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Comparative Evaluation of Metformin and Letrozole in a Rat Model of Polycystic Ovary Syndrome

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Abstract

Background: A number of rat models have been proposed to investigate the heterogeneity of polycystic ovary syndrome (PCOS), hyper-androgenized routine being the most popular one. Although, effects of androgen excess have been widely investigated, the ameliorating potential of the ovulogens on different aspects of the treatment on a single model is yet to be explored. *Aims:* This particular experimental study ends to investigate the effects of metformin and letrozole on experimentally induced rodent model of PCOS. *Methods and materials:* After neonatal androgenisation (1.5 mg/animal), rats were given metformin (200 mg/kg body weight for 4 weeks) or letrozole (0.5 mg /kg/day for 15 days) from ~53 day of age. Ovarian histology, response to exogenous gonadotropins, hormonal profile, glucose tolerance, and dyslipidaemic features with RT-PCR of specific steroidogenic and hyperinsulinemic genes were carried out to compare and evaluate the therapeutic response of the drugs. *Results and conclusion:* Androgenized rats had polycystic ovaries characterized by increased theca interna and fewer or no functional corpora lutea. Metformin improved ovarian responses and was superior in attenuating testosterone status, hyperinsulinemia, dyslipidaemia along with a significant improvement in expression of the genes assessed. In conclusion, metformin reverted the hyperinsulinemia-associated hyperandrogenemia to reinstate ovulatory function with parallel improvements in the metabolic corners of the syndrome; however, letrozole appears to have no beneficial effect with reference to restoration of ovulation or amelioration of polycystic state of the ovary.

Keywords: polycystic ovary syndrome; ovulation induction; hyperinsulinemia; hyperandrogenemia; animal model.

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Introduction

Polycystic ovary syndrome (PCOS) is a major form of dysovulatory infertility that affects 5-10% of women of reproductive age (Sattar et al., 2009). The syndrome is a heterogeneous entity comprising broad spectra of ovarian disorders and metabolic syndrome (MS) including anovulation, hyperandrogenism with frequent association of insulin resistance (IR), obesity and dyslipidaemia (Escobar-Morreale et al., 2005) to name a few. Hyperandrogenism is believed to be at the heart of the syndrome since excess androgens from multiple small follicles results anovulation and IR (Homburg et al., 2009).Therefore, medical therapy is often aimed at lowering the hyperandrogenic and infertile status.

PCOS women, in general have difficulty becoming pregnantdue to anovulation. Induction of ovulation is the first-line of treatment for these women and is aimed at the introduction of an endocrine milieu that promotes growth and ovulation of a single dominant follicle with consequent singleton pregnancy.

Clomiphene citrate (CC) has been the gold standard treatment option for induction of ovulation in women with PCOS for many decades (Neveu et al., 2007). Discrepancy between ovulation and pregnancy rates with CC has been attributed to its anti-estrogenic action and estrogen receptor depletion at the endometrium level. In this context aromatase inhibitors (AI) have emerged as an alternative treatment to clomiphene. Letrozole, the most prevalently used anti aromatase for this indication, has been shown to be effective. The drug is non-steroidal, highly potent, well tolerated competitive inhibitor of aromatase enzyme system (Casper et al., 2006).

As hyperinsulinemia plays a significant role in anovulation in PCOS, clinical improvements can be anticipated following the reduction of serum insulin concentrations (Tang et al., 2012). Metformin acts by decreasing gluconeogenesis as well as increasing peripheral utilization of glucose in the presence of endogeneous insulin. A number of studies (Genazzani et al., 2007; Elter et al., 2005; Pasquali et al., 2006) have shown significant improvements in insulin sensitivity and hyperinsulinemia in non-obese PCOS women after metformin administration. Furthermore, these changes were associated with a reduction in serum androgen and a concomitant increase in serum sex-hormone-binding globulin concentrations. Recent report also document metformin usage for ovulation induction in PCOS women promote ovulation and pregnancy rate which is comparable to that of clomiphene (Xiao et al., 2012).

A number of rat models have been proposed to investigate the heterogeneity of the syndrome, hyper-androgenized routine being the most popular one (Amalfi et al., 2012). To our knowledge, no studies have been performed to compare the effects of metformin and letrozole on testosterone-induced PCOS in an animal model. Additionally, there has been no research to determine the effect of letrozole on the metabolic corner of PCOS in a rat model. Thus, the aim of the present study was to evaluate the potential of metformin and letrozole in improving both endocrine as well as metabolic milieu in anandrogen induced rodent PCOS model.

