Effect of Crocin on Letrozole-Induced Polycystic Ovarian Syndrome

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Abstract

Background: Letrozole (LET), a non steroidal aromatase inhibitor, produce an animal model to study Polycystic Ovarian Syndrome (PCOS). Clomiphene Citrate (CC), first-line in treatment of PCOS. Crocin is a carotenoid used as traditional medicine to treat many diseases.

Aim of Study: Study effect of crocin on letrozole-induced polycystic ovarian syndrome.

Material and Methods: Forty local strainfemale rats were used in this study; these animals were divided into four main groups (ten rats each). Group I: Normal group. Group II (Letrozole-induced PCOS group): These animals were adminstered LET once daily in a dose of 1mg/kg for 21 consecutive days by oral gavage. Group III; Crocin-treated group: Received LET as group II, then crocin 50mg/Kg once daily by intraperitoneal injection for 6 weeks. Group IV (CC treated group): Received LET as in group II, then CC 1mg/kg once daily by oral gavage for 6 weeks. Blood samples were collected by cardiac puncture for analysis of serum testosterone, FSH, LH, estradiol, glucose, insulin and total cholesterol. HOM-IR where calculated. Ovaries were be dissected out. One ovary from one animal of each group was preserved in 10% formalin for histopathological study.

Results: Letrozole treatment resulted in, increase in serum testosterone, FSH, LH, glucose, insulin, cholesterol levels and also, decrease in serum estradiol level. However, after injection of crocin, all parameters studied returned back to nearly normal levels as in control group there were no changes between crocin treated or CC treated groups. These results were confirmed by histopathological examination.

Conclusion: These findings highlighted the important role played by crocin in treatment of polycystic ovarian syndrome.

Key Words: Polycystic ovarian syndrome – Crocin – Clomiphene citrate.

Introduction

POLYCYSTIC Ovarian Syndrome (PCOS) is recognized as the most common endocrinopathy of women. The causes of PCOS are not fully understood [1] and its management is often unsatisfactory or requires a diversified approach.

Letrozole (LET), a non steroidal aromatase inhibitor, produces an animal model to study PCOS. This animal model in several ways is similar to the human polycystic ovary syndrome [2].

Clomiphene citrate (CC) is considered as first-line in treatment of PCOS women [3]. It works by blocking estrogen receptors at the hypothalamus which is stimulated to release follicle stimulating hormone and luteinizing hormone.

Nowadays people are focusing towards allopathic medicines to get the temporary relief for many diseases. However, women with PCOS can find alternative therapy for permanent cure. Herbal medicines are the most popular alternative therapy among all [1].

Crocin is a carotenoid obtained commercially from the dried trifid stigma of the culinary spice crocus sativus L. (saffron) and is responsible for the red color of saffron. It is used as a traditional medicine, throughout history [4].

The aim of the present work is to study effect of crocin on letrozole-induced polycystic ovarian syndrome.

Material and Methods

Drugs: Crocin was obtained from Sigma Aldrish as a powder and dissolved by addition of normal saline. Letrozle was obtained from Novartis and dissolved by addition of normal saline.

Experimental animals: The present work was carried out on 40 female adult rats of local strain
weighing (180-200gm). These rats were housed in isolated animal cages, ten in each cage, in a standard animal laboratory room in Faculty of Medicine, Tanta University during 2018 and had free access to water and food ad libitum all over the period of the work, and were kept at room temperature. Rats were acclimatized to the new environment for 7 days prior to the start of the experiments. All procedures were done according to the instructions of the ethical committee of Tanta University.

Experimental design and animal grouping:
The animals were divided into four groups (10 rats for each):
1- **Group I:** (Normal control group): The animals of this group received 0.5ml saline daily by intraperitoneal injection (IP) and 0.5ml saline by oral gavage for 21 days.

2- **Group II:** (LET-induced PCOS group): The animals of this group received LET once daily in a dose of 1mg/kg for 21 consecutive days by oral gavage to induce PCOS [5].

3- **Group III:** (Crocin-treated group): The animals of this group received LET as group II to induce PCOS, then crocin 50mg/Kg once daily by (IP) injection for 6 weeks [6].

4- **Group IV:** (CC-treated group): The animals of this group received LET as group II to induce PCOS then, CC 1mg/kg once daily by oral gavage for 6 weeks [7].

Blood and tissue sampling:
At the end of experimental period, all animals were fasted for 12 hour, deeply anesthetized by diethyl ether, then sacrificed by cervical decapitation and blood samples were collected.

Ovaries were dissected out, one ovary from one rat in each group.

