



# EFFECT OF CANAGLIFLOZIN ALONE AND IN COMBINATION WITH METFORMIN ON HORMONAL DERANGEMENTS AND ESTROUS CYCLE IN A POLYCYSTIC OVARY SYNDROME RAT MODEL

Akfish Zaheer<sup>1,2✉</sup>, Rabab Miraj<sup>1,3</sup>, Nada Azam<sup>1,4</sup>, Amir Hassan<sup>1</sup>, Sadia Chiragh<sup>1,5</sup>

## ABSTRACT

**OBJECTIVE:** To evaluate the effect of canagliflozin and metformin on the estrous cycle and hormonal derangement of letrozole induced polycystic ovarian syndrome (PCOS) in Sprague Dawly rats.

**METHODS:** Thirty six female adult non-pregnant Sprague Dawly rats were divided into six groups and all, except normal control (A), were treated with letrozole 1mg/kg daily for 21 days. Treatment was started to all rats except normal control (group-A) and disease control (group-B). Canagliflozin 10 mg/kg, metformin 100 mg/kg and their combinations in high and low doses were given daily to rats of groups-C (canagliflozin-alone), group-D (metformin-alone), group-E (high dose combination) and group-F (low dose combination) respectively. Vaginal smears were taken daily, to observe the estrous cycle, till the 48<sup>th</sup> day of study. Blood samples were collected on the 22<sup>nd</sup> and 48<sup>th</sup> day for hormonal assay.

**RESULTS:** Significant increase in serum estradiol levels (ng/dl) [group-A=46.53±9.40, group-B=18.75±2.48, group-C=23.00±10.15, group-D=37.41±8.54, group-E=57.58±12.88, group-F=41.93±10.32] and Follicle Stimulating Hormone levels (IU) [group-A=22.90±5.98, group-B=9.30±2.88, group-C=25.73±6.70, group-D=17.60±4.89, group-E=27.36±4.12, group-F=16.11±3.56] and decrease in testosterone levels (ng/dl) [group-A=4.48±1.21, group-B=10.12±2.90, group-C=2.85±1.00, group-D=4.41±2.30, group-E=2.50±1.28, group-F=4.15±0.79] and Luteinizing Hormone levels(ng/dl) [group-A=24.55±7.07, group-B=31.25±12.88, group-C=7.71±2.89, group-D=11.43±7.17, group-E=6.98±2.28, group-F=15.86±16.35]. Number of estrous cycles were significantly less in group-B (3-4 cycles/rat). Group-E (10-11 cycles/rat) resulted in significantly high estrous cycles number as compared to group-C (8-9 cycles/rat), group-D and group-F (7-8 cycles/rat).

**CONCLUSION:** Canagliflozin is effective alone and augment the effect of metformin for the regularization of estrous cycles and reversal of hormonal derangements in a rat model of PCOS.

**KEY WORDS:** Estradiol (MeSH); Estrous Cycle (MeSH); Follicle Stimulating Hormone (MeSH); Letrozole (MeSH); Luteinizing Hormone (MeSH); Testosterone (MeSH); Canagliflozin (MeSH); Metformin (MeSH).

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## INTRODUCTION

Polycystic ovary syndrome (PCOS) is the most common endocrinopathy in women of reproductive age. Two decades back, 10-15% of women were affected worldwide, now

the incidence of the disease has been increased to 21% either due to changes in eating habits or sedentary lifestyles.<sup>1</sup> Women with PCOS mostly present with menstrual irregularities, hirsutism due to hyperandrogenism and infertility.<sup>2</sup>

- 1: Department of Pharmacology, Post Graduate Medical Institute, Lahore, Pakistan
  - 2: Department of Pharmacology, University of Faisalabad, Pakistan
  - 3: Department of Pharmacology, Amna Anayat Medical College, Lahore, Pakistan
  - 4: Department of Pharmacology, Central Pak Medical College, Lahore, Pakistan
  - 5: Department of Pharmacology, Al Aleem Medical College, Lahore, Pakistan
- Email ✉: akfishzaheer80@gmail.com  
Contact #: +92-304-1792797  
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In PCOS, increased production of testosterone and its precursor's androgens results from persistently raised GnRH pulses from anterior pituitary that lead to an increase in the level of luteinizing hormone (LH) and a decrease in follicle stimulating hormone (FSH). The increased level of LH becomes resistant to the negative feedback of estrogen and stimulates theca cell of the ovary to produce more androgens, which causes abnormal follicular development.<sup>3</sup>