Materials and Methods

Animals: Maintenance and Care

Adult healthy female Sprague Dawley rats (*Rattus norvigecus*, 180-220g) were housed in controlled conditions of room temperature (23±2°C), humidity (50±5%), and a 12 h-12 h light-dark cycle. The animals were kept in sanitized polypropylene cages and fed with standard rat pellet diet and drinking water *ad libitum*. All experiments were performed as per the national guidelines formulated by the Committee for the Purpose of Control and Supervision of Experiments on Animals, Ministry of Culture, India with approval from the Animal Ethics Committee of Institute of Reproductive Medicine.

Chemicals

Unless otherwise stated, chemicals used for the preparation of reagents were of analytic grade and purchased from Sigma Aldrich (Sigma Aldrich, St. Louis, M.O., USA). HEPES, Dulbecco's Modified Eagle's Medium and antibiotics were purchased from Invitrogen (Life Technologies, Grand Island, NY, USA). Letrozole was purchased from (Femara, Novartis, US) Radioimmunoassay (RIA) kits for total testosterone (DSL-4000 Active® Testosterone Coated-Tube RIA Kit) and estradiol-17β (DSL-4400 17β-Estradiol RIA Kit) were obtained from Diagnostic Systems Laboratories (DSL, Webster, Texas, USA). Follicle stimulating hormone (RK-550) and luteinizing hormone (RK-552) kits were procured from IZOTOP (Institute of Isotopes Ltd, Budapest, Hungary), while RIA kit for rat insulin was procured from BIOTRAK (AP Biotech, Amersham, UK).

Induction of PCO by neonatal androgenisation and subsequent treatment design

A single subcutaneous (SC) injection of testosterone propionate (TP) (Nutritional Biochemical Corporation, Cleveland, USA) dissolved in olive oil was administered at a dose of 1.5 mg/animal on day 5 to induce PCO Ota et al, 1986. Control animals were injected with olive oil alone. At 53±1 day of age, the TP-treated rats were divided into 3 groups each comprising 10 rats. Group 1 (PCO) was given no medication. The other two groups were orally treated with 200 mg/kg body weight of oral metformin for 4 weeks or 0.5 mg/kg/day of oral letrozole for 15 days. A separate group (Group 4) consisting of 10 rats was maintained as control. The medications were given via orogastric tubes.

Evaluation of growth rate

The litters from different groups were weighed every seventh day since birth, continuing throughout the course of experiments.

Histoarchitecture of the ovary

After completion of the treatment regimen a section of animals from each group were killed under ether anaesthesia. The ovaries were dissected out and fixed by immersion in 3.7% buffered formaldehyde in 0.1M phosphate buffer, pH 7.2. After fixation, the tissues were processed, embedded in paraffin and serially sectioned at 5µm with a Leica RM2155 rotator microtome. Sections from the ovaries were used for routine haematoxylin-eosin staining and photographed under a Biomed light microscope enabled with a Leica RM2235 digital camera.

Ovarian response to exogenous gonadotropins

At 10-12 weeks of age, rats (n = 5) from control and experimental groups were induced to super-ovulate by s.c. injection of 40 IU PMSG at '0' h and 25 IU hCG at '52' h after the completion of respective treatments. Oviducts were flushed with normal saline after '20' h when the presence of oocytes and their numbers were recorded. Follicular response was assessed both in respects of the number of rats (%) responded and the number of oocytes retrieved.

Hormonal analyses

At ~ 12 weeks of age blood samples were collected by cardiac puncture under ether terminal anaesthesia in vaccutainer with or without anticoagulant. Hormones were measured by RIA using the protocol described in the respective instruction manuals. The intra- and inter-assay coefficients of variation were within the acceptable limits (<10%). Hormone levels were expressed as ng/ml or pg/ml, as applicable.