Biochemical analysis:
Free serum testosterone [8], estradiol [9], FSH [10], LH [11], fasting glucose [12] and insulin [13] levels were measured. Insulin resistance (HOMA IR%) was also calculated [14]. Serum total cholesterol level, was measured by BioMed-cholesterol-LS kits [12].

Histopathological study:
Ovaries were fixed in Bouin’s fixative then subsequently washed and stored in 10% formalin until processing and embedding in paraffin wax. After embedding, each ovary was serially sectioned at the diagonal plane at 4 µm thickness and subsequently stained with hematoxylin and eosin [15] using standard techniques for histopathological examination.

Statistical analysis:
Data were analyzed using Statistical Program for Social Science (SPSS) version 17 using one way Analysis of Variance (ANOVA) followed by Tukey-Kramer multiple test for comparison between individual groups. Data were expressed as mean ± Standard Deviation (SD) [16].

Results
The results of this work showed that LET administration caused significant increase in all studied parameters except serum estradiol which showed significant decrease compared to control group (*p*≤0.05). Treatment of animals with crocin or CC resulted in decrease in all studied parameters except serum estradiol level which showed significant increase when compared to LET-induced PCOS group (*p*≤0.05). There was no change in studied parameters in both crocin-treated and CC-treated groups when compared to control group (*p*≥0.05) (Tables 1,2).

Table (1): Serum free testosterone (ng/ml) estradiol (pg/ml) FSH (mlu/ml) LH (mlu/ml) in all studied groups (mean values ±SD).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Groups</th>
<th>GI Control</th>
<th>GII polycystic ovary</th>
<th>GIII Crocin treated</th>
<th>GIV clomiphene citrate treated</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum free testosterone (ng/ml)</td>
<td>2.41±0.12</td>
<td>4.16*±0.47</td>
<td>2.44#±0.13</td>
<td>2.44#±0.13</td>
<td>109.651</td>
<td></td>
</tr>
<tr>
<td>Serum estradiol (pg/ml)</td>
<td>90.48±1.09</td>
<td>81.40*±0.80</td>
<td>90.32#±1.10</td>
<td>90.05#±0.79</td>
<td>216.425</td>
<td></td>
</tr>
<tr>
<td>Serum FSH level (mlu/ml)</td>
<td>0.04±0.01</td>
<td>0.12*±0.03</td>
<td>0.05#±0.02</td>
<td>0.05#±0.02</td>
<td>24.840</td>
<td></td>
</tr>
<tr>
<td>Serum LH level (mlu/ml)</td>
<td>0.27±0.04</td>
<td>0.47*±0.02</td>
<td>0.28#±0.05</td>
<td>0.28#±0.04</td>
<td>67.333</td>
<td></td>
</tr>
</tbody>
</table>

*: Denotes statistical significance (*p*≤0.05) compared with the control group.

#: Denotes statistical significant (*p*≤0.05) compared with the PCOS group.
Table (2): Serum glucose (mg/dl), insulin (µIU/ml), HOMA IR cholesterol (mg/dl) in all studied groups (mean values ±SD).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Groups</th>
<th>G1 Control group</th>
<th>GII polyctic ovary group</th>
<th>GIII Crocin treated group</th>
<th>GIV clomiphene citrate treated group</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum glucose (mg/dl)</td>
<td>91.25±9.98</td>
<td>196.22±12.24</td>
<td>91.32±9.94</td>
<td>92.88±10.03</td>
<td>173.908</td>
<td></td>
</tr>
<tr>
<td>Insulin level (µIU/ml)</td>
<td>12.70±2.26</td>
<td>21.55±2.23</td>
<td>12.38±1.9</td>
<td>13.68±2.01</td>
<td>42.083</td>
<td></td>
</tr>
<tr>
<td>HOMA IR</td>
<td>2.81±0.23</td>
<td>10.43±1.1</td>
<td>2.83±0.16</td>
<td>3.00±0.26</td>
<td>152.032</td>
<td></td>
</tr>
<tr>
<td>Serum cholesterol (mg/dl)</td>
<td>118.91±15.4</td>
<td>144.42±14.78</td>
<td>119.45±13.95</td>
<td>120.07±13.01</td>
<td>7.122</td>
<td></td>
</tr>
</tbody>
</table>

*: Denotes statistical significance (p≤0.05) compared with the control group.
#: Denotes statistical significant (p≤0.05) compared with the PCOS group.

Fig. (1): Control group, normal ovarian structure showing primary follicle (yellow arrow) and Graafian follicle (red arrow) under the surface epithelium (H & E 100).

Fig. (2): PCO, section in the ovary showing multiple cystically dilated follicles (red arrow) (H & E 200).

