciences, Tehran, Iran
2 Department of Anatomical Sciences and Pathology, Lorestan University of Medical Sciences, Khorramabad, Iran
3 Razi Herbal Medicines Research Center, Lorestan University of Medical Sciences, Khorramabad, Iran
4 Student Research Committee, Lorestan University of Medical Sciences, Khorramabad, Iran
5 Neuroscience Research Center, Iran University of Medical Sciences, Tehran, Iran
6 Pediatric Growth and Development Research Center, Institute of Endocrinology and Metabolism, Iran University of Medical Sciences, Tehran, Iran

*Corresponding author: Ahmadi.say@iums.ac.ir, Mandana.beigi@lums.ac.ir, naserpajoh@yahoo.com, Amin.hasanvand@lums.ac.ir, afshinhasanvand@yahoo.com, Jameie.sb@iums.ac.ir, Dr.aaliehpour@gmail.com, nekouian.r@iums.ac.ir

ORCID ID: https://orcid.org/0000-0001-9274-7408, https://orcid.org/0000-0003-2276-3554, https://orcid.org/0000-0002-3051-0015, https://orcid.org/0000-0003-0837-7542, https://orcid.org/0000-0003-0837-7542, http://orcid.org/0000-0003-0991-2935, https://orcid.org/0000-0003-0303-2801, https://orcid.org/0000-0002-8348-8368

Received 13/6/2020, Accepted 16/12/2020, Published Online First 21/2/2021

This work is licensed under a Creative Commons Attribution 4.0 International License.

Abstract:
There are many animal models for polycystic ovary (PCO); using exogenous testosterone enanthate is one of the methods of induction of these models. However, induction of insulin resistance should also be studied in the modeling technics. Therefore, the present study aims to investigate the expression of insulin receptor substrate (Irs)-2 mRNA in the liver tissue of rat PCO model. Nineteen Wistar rats were divided into three groups; (1) PCO modeling group (N = 7) received daily 1.0 mg/100g testosterone enanthate solved in olive oil along with free access dextrose water 5%, (2) vehicle group (N = 6), which handled like the PCO group, but did not receive testosterone enanthate, (3) control group (N = 6) with standard care. All the animals were administered via intraperitoneal injection for 14 days. Expression of Irs-2 mRNA was studied with real-time PCR and fold changes (FC) were reported. The average of expression in the control group was considered as the calibrator. About 13.4% expression reduction was found in the PCO group (FC = 0.874, P-value = 0.043). No significant reduction was found in the vehicle group (FC = 0.951, P-value = 0.076). However, analysis of variance did not show a significant difference between all the groups of study (P-value = 0.085). The present model of PCO might induce insulin resistance at liver level with a low effect size via reduction in the mRNA expression of Irs-2. Study of the involved genes and molecules in other tissues of PCO animal models is suggested.

Key words: Animal model, Insulin receptor, Polycystic ovary.

Introduction:
Insulin resistance is a condition in which the body cells are not sensitive to absorb glucose and the insulin receptors have decreased. In basic medical sciences, the expression of insulin receptor substrates (IRS) is studied to investigate insulin resistance at molecular level. Studies have shown that among IRSs, IRS-2 is more associated with insulin resistance especially in the liver (1, 2). IRS-2 is responsible for insulin resistance at liver level in patients with type 2 diabetes mellitus. In human, genetic polymorphisms of IRS-2 affects the risk of type 2 diabetes mellitus (3).

Hormonal and metabolic disturbance affects women's reproductive system. Polycystic ovary...
(PCO) syndrome (PCOS) is a disorder resulting in health problems and infertility. PCOS includes symptoms and problems such as lack of ovulation, hirsutism, and insulin resistance. Type 2 diabetes mellitus, metabolic syndrome, hypertension etc. are considered as risk factors of PCOS. According to the role of insulin resistance, metformin is used for the management of PCOS (4-7). Other than an effect on insulin sensitivity, metformin has antioxidant effects on liver (8, 9). In addition to using metformin, there are many other chemical and herbal drugs such as cinnamon, ginger and spearmint that may be used for management of PCOS (10-13). The exact pathophysiology of PCOS is not clear. In other words, it is not obvious whether the mentioned insulin resistance and metabolic syndrome are the cause or the effect. Nevertheless, the hypothesis of hormonal imbalance is accepted. Hormonal disturbance results in incomplete growth of the immature follicle and remaining in the ovary (anovulation) (14). This unreleased follicle is called as a cyst. These cysts are not necessarily macroscopic; therefore, imaging (ultrasoundography) is merely one item in the diagnostic criteria of PCOS (15, 16).