Oral Glucose Tolerance Test (OGTT)

At 10-12 weeks of age, insulin sensitivity was examined by performing OGTT. OGTT was performed according to the standard protocol Du Vigneaud et al, 1925. Briefly, the rats (n = 7 from each group) were fasted overnight and next day bled at '0' hr to estimate the fasting glucose level followed by an oral glucose challenge (2 g/kg body weight). Animals from the either group had ~0.02 mL of blood drawn (by tail nick) at 30, 60, 90, 120 and 180 minutes (min) after glucose administration and blood glucose levels were measured using a commercial glucometer (Acku-check, sensor comfort, Roche Diagnostics, Germany).

Biochemical Analysis of Lipid Profile

At 10-12 weeks of age blood was obtained by cardiac puncture after an overnight fasting to assess the lipid profile. Serum total cholesterol, triglyceride and lipoprotein levels were measured by automatic enzymatic methods in all groups. HDL was determined after precipitation of the lipoproteins by dextran sulphate Ruel, et al. 2003. The serum levels of total cholesterol, HDL and triglyceride (TG) levels were measured using reagents purchased from Randox Laboratories Ltd, London, UK by Daytona. Lipoprotein levels were expressed in mg/dl. LDL cholesterol was calculated using the Friedewald formula: LDL-C = cholesterol-HDL-C-(TG/5). Serum samples were stored at -20°C until assayed.

Semiquantitative Polymerase Chain Reaction

Total RNA was extracted from five ovaries for each group using Trizol reagent (Invitrogen, California, USA) and was reverse transcribed into complementary DNA (C_{DNA}) in a total volume of 20µL reaction mixture containing 1µl oligo $(dT)_{12-18}$ (500 ng/µl) (Applied Biosystems). Reverse transcriptase was performed with 200U/µl superscript II reverse transcriptase (Invitrogen) at 42°C for 60 min. C_{DNA} amplification was carried out in an automated thermal cycler (iCycler; Bio-Rad) using the appropriate conditions for StAR (forward 5′–ggcatccttagcaaccaaga-3′; reverse 5′–tctccttgacattggggttc-3′; Tm 56°C), insulin-like growth factor-1 (IGF-1)(forward 5′– tcgtcttcacatctcttctacctg-3′; reverse 5′–acatctccagcctcctcaga-3′; Tm 55°C) and Cyp19 (forward 5′– attcttgtggatggggattg -3′; reverse 5′–acaggctcgggttgttgtta -3′; Tm 55°C) primers. β-actin was used as positive control. An aliquot of each C_{DNA} sample was amplified by PCR with β-actin gene-specific primers (forward 5′–catgtcagtggacagatgct -3′; reverse 5′–taacttcagacatcatttccgg -3′; Tm 55°C). Upon completion of the PCR reaction, products (10µl) were subjected to electrophoresis on 1.5% agarose gel and in Tris acetate- EDTA buffer and stained with ethidium bromide (0.5µg/ml). The relative amount of the transcript was calculated by dividing the intensity of the band by the intensity of β-actin.

Statistical Analysis

The data were expressed as mean±standard error of the mean (SEM), where 'n' refers to the number of animals or determinations. The results were analyzed by paired two-tailed t-test using the GraphPad Prism 3.0 software (Graph Pad Software, Inc, San Diego, CA). Densitometric quantification of

signals by RT-PCR was done by Image-J software. All values were normalized with internal control, i.e. β-actin, and represented in the form of bar diagram. $p<0.05$ was considered significantly different.

Results

Reduction of body weight after metformin regimen

Body weights (BWs) of the litters at birth and subsequent growth rates have been presented in Figure 1. At birth the BWs of the pups were statistically indifferent between the groups, although during the course of time, PCO pups showed relatively higher rate of weight gain at different points of time from 7th week onwards (Fig. 1A). The metformin treated group, by contrast, showed reduced growth rate ($p<0.04$) as compared to that of the TP-intervened study group in the later stages of treatment.

Ovarian response to gonadotropins

Ovarian response to gonadotropins was evaluated with respect to the number of animals (%) exhibiting the presence of ovulated eggs in the oviduct, as well as the number of ovulated eggs per stimulated rats. All control rats (100%) responded to stimulation at a rate of 34.42±3.52 ovulated eggs per rat. By contrast, PCO group of rats responded very poorly ($p < 0.0002$) to the stimulation. PCO group, 20% of the rats did not respond to stimulation, while the rest responded with 9.33±1.85 of ovulated eggs per rat (Fig. 1B). All rats responded well with metformin treatment significantly improved ($p<0.002$) the ovarian response (26.2±3.05) in comparison to 60% of the rats that received letrozole treatment displaying marginal beneficial effect ($p < 0.05$) when compared to the androgenised set (16.6±2.51).