Obesity is one of the leading causes of PCOS, hyperinsulinemia and increase insulin resistance. Insulin resistance and hyperinsulinemia can either directly lead to increase androgen production or indirectly by increasing in GnRH pulses from pituitary and increase LH production. Insulin also decreases the sex hormone-binding globulin (SHBG) production from the liver thus increasing the free testosterone levels in the body.<sup>4</sup>

PCOS is a complex disorder and the choice of treatment is determined mainly by the symptoms and clinical presentation of patients.<sup>5</sup>

Metformin is an oral antidiabetic drug that reduces insulin resistance and has a beneficial effect on many features of polycystic ovaries (hyperinsulinemia, hyperandrogenism and obesity) by

**TABLE I: EFFECT OF CANAGLIFLOZIN AND METFORMIN ON NUMBER OF ESTROUS CYCLES OF RATS IN EACH GROUP OF LETROZOLE INDUCED PCOS RAT MODEL**

Groups (n=6 in each group)	Number of Estrous Cycle (Mean±SD)	ANOVA Sig.#
A (Normal control)	11.17±0.75*	<0.001
B (Disease control)	4.66±0.52	
C (Canagliflozin)	8.17±1.16*	
D (Metformin)	7.61±0.85*	
E (High Dose Combination)	10.13±1.73*§	
F (Low Dose Combination)	7.50±1.04*	

#One way ANOVA followed by post hoc Tukey's test; \* = 0.05 (as compared to disease control group); §: group-E has a significant increase in the number of estrous cycles as compared to Group C & D

increasing insulin sensitivity which is due to inhibition of gluconeogenesis and increasing uptake of glucose in the liver, muscles, adipocytes and ovaries. Perhaps metformin proved to be the safest drug among all other treatments but on the other hand, a metanalysis conducted by Tang T et al, (2010) on use of insulin-sensitizing drugs (metformin, pioglitazone, rosiglitazone) for women with PCOS, oligomenorrhea and infertility, showed that there is limited benefit of metformin use regarding fertility in women and there was no evidence that metformin increases live birth rate.<sup>6</sup> Due to the treatment failure in PCOS after using multiple therapies, there is still a need to probe for new agents that can be used either alone or as an adjunct with previously available therapies.

Canagliflozin, an oral hypoglycemic agent, approved by Food and Drug Administration on March 29, 2013. It is a sodium glucose co-transporter (SGLT2) inhibitor. SGLT2 is expressed in the proximal tubules of the kidney and causes the reabsorption of glucose from the tubular lumen. By inhibiting SGLT2 in the kidney, a large amount of the filtered glucose is excreted.<sup>7</sup> It also activates adenosine monophosphate kinase (AMPK) by inhibiting mitochondrial function and increasing adenosine monophosphate (AMP) and reduces fatty acid synthesis.<sup>8</sup>

Besides these molecular mechanisms, canagliflozin reduces the insulin resistance in diabetic obese rodents<sup>9</sup> and reduces body weight in non-diabetic subjects<sup>10</sup> These effects are similar to that of metformin and can be used in PCOS patients with the advantage of renoprotective and hepatoprotective effects of canagliflozin.<sup>11</sup>

Therefore, the current study was planned to determine, whether canagliflozin is effective in different hormonal and menstrual derangements appearing as a consequence of PCOS and whether there is an additive effect of canagliflozin with metformin when given in combination.

## METHODS

The study was conducted from March 2018 to March 2019 at Postgraduate Medical Institute, Lahore after approval from the institutional ethical committee. This experimental study was conducted on female Sprague Dawley rats. Animals were bred in the animal house of Postgraduate Medical Institute, Lahore. Thirty Six (36) Sprague Dawley adult non-pregnant female rats of 7-8 weeks age and 90-120 g weight were included in the study after 2 weeks of acclimatization. Rats showing any signs of disease (skin infections, vaginal or urinary tract infections) were to be excluded, which were none in this study. After selection according to these criteria they were divided into six groups randomly by the lottery method.

For induction of PCOS, all rats (group B, C, D, E, F) were treated with letrozole (Famera, 2.5 mg, Novartis pharmaceutical, Pakistan) 1mg/kg/day per oral for 21 days<sup>12</sup> except those in normal control group A as they were given an equal amount of distilled water.