Animal models are needed for the preclinical phase study of novel drugs. So far, different models for the induction of PCO and PCOS have been designed (17-19). The limitation of these models is that each of them simulates only a part of the pathogenesis. Some models emphasize on the induction of metabolic syndrome, while some others emphasize on the induction of hyperandrogenism. Most models are based on hormonal methods. Obviously, all the models observe Rotterdam criteria (17-19). The animal model of PCO through testosterone enanthate has been previously validated histologically and biochemically by some researchers (20-23). Since the mechanisms of PCO induction are different, each model is not necessarily representative for investigation of the efficacy of all drugs, because each drug targets a specific part of the pathogenesis. For instance, metformin targets insulin resistance, whereas spironolactone targets hyperandrogenism.

A way to resolve this limitation is to design combined models or to investigate induction the other part of the pathogenesis. For this reason, we aimed to study the insulin resistance in a hormone-based model. This study was designed to investigate the expression of Irs-2 mRNA at the liver level in rat on the paraffin-embedded samples in the researchers archive.

Material and Methods:

Study design

The present mRNA expression study was performed on paraffin embedded samples of a previously unpublished experimental study using rat model of PCO with testosterone enanthate and unlimited access to glucose regimen.

Laboratory study

Nineteen Wistar rats with 8-10 weeks of age were studied under standard condition of light and temperature in animal laboratory of Razi Herbal Medicines Research Center, Lorestan University of Medical Sciences, Iran. The rats were divided into three groups; 1) PCO modeling group (testosterone group) received daily 1.0 mg/100g testosterone enanthate solved in olive oil 300 µL per injection along with free access dextrose water 5% (N =7), 2) vehicle group, which handled like the PCO group, but did not receive testosterone enanthate (N =6), 3) control group with standard care of light, temperature and moisture (N =6). All the treatments were given for 14 days via sub-dermal injection at the back of neck.

The paraffin-embedded livers of the experimental animals were used for the mRNA expression study. The liver samples were removed from the paraffin blocks and about 100-200 mg of them were used for RNA extraction. These samples were put in xylene 2 times for 3 minutes at 50°C and then they were washed in ethanol 100% 2 times. The deparaffinized samples were dried and chopped by a scalpel. Then the samples were put in Eppendorf tubes and 1.0 ml Trizol reagent (Maxcell, Iran) was added and we waited for 5 minutes. Then 0.3 ml chloroform was added followed by 15 seconds vortex. The Eppendorf tubes were centrifuged up to 15000 g for 10 minutes at 4°C. The superior phase was separated and 400 µL isopropyl alcohol was added followed by a brief vortex. Then the Eppendorf tubes were centrifuged up to 15000 g for 10 minutes at 4°C (UNIVERSAL320/320R, Cat. no. 1789-a, Hettich, Germany). The Eppendorf tubes were emptied and 1 ml ethanol 75% was added. Then the Eppendorf tubes were centrifuged up to 6000 g for 5 minutes at 4°C. The Eppendorf tubes were emptied and dried on sterile gauze. Finally 50 µL sterile water was added, and the Eppendorf tubes were incubated at 65°C for 3 minutes. Concentration of the extracted RNA was investigated using nanodrop spectrophotometry and after putting on ice the samples were transferred to -80°C refrigerator.

cDNA was synthesized using a kit (Yekta Tajhiz, Iran) according to the manuals of the manufacture. Real-time polymerase chain reaction (real-time PCR) was used for the amplification of Irs-2 cDNA using SYBR green master mix (Norgen, Biotek Corp., Canada) using the 2-step
method. The primers were chosen from previous literature and re-checked in NCBI data base and Oligo7 software (24, 25) (Table 1).

Table 1. Sequences of the primers used for real-time PCR.

<table>
<thead>
<tr>
<th>Target</th>
<th>Sequence</th>
<th>Product size</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>Forward: CCAGGGCTGCC</td>
<td>168</td>
<td>Jeckel et al. (24)</td>
</tr>
<tr>
<td></td>
<td>TTCTCTTGT</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Reverse: TGATGGGTTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CCGTTGAT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Irs-2</td>
<td>Forward: TCATACCCAGC</td>
<td>70</td>
<td>Gyte et al. (25)</td>
</tr>
<tr>
<td></td>
<td>CTCATCACTA</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Reverse: CCTAGGTACGC</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CAACGAT</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Protocol of real-time PCR

After extraction of RNA from the paraffin-embedded tissues, the concentration of RNA was obtained 70-80 ng/µL. About 100-300 ng of RNA was used for cDNA synthesis using random hexamer primers. PCR reaction mixture consisted of 10 µL SYBR green master mix, 2 µL cDNA, 1 µL 10 pmol forward and reverse primers, and water up to the total volume of 20 µL. The 2-step cycling method consisted of initial denaturation at 95°C for 2 min, and 35 cycles of denaturation at 95°C for 12 seconds followed by annealing/extension at 60°C for 20 seconds.