Fig. (3): PCO, section in the ovary showing a large cystically dilated follicle (red arrow) and multiple small cystic follicles (yellow arrow) (H & E 100).

Fig. (4): Crocin treated group, section in the ovary showing no cystic changes with many blood vessels (red arrow) in the medulla (H & E 200).

Fig. (5): CC treated group, section in the ovary showing normal ovarian structure with ovarian follicles (red arrow) and no cystic changes (H & E 200).

Fig. (6): Serum free testosterone level ng/ml in all studied groups.
Effect of Crocin on Letrozole-Induced Polycystic Ovarian Syndrome

Discussion

The results of the present study showed that in letrozole-induced PCOS group, there was significant increase in serum free testosterone, LH and serum FSH level. On the other hand serum estradiol level showed significant decrease compared to control group.

Pandey et al., further supports the role of oxidative/nitrosative stress in the pathogenesis of letrozole-induced hyperandrogenic and hormonal changes in PCO rats [17]. The mechanism by which letrozole non-steroidal aromatase inhibitor produces
these changes in the hormonal profile could be explained by altering reproductive gene expression at multiple levels of the hypothalamic-pituitary-gonadal axis [18]. The significant increase in testosterone and decrease in estradiol could be also explained by blocking conversion of testosterone and androstenedione to estradiol and estrone respectively [19]. This decreases the estrogenic activity releasing the hypothalamus from its negative feedback leading to an increase in FSH release [20]. The hypothalamus becomes hyperactive in PCOS and secretes gonadotropins releasing hormone (GnRH) more often than usual without identified reason that leads to excess production of Luteinizing Hormone (LH) and Follicle Stimulating Hormone (FSH) [18].

Steroidogenic enzymes are required for formation of active sex steroids as dihydrotestosterone (DHT) and the estrogens 17β-estradiol (E2). In PCOS, estrogen synthesis is inhibited by the use of LET the 3β-HSD activity is higher compared to 17β HSD activity, and androgen production will be higher than estrogen production with a secondary change LH: FSH hormonal balance [21].

CC is an anti-estrogen it works by blocking estrogen receptors at the hypothalamus which is stimulated to release Follicle Stimulating Hormone (FSH) and luteinizing hormone [22].

Crocin administration caused significant decrease in serum free testosterone level, FSH level, LH level and significant increase in serum estradiol level in letrozole induced PCOS. Treatment with clomiphene citrate to PCO rats induced by letrozole resulted in the same changes caused by treatment with crocin.

Crocin may reduce hypophysis-hypothalamus sensitivity to testosterone and subsequently the negative feedback control on Luteinizing Hormone (LH) secretion [23]. Another cause for the therapeutic effect of crocin is demonstrated by Sajjadi and Bathaie, who observed that growth in basophil cells of the anterior pituitary which responsible for LH and FSH production [24]. Krismanovic et al., also showed that crocin is capable of releasing LH hormone by affecting hypothalamic axis and increasing the secretion rate of GnRH hormone [25]. Zohrabi et al., observed that crocin reduces plasma levels of total cholesterol therefore, the reduced estrogen level reported in the present study could be due to a reduction in cholesterol levels [26].

It was evident from results of the present work that LET administration to female rats caused significant increase in serum glucose level, serum insulin level and HOMA IR. The hyperglycemia and hyperinsulinemia secondary to insulin resistance in LET induced PCOS animals, the significant elevation of androgen levels in LET treated groups are partly responsible for insulin resistance [27]. Another mechanism for insulin resistance may be chronic low-grade inflammation which emerged as a key contributor to the pathogenesis of Polycystic Ovary Syndrome (PCOS). The pro-inflammatory cytokine tumor necrosis factor-α (TNF-α) is a known mediator of insulin resistance [28]. Another cause of insulin resistance in letrozol induced PCOS is the paracrine dysregulation of adipokine (for example, adiponectin) production by macrophage-secreted cytokines in PCOS favors development of insulin resistance [29], plasma leptin levels were higher in letrozole treated rats than in controls Hyperleptinemia is thought to indicate leptin resistance, which may be strongly connected to insulin resistance [30].

Results of the present work reveal that LET administration to rats resulted in significant increase in serum cholesterol level. This could explained by the aromatase inhibitors, LET may interfere with enzymatic pathways in the liver or with bile acid secretion [31]. The significantly decreased serum cholesterol as a result to crocin administration to LET induced polycystic ovary animal could explained by competitive inhibition of pancreatic lipase. Leading to the malabsorption of cholesterol [32]. Clomiphene citrate treatment in polycystic ovary female rats causes also improvement of serum cholesterol, which may be attributed to the estrogenic biological activities of clomiphene citrate [33].