On 22<sup>nd</sup> day treatment with daily administration of drugs was started to all rats except normal control group A and disease control group B which were given an equal amount of distilled water. Canagliflozin (Invokana, 100 mg from Janssen Pharmaceutical, UAE) 10 mg/kg

(group C), metformin (Glucophage, 500 mg from Merk Pharmaceutical, Pakistan) 100 mg/kg (group D), and their high dose combinations i.e. canagliflozin 10 mg/kg/day with metformin 100 mg/kg/day (group E), low dose combination i.e. canagliflozin 05 mg/kg/day with metformin 50 mg/kg/day.<sup>13</sup>

Stages of estrous cycle observed during vaginal cytology were used to calculate the number of estrous cycles and the response of drugs. Vaginal smear was taken daily from 0- 48 days at same time in the morning. Samples were collected by vaginal lavage. This method yields higher cellularity as compared to swab methods. Slides were prepared and stained with hematoxylin (Scharlau, Hematoxylin according to Harris) and eosin (Victor lines, Pakistan) to see the estrous cycle phase<sup>14</sup> and number of ester cycles were recorded by using digital microscope Camera control unit digital sight DS-L3 (Eclipeci by Nikon).

On 22<sup>nd</sup> and 48<sup>th</sup> day of study, 2ml and 5ml of blood sample was collected by tail vein and intracardiac puncture respectively,<sup>15</sup> under light anesthesia using 5ml syringe. Blood was allowed to clot and then centrifuged at 2500 rpm for 10 minutes. Sera were separated and stored in the freezer at -20°C for further analysis.

Hormonal assay (testosterone, estradiol, LH, FSH) measured by using rat specific sandwich enzyme linked immunosorbent assay (ELISA) detection method (ELISA kits by International immune-diagnostic USA) that quantitatively measured hormonal concentrations. The data collected was analyzed by using the Statistical Package of Social Sciences (SPSS 22). Data were checked for normality and homogeneity of variance by Levene's test. As data was normally distributed, it was presented as mean±standard deviation (SD). Paired t-test was applied to check the significance of results within each group for where two readings are compared. ANOVA was used for multiple group comparisons, to test the significance of the result of quantitative data between groups followed by post hoc Tukey's test to check mean difference between each group. A p-value of ≤ 0.05 was considered statistically significant.

**TABLE II: EFFECT OF CANAGLIFLOZIN AND METFORMIN ON SERUM TESTOSTERONE AND SERUM ESTRADIOL LEVELS OF LETROZOLE INDUCED PCOS RAT MODEL**

Groups (n=6 in each group)	Testosterone levels (ng/dl) [Mean±SD]			Estradiol levels (ng/dl) [Mean±SD]		
	Day 22	Day 48	P value Paired t-test	Day 22	Day 48	P value Paired t-test
Normal control (A)	5.81±1.44	4.48±1.21 <sup>a</sup>	0.082	44.86±7.89	46.53±9.40 <sup>a</sup>	0.80
Disease control (B)	15.71±6.54*	10.12±2.90 <sup>a</sup>	0.110	17.11±8.38*	18.75±2.48	0.712
Canagliflozin(C)	14.50±2.92*	2.85±1.00 <sup>a</sup>	<0.001	15.11±4.72*	23.00±10.15	0.100
Metformin (D)	12.95±2.75*	4.41±2.30 <sup>a</sup>	0.002	18.40±6.07*	37.41±8.54 <sup>b</sup>	0.100
High dose combination (E)	12.93±1.79*	2.50±1.28 <sup>a</sup>	<0.001	11.63±5.16*	57.58±12.88 <sup>a</sup>	0.001
Low dose combination (F)	12.98±1.98*	4.15±0.79 <sup>a</sup>	<0.001	14.26±7.13*	41.93±10.32 <sup>a</sup>	0.001
P value ( One Way ANOVA ) <sup>#</sup>	0.001	<0.001		<0.001	<0.001	

<sup>#</sup>Multiple comparisons by post hoc analysis with significance level =0.05. \*significant as compared to Group A, B & E within the same duration of treatment.

## RESULTS

### Effect of treatment on estrous cycle:

Number of estrous cycles were significantly less in the disease control group (3 to 4 cycle per rat) as compared to all other groups, while high dose combination (10 to 11 cycles per rat) resulted in significantly higher number of estrous cycles as compared to canagliflozin and metformin alone groups (Table I).

### Effect of Treatment on hormonal levels:

On day 22<sup>nd</sup>, all groups receiving letrozole recorded a higher serum testosterone level and lower estradiol level (Table II) and higher LH and lower FSH level (Table III) as compared to the normal control group.