Glucose tolerance

Figure 1C represents the outcome of OGTT. PCO group had 92.46±2.31 mg/dL of basal blood glucose, which was significantly greater ($p<0.03$) than that of control (83±1.86). As compared with control, the glucose levels at all-time points were higher in the androgenised group. However, in both control and PCO groups plasma glucose levels reached their

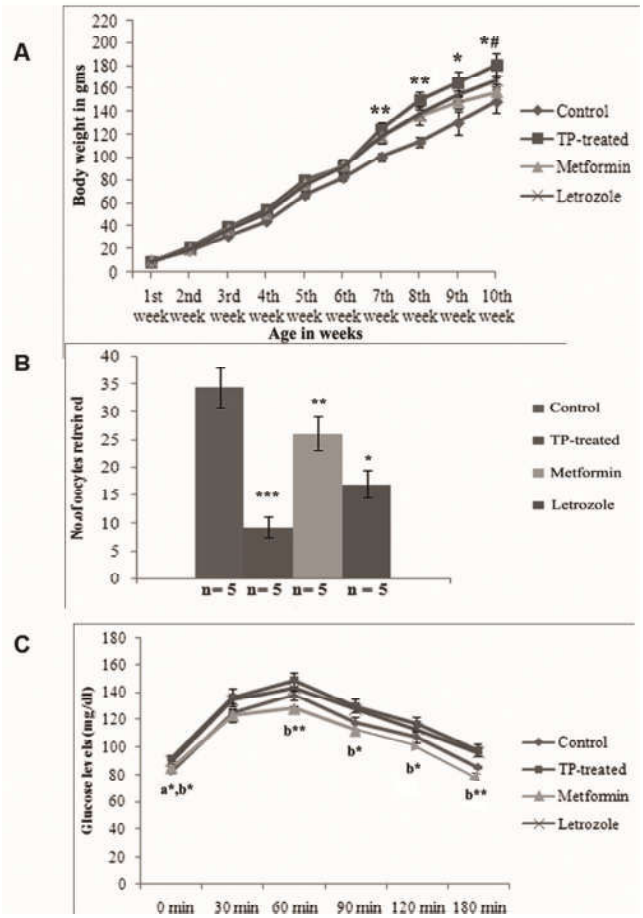

Fig. 1: Comparative evaluation of growth rate, ovarian response to gonadotropins and glucose level in different groups

A. Postnatal growth rates in the control, TP-treated and drug treatment groups. As compared to controls the rate of growth in the androgenised rats are comparatively higher ($p < 0.002$; $p<0.0006$; $p<0.03$) during the 7-10 postnatal weeks. However, metformin management showed reduced growth rate ($p<0.04$), when compared to that of control. Each point represents the mean±SEM values of 8-10 different animals.*$p<0.03$: PCO vs Control; **$p<0.002$: PCO vs Control; ***$p<0.0006$: PCO vs Control, #$p<0.04$# PCO vs Metformin.

B. Ovarian response to standard PMSG/hCG simulation is presented as number of ovulated oocytes (mean ±SEM)/rat. The androgenised group shows significantly ($p<0.0002$) lower number of ovulated oocytes/ rat compared to control; however, rats treated with metformin ($p<0.002$) or letrozole ($p<0.05$) significantly improved the ovarian response. Each bar represents the mean±SEM of five individual observations of the respective group.***$p<0.0002$: PCO vs control; **$p<0.002$: PCO vs metformin; ***$p<0.05$: PCO vs letrozole.

C. Plasma glucose levels after glucose challenge at 2 g/kg body weight after different treatment regimens with comparison to control set. The androgenised group exhibit poor glucose tolerance. Metformin could only recover the poor glucose tolerance of the androgenised set. Each datum point represents the mean±SEM of 5-7 observations in the respective group. */** mark the level of significance, while the superscripts designate the groups compared. [a]control vs TP; [b]TPvs metformin; *$p<0.03$; **$p<0.006$; ***$p<0.0002$.

peak at 60 min and came down at a comparatively lower rate to maintain significantly higher ($p<0.03$) glucose levels over their basal values until 180 min. However, significant improvement were observed in fasting blood glucose level on completion of metformin treatment ($p<0.03$) which continued during the course of glucose tolerance over other groups. The metformin-treated group showed the peak sugar level at 60 min that decreased to the basal level by 120 min, while letrozole-treated group maintained high levels at least until 180 min.