Data analysis

For calculation of ∆CT, GAPDH was used as a housekeeping gene. The average of ∆CTs of the control group was considered as our calibrator for calculation of ∆∆CT. Statistical analyses were performed on the amounts of -Δ∆CT. One sample t-test was used for each experimental group (H0: -Δ∆CT =0). One way analysis of variance (ANOVA) was used for group comparison with Dunnett's post hoc test. After statistical analysis, the amounts of -Δ∆CT were converted to fold change (FC) via 2^Δ∆CT and 95% confidence interval (CI) of FC was calculated through conversion of lower and upper limits of -Δ∆CT to FC. Therefore, the 95% CIs for FCs were obtained geometrically. Sta 14 (StataCorp LLC, US) was used for statistical analysis and P-value less than 0.05 was considered as significance level.

Ethical considerations

The ethics of working with laboratory animals had been regarded during the background animal study. The ethic committee of Iran University of Medical Sciences permit us to use the paraffin blocks for gene expression study (registration number: IR.IUMS.REC.1398.446).

Results:

About 13.4% of Irs-2 mRNA expression reduction was found in the PCO group (FC =0.874, 95% CI =0.768-0.994, P-value =0.043, power =0.728). No significant reduction was found in vehicle group (FC =0.951, 95% CI =0.899-1.007, P-value =0.076, power =0.617). However, analysis of variance did not show significant difference between the groups (P-value =0.085). Only the 95% CI of PCO group did not cross the null hypothesis (FC =1) (Table 2, Fig 1).

Table 2. Fold changes of Irs-2 mRNA expression.

<table>
<thead>
<tr>
<th>Group</th>
<th>Fold change (95% CI)</th>
<th>One sample P-value</th>
<th>ANOVA P-value</th>
<th>Dunnett's test P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Testosterone</td>
<td>0.874 (0.768-0.994)</td>
<td>0.043*</td>
<td>0.085</td>
<td>0.057</td>
</tr>
<tr>
<td>Vehicle</td>
<td>0.951 (0.899-1.007)</td>
<td>0.076</td>
<td>0.639</td>
<td>Reference</td>
</tr>
<tr>
<td>Control</td>
<td>1.000 (0.918-1.085)</td>
<td>Reference</td>
<td>Reference</td>
<td></td>
</tr>
</tbody>
</table>

* Significant at P <0.05 according to one sample t test; Fold change =1 (-Δ∆CT =0) is the null hypothesis.

Figure 1. Graphing fold changes of Irs-2 mRNA expression with geometrical 95% CI.

* Significant at P <0.05 according to one sample t test; Fold change =1 (-Δ∆CT =0) is the null hypothesis shown as hyphenated reference line.
Discussion:
According to the results, the down-regulation of Irs-2 mRNA in PCO (testosterone) group was statistically significant. Lack of significant association in the vehicle group showed that using dextrose water regimmen with high dose olive oil (as a highcalorie diet) could not induce insulin resistance at liver level per se without adding testosterone enanthate. According to our findings, it seems that in the animal models of PCO, testosterone may facilitate induction of insulin resistance.

There was no other study investigated directly liver level of insulin resistance in a hormone-based animal model of PCO. Demissie et al. (2008) showed that perinatal exposure to androgens result in induction of metabolic syndrome in adult female rats. However, liver level of IRS-1 and IRS-2 did not show a significant different via western blot study (26). Zhang et al. (2001) showed that sustained exposure to insulin results in reduction in liver mRNA expression of Irs-2 (27). Dominici et al. (2002) in insulin hypersensitivity model of mice (via depletion of growth hormone, prolactin, and thyroid stimulating hormone, called as hypopituitary Ames dwarf mice) showed that hypoglycemic and insulin hypersensitivity status was associated with increased liver IRS-1 and IRS-2 expression (28). In a human study, Yen et al. (2004) investigate wedge samples of 11 women with PCOS and 10 women as control. They extracted total protein and used western blot. IRS-1 and IRS-2 showed increase in theca cells of PCOS patients, but not in granulosa cells. Gathering their results with the results of ours indicates that insulin signaling pathway may be different in ovary and liver (29).

A systematic review has shown that non-alcoholic fatty liver disease (NAFLD) has coexistence with PCOS. The involvement of the insulin-signaling pathway has existed in the pathogenesis of both NAFLD and PCOS. Hyperinsulinism and insulin resistance perform important roles in the pathogenesis of PCOS. IRS-1 is more closely associated with glucose metabolism whereas IRS-2 is more closely associated with lipid metabolism (30). IRS-2 is a component of many biological pathways. A study has shown that the deletion of IRS-2 is associated with female infertility in mice. They discussed about the role of this pathway in the growth signaling of the ovary; however, in our study we emphasized the metabolic role in the liver (31).