At 48<sup>th</sup> day of study, serum testosterone level decreased and estradiol level increased in all treatment groups as compared to the disease control group (Table II). Similarly serum LH level decreased and FSH level increased as compared to day 22<sup>nd</sup> and disease control.

## DISCUSSION

PCOS is a complex disease affecting multiple systems of the body. There are multiple phenotypic parameters of PCOS including neuroendocrine, ovarian and metabolic features.<sup>16</sup> Objective of this study was to investigate endocrine and cyclical changes of rat model of PCOS. It was observed that canagliflozin not only improve the endocrine

abnormalities but also restore the estrous cycle and enhance the effect of metformin. The development of rodent models to study mechanisms of PCOS is proven challenging due to the heterogeneity of the disease. Similarly, multiple drugs have been used for PCOS induction in rats and other murine models, including pre and postnatal dihydrotestosterone, estradiol valerate, progesterone receptor antagonist and letrozole. All these models recapitulate different aspects of PCOS phenotype. In this study, we used letrozole to induce polycystic ovarian disease in rats because it exhibits raised LH and androgen levels similar to humans and therefore appropriate for investigating and treating different ailments of human PCOS.<sup>17</sup>

Induction of PCOS was confirmed by dysfunctional estrous cycles in the rats. Vaginal smears revealed an acyclic estrous cycle i.e. persistence of the diestrous phase in rats after induction of PCOS. Similar results were found with letrozole in a previous study in which after being acyclic, rats were evaluated for fertility but none of the letrozole treated rats gave birth revealing infertility in PCOS model.<sup>18</sup> It was also observed that during the induction of PCOS, vaginal secretions became thick mucus-like difficult to pipette while taking the smear. It represents the diestrous phase. Microscopically, these thick secretions impeded with darkly basophilic cells, clumped together in groups. A study conducted to elaborate the histological guide to staging of

female rat reproductive cycles also reveal little thick mucus with leukocytes, nucleated basophilic cells.<sup>19</sup>

An increase in androgen levels and LH is found to be the most consistent feature of letrozole induced PCOS.<sup>18</sup> The same changes were observed in the current study when hormonal levels were measured on the 22<sup>nd</sup> day of study. Testosterone and LH levels were raised significantly in all groups as compared to the normal control group. An increase in LH level can be due to inhibition of negative feedback resulting from low estradiol levels after aromatase inhibition by letrozole.<sup>20</sup> The decrease in FSH level is pronounced in all letrozole treated groups, as has also been observed in human PCOS.<sup>21</sup> Contrary to this, it has been observed in a previous study that level of FSH increased after letrozole treatment in rats,<sup>12</sup> while variable results were observed with different inducing agents because animals are polyovulatory. Despite the hypothalamic pituitary ovarian axis similarities FSH dependent follicle selection process altered with different inducing agents.<sup>22</sup>

The objective of the current study was to observe the effect of canagliflozin and metformin on PCOS derangements. Canagliflozin was used in this study because its mechanism of action is similar to metformin, which is approved for PCOS treatment. In this study, the effect of canagliflozin on estrous cycle and hormonal derangements was compared with metformin. Both drugs were also used in low dose and high

**TABLE III: EFFECT OF CANAGLIFLOZIN AND METFORMIN ON SERUM LUTEINIZING HORMONE AND SERUM FOLLICLE STIMULATING HORMONE LEVELS OF LETROZOLE INDUCED PCOS RAT MODEL**

Groups (n=6 in each group)	Luteinizing Hormone Levels (ng/dl) [Mean±SD]			Follicle Stimulating Hormone Levels (IU) [Mean±SD]		
	Day 22	Day 48	P value (Paired t-test)	Day 22	Day 48	P value (Paired t-test)
Normal control (A)	22.45±3.98	24.55±7.07 <sup>b</sup>	0.60	17.75±6.54	22.90±5.98 <sup>a</sup>	0.14
Disease control (B)	48.8±8.79*	31.25±12.88	<b>0.01</b>	2.50±0.53*	9.30±2.88	<b>0.003</b>
Canagliflozin(C)	51.85±15.26*	7.71±2.89 <sup>a</sup>	<b>0.002</b>	2.15±1.20*	25.73±6.70 <sup>a</sup>	<b>&lt;0.001</b>
Metformin (D)	47.30±18.19*	11.43±7.17 <sup>a</sup>	<b>0.002</b>	2.45±2.60*	17.60±4.89 <sup>b</sup>	<b>0.001</b>
High dose combination (E)	44.98±14.32*	6.98±2.28 <sup>a</sup>	<b>0.002</b>	6.50±4.66*	27.36±4.12 <sup>a</sup>	<b>&lt;0.001</b>
Low dose combination (F)	46.16±3.82*	15.86±16.35	<b>0.003</b>	3.98±2.40*	16.11±3.56 <sup>c</sup>	<b>0.002</b>
P value ( One Way ANOVA ) <sup>#</sup>	0.003	<0.001		<0.001	<0.001	