Histopathological data reveals that LET induced PCOS. It showed numerous subcapsular cysts, with no granulosa layer. Corpora lutea were completely absent indicating anovulation. In addition, they were accompanied with atretic follicles containing fluid filled antrum and higher incidence of pyknotic granulosa cells. These signs were ameliorated after crocin treatment.

**Conclusion:**

These findings highlight the important role played by crocin in treatment of polycystic ovarian syndrome.

Further studies in different species, including man, are necessary to clarify the role of the crocin in pathophysiology in those people suffering from polycystic ovarian syndrome.

**Conflict of interest statement:** Authors declare no conflict of interest.
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Yasmin M. Eid, et al. 5243


تأثير الكروسين على تكييس المياض المستحثة بالليتروزول

الهدف من البحث: يهدف هذا البحث إلى دراسة تأثير الكروسين على تكييس المياض المستحثة بالليتروزول.

طرق البحث: تم إجراء هذا البحث على عدد (40) من إناث فئران التجربة قسموا إلى أربعة مجموعات كل مجموعة تحتوي على عدد (10) فئران.

• المجموعة الأولى (المجموعة الطبيعية الضابطة): هذه المجموعة تم حقنها بمحلول ملحي نصف مليتر عبر الفم البريتوتي ونصف مليتر محلول ملحي عن طريق الفم يوميا لمدة (2) أسابيع.
• المجموعة الثانية: مجموعة تم إعطاؤها عقار الليتروزول لمدة (21) يوم بجرعة (1 مليجرام لكل كيلوغرام) عن طريق أنبوب داخل المعدة.
• المجموعة الثالثة: مجموعة تم إعطاؤها عقار الليتروزول بنفس الجرعة كما في المجموعة الثانية ثم عقار الكروسين بجرعة (0.5 مليجرام لكل كيلوغرام) يوميا لمدة (3) أسابيع عبر الفم البريتوتي.
• المجموعة الرابعة: مجموعة تم إعطاؤها عقار الليتروزول بنفس الجرعة كما في المجموعة الثانية ثم عقار سترات الكوليفين بجرعة (1 مليجرام لكل كيلوغرام) يوميا لمدة (3) أسابيع عن طريق أنبوب داخل المعدة.

في نهاية التجربة، تم تخدير الحيوانات بواسطة إجراء مختفي، ثم ذبح الحيوانات عن طريق قطع الرأس وتم قياس الهرمونات الآتية: التستوستيرون، والهرمون المنبه ل hüيئيات المبيض والهرمون المنبه للإستراديل والأنستاينون. وحسب درجة معاينة الأنساينون وكذلك نسبة الكوليفين في الدم. وعلاقة على ذلك تم فحص عينة من أنسجة المبيض من كل مجموعة.

النتائج: قد تانت نتائج البحث على الآتي:

1- بالنسبة للمجموعة المعالجة بالليتروزول: إن الحقن بالليتروزول أدى إلى إرتفاع نزولا داليا إحصائيًا في كل من هرمون التستوستيرون، والهرمون المنبه ل hüيئيات المبيض والهرمون المنبه للإستراديل في مصل الدم بينما أدى إلى إنخفاض في الإستراديل بالمقارنة بالمجموعة الضابطة. وفي نفس الوقت كانت هناك عدد من النكتياس في أنسجة المبيض بالمقارنة مع المجموعة الضابطة.

2- بالنسبة للمجموعة المعالجة بالكروسين بعد الحقن بالليتروزول: فقد أظهرت النتائج أن حقن الكروسين أدى إلى إنخفاض نزولا داليا إحصائيًا في كل من هرمون التستوستيرون، والهرمون المنبه ل hüيئيات المبيض والهرمون المنبه للإستراديل والكوليفين في الدم بالمقارنة بالمجموعة المعالجة بالليتروزول. وفي نفس الوقت كان هناك ارتفاع داليا إحصائيًا في مستوى الإستراديل مع عدم وجود أي نكتياس مرضية في أنسجة المبيض بالمقارنة مع المجموعة المعالجة بالليتروزول.

3- بالنسبة للمجموعة المعالجة بمسترترات الكوليفين بعد الحقن بالليتروزول: أظهرت النتائج أن العلاج بمسترترات الكوليفين أدى إلى نتائج متطابقة مع المجموعة المعالجة بالكروسين.

الاستنتاج: تُستخلص من هذا البحث أن الكروسين يلعب دورًا فعالًا في المقاومة ضد تكييس المياض المستحثة بالليتروزول.