From the limitations of the study, it can be mentioned that the effect size of the positive result is not clinically representative and comparable with human. In other words, it is not clear whether a 13.4% reduction in liver expression of IRS-2 in human cause clinical manifestations or not. Future human studies help to fill this evidence gap.

Conclusion:
The present model of rat PCO might induce insulin resistance at liver level with a low effect size via reduction in mRNA expression of Irs-2. Study of the involved genes and molecules in other tissues of the animal models of PCO such as skeletal muscles is suggested to find out which part of the model can induce which part of the pathogenesis of the disease. It helps to study the efficacy of new treatments with animal models.

Acknowledgements:
This study was supported by Student Research Committee of Iran University of Medical Sciences with grant number 98-1-15-14942 and ethical registration number IR.IUMS.REC.1398.446.

Authors' declaration:
- Conflicts of Interest: None.
- We hereby confirm that all the Figures and Tables in the manuscript are mine ours. Besides, the Figures and images, which are not mine ours, have been given the permission for republication attached with the manuscript.
- The author has signed an animal welfare statement.
- Ethical Clearance: Approved by Iran University of Medical Sciences with registration number: IR.IUMS.REC.1398.446.

References:
4. Keshavarz M, Moradi S, Emami Z, Rohani F. Association between serum 25 (OH) vitamin D and
تأثير نمذجة التستوستيرون إينونثات للمبيض متعدد التكيسات على تعبير الكبد

 этап تقرير موجز

أيمن حسنوند 1
ردام نكونيان 2
ناصر بيجهي 2
ماهنا بيجي بروجنی 1
حيدر الهی جامعی 6
افشین حسنوند 5

1 لجنة أبحاث الطلاب ، جامعة إيران للعلوم الطبية ، طهران ، إيران
2 قسم العلوم التشريحية وعلم الأمراض ، جامعة لورستان للعلوم الطبية ، خرم آباد ، إيران
3 مركز أبحاث التهاب الكبد ، جامعة لورستان للعلوم الطبية ، خرم آباد ، إيران
4 مركز أبحاث الأدوية العشبية الرازي ، جامعة لورستان للعلوم الطبية ، خرم آباد ، إيران
5 لجنة أبحاث الطلاب ، جامعة لورستان للعلوم الطبية ، خرم آباد ، إيران
6 مركز أبحاث علم الأعصاب ، الجامعة الإيرانية للعلوم الطبية ، طهران ، إيران
7 مركز أبحاث نمو وتطور الأطفال ، معهد الغدد الصماء والتمثيل الغذائي ، جامعة إيران للعلوم الطبية ، طهران ، إيران

الخلاصة: هناك العديد من النماذج الحيوانية لتكيس المبيض (PCO). يعد استخدام إينونثات التستوستيرون الخارجي إحدى طرق تحريض هذه النماذج. ومع ذلك، يجب أيضًا دراسة تحريض مقاومة الأنسولين في تقنيات النماذج. لذلك، تهدف الدراسة الحالية إلى التحقق من تعبير ركيزة مستقبلات الأنسولين (Irs) -2 mRNA في أنسجة الكبد لنموذج PCO الفئران. تم تقسيم تسعة عشر من قئران ويستار إلى ثلاث مجموعات (1) تلقت مجموعة نمذجة PCO (N = 7) يوميًا 1.0 مجم / 100 جرام من إينونثات التستوستيرون المذاب في زيت الزيتون جنبًا إلى جنب مع الوصول الحر إلى ماء الدكستروز ذي التركيز 5٪ (2) مجموعة المركبات (N = 6) مع الرعاية المنتظمة. تم حقن جميع الحيوانات داخل الصفاق لمدة 14 يومًا. تم دراسة تعبير عن Irs-2 mRNA باستخدام التحقيق بال الوقت الفعلي وتم الإبلاغ عن تغييرات الطي (FC) في مجموعات الدراسة. تم تحديد اختلافات عقدية مع تجربة في مجموعة الوردي (FC = 0.874، قيمة P = 0.043). لم يتم العثور على تأثير كبير في مجموعة المركبات (FC = 0.951، قيمة P = 0.076). ومع ذلك، لم يظهر تحليل التباين فرقًا معينًا بين جميع مجموعات الدراسة (قيمة P = 0.085). لذلك، يقترح النموذج الحالي لـ PCO إينونثات التستوستيرون كنموذج منخفض للذكور بحمالات مختلطة عن طريق تقليل تعبير عن Irs-2 mRNA، يقترح دراسة الجينات والجزيئات المعنية في الأنسجة الأخرى للنماذج الحيوانية.

الكلمات المفتاحية: نموذج حيواني، مستقبل الأنسولين، بيض متعدد التكيسات.