Ovarian architecture

Light microscopic analysis of the control ovary showed follicles and corpus lutea in different stages of development and regression (Fig. 2A). Polyhedral theca and cuboidal granulosa cells were observed in control. By contrast, TP rat ovaries showed increased number of follicular cysts characterized by large fluid filled cavity with disorganized granulosa cell layers and thickened theca interna with the presence of primary and early secondary follicles (Fig. 2B). Treatment with metformin (Fig. 2C) made considerable recovery of ovarian architecture as marked by the presence of corpus luteum as a sign of ovulation. Letrozole (Fig. 2D) led to arrest of follicle growth with comparable number of pre-antral follicles and cysts to that of the hyper androgenised set.

Serum hormone analysis and correction of dyslipidemia

The serum gonadotropin profile of the PCO group demonstrated a significant decrease in FSH concentration (PCO vs. control $p<0.04$) with an increase ($p<0.0001$) in testosterone level. However, LH and estradiol level remained statistically comparable to controls with an increase ($p<0.04$) in insulin (Table 1). Treatment with metformin significantly ($p<0.03$) reduced the serum testosterone levels of the PCOS rats; however, letrozole had no effect on the same. A significant decrease in insulin ($p<0.03$) and estradiol ($p<0.01$) was observed after treatment with metformin and letrozole, respectively, as compared to the androgenised set.

Table 2 summarizes the lipid profiles in the control and study groups. PCO rats had higher levels of triglyceride ($p<0.003$) compared with controls. Total cholesterol was comparable in PCO group. As compared with the control, PCO set exhibited comparatively lower level of HDL ($p<0.02$) and higher level of LDL, however the difference in the latter was not significant enough. Letrozole treatment had no influence on the lipid profile of the TP-treated rats. Metformin treatment, however, changed the lipid profile significantly by reduction in triglyceride ($p<0.02$), with an increment in HDL cholesterol ($p<0.02$).

Expression of StAR, IGF-1and Cyp19

The PCO group demonstrated increased ovarian expression of StAR, Cyp19 ($p<0.001$) and IGF-1 ($p<0.001$)(Fig. 3) when compared to control. However, a significant decrease in StAR expression ($p<0.01$) with a simultaneous diminution ($p<0.02$) in Cyp 19 profile was observed in the metformin-treated group. Letrozole treatment

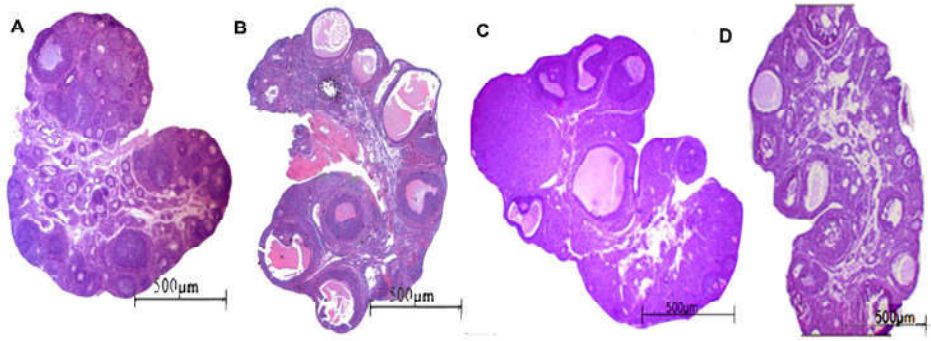

Fig. 2: Ovarian histology

Histological sections through ovaries of ~70-day old control (A), TP- treated rats (B), metformin rats (C) and letrozole group (D). The Control ovary exhibits the presence of small and medium-sized antral follicles and functional corpus luteum (not shown in the picture). Androgenised group exhibited polycystic changes characterized by accumulation of pre-antral follicles with 2-3 cysts mostly distributed towards the cortex. Letrozole treatment leads to withdrawal of maturation arrest of follicles with a reduction in number of cysts. However, metformin conduct also made sizeable changes with occasional presence of graffian follicle and a reduction in ovarian volume almost comparable to that of the control. Each image is one representative of five replicates of the concerned subsets; bar = 500 µm.