<sup>#</sup>Multiple comparisons by post hoc analysis with significance level =0.05. \*Significant as compared to Group A, B, E and Group C alone within the same duration of treatment.

dose combination to observe their synergistic effect on PCOS.

The estrous cycle of rats became acyclic after letrozole treatment but when treated with canagliflozin and metformin, the stages of the estrous cycle gradually began to regularize. At the end of study, numbers of estrous cycles were highest in rats receiving the high dose of both drugs (group E). The possible explanation behind this beneficial effect is that ovaries resume their function after correction of hormonal derangements. Role of metformin for correction of menstrual irregularities in human PCOS patients has already been established.<sup>23</sup>

Canagliflozin is reported to improve the  $\beta$ -cell function by non-insulin dependent mechanism;<sup>9</sup> it decreases the insulin demand and hyperinsulinemia, which is a key factor to increase the androgen levels in PCOS.<sup>24</sup> In the current study, there was a significant decrease in testosterone level in all treatment groups. Rats that received combined treatment with both drugs did not show statistically better results but numerically there is more decrease in testosterone level indicating that insulin lowering has a normalizing effect on androgen abrasions of PCOS.

The increase in estradiol levels was observed in all treatment groups as compared to disease control group. Both combination groups revealed a highly significant increase and the high dose combination group was found more effective as compared to metformin. In a previous study, it has

been observed that metformin increases the estradiol levels<sup>25</sup> but no study is available with canagliflozin.

The decreases in LH levels and an increase in FSH were observed after treatment with both drugs. The effect of canagliflozin on the LH decrease was significantly pronounced as compared to metformin. Metformin effect on lowering LH levels is already established in the study on women that showed, metformin induces a prompt decrease in LH-stimulated testosterone secretion after only several days of use. This action precedes the medication's effects on insulin sensitivity or weight loss.<sup>26</sup>

In this study effect of canagliflozin and metformin alone and combination were studied on hormonal and ovarian derangements of PCOS but no parameter was studied to assess the possible mechanism, due to lack of facilities. Other studies reveal some of the possible mechanisms for metformin, while no study has been conducted with canagliflozin on PCOS. Metformin reduces the level of insulin-like growth factor and increases the level of insulin-like growth factor binding protein and may modify the hyperandrogenic follicular development.<sup>27</sup> There is a complex relationship between insulin resistance, hyperandrogenism and PCOS. Insulin resistance can directly increase the risk of PCOS in obese patients due to an increase in androgen secretion from ovaries and the adrenal cortex.<sup>28</sup>

To the best of our knowledge, this is first report showing beneficial effect of

canagliflozin and its additive effect with metformin for correction of hormonal derangements and restoration of estrous cycle in a PCOS model. Study of ovarian histology is recommended for future study.

## CONCLUSION

Based on the results of this study, it is concluded that the effect of canagliflozin on rectification of hormonal derangement is the same as that of metformin, while a high dose combination of metformin and canagliflozin has better outcome on hormonal derangements of PCOS. High dose combination of both drugs also has a more rapid effect on normalizing irregular estrous cycle as compared to metformin, so it can be tested for PCOS patients with menstrual irregularities.

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### AUTHOR'S CONTRIBUTION

Following authors have made substantial contributions to the manuscript as under:

**AZ:** Study design, acquisition of data, drafting the manuscript, approval of final version to be published

**RM & AH:** Conception, analysis and interpretation of data, drafting the manuscript, critical review, approval of final version to be published

**NA:** Acquisition of data, drafting the manuscript, approval of final version to be published

**SC:** Conception and study design, acquisition of data, critical review, approval of final version to be published

*Authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.*

### CONFLICT OF INTEREST

Authors declared no conflict of interest

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### DATA SHARING STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.



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KMUJ web address: [www.kmuj.kmu.edu.pk](http://www.kmuj.kmu.edu.pk)  
Email address: [kmuj@kmu.edu.pk](mailto:kmuj@kmu.edu.pk)