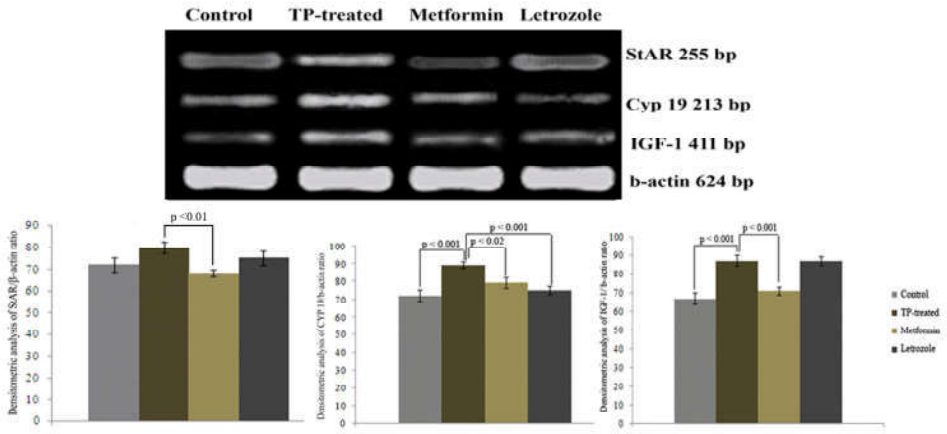

Fig. 3: Ovarian expression of gens following treatment regimen/s

A. RT-PCR analysis of ovarian expression of StAR, Cyp 19 and IGF-1 of hyperhomocysteinemic rats following different treatment regimens. β-Actin is showed as a loading control. B. Densitometric analyses of the RT-PCR by Image J software.

also documented a significant decrease in Cyp19 ($p < 0.001$) expression. An up-regulation of IGF-1 expression was noted in the hyperandrogenized group, which got significantly decreased after treatment with metformin ($p<0.001$) (Fig 3). Nevertheless, no change in IGF-1was observed after letrozole regimen.

Discussion

Medical treatments for PCOS usually revolve around the correction of hyperandrogenemia and improvement of ovulation rates. The present results showed comparative superiority of metformin as an ovulogen over letrozole in PCOS.

The PCO set of animals developed ovarian-endocrine abnormalities with elevated serum testosterone (T) concentrations consistent with previous findings. (Franks et al., 1995; Mannerås et al., 2007). Our findings suggest supremacy of metformin over letrozole in reducing hyperandrogenicity with improving the metabolic set-backs of androgenised set.

Sex steroids appear to be important in regulating adiposity cueing the result of TP treatment to a significant increase in body weight. Moreover, administration of testosterone has got its direct correlation with IR attributed to effects on glucose transport and/or glucose intolerance (Rincon et. al., 1996). Metformin reduces the body weight via a direct inhibition in StAR or IGF-1 as well as brought down glucoseto basal level by 120 min indicating improved glucose tolerance as perceived in corresponding human studies (Lehtovirta et. al., 2001) however with no improvement after letrozole regimen. This may render a strong, more direct effect of metformin on glucose tolerance so as to reduce hyperandrogenicity with a concomitant improvement in response to exogenous gonadotropins.

Our androgen-induced rodent model displayed an elevated LH/FSH ratio which was achieved through lowering of FSH with unaltered serum LH levels and a significant increase in insulin level. A well-known positive correlation exists between insulin and androgenin women with PCOS (Wang et al., 2001). Metformin via its insulin sensitizing effect and by direct inhibition of androgens improves insulin sensitivity (Mansfield et al., 2003). The inhibition of aromatase by metformin might be via an extracellular signal regulated kinase pathwayas recently proposed by (Rice et. al., 2009). Approximately 70% of PCOS patients have dyslipidaemia characterized by increased total and low density lipoprotein-cholesterol (LDL-C) and triglyceride (TG) (King et al., 2007; Legro et al., 2001) observed that exposure to excess T may alter serum TG and LDL levels in a manner similar to that of lean PCOS women irrespective of obesity (Legro et al., 2001). Augmented TG level was reversed to normal after metformin treatment. This might have led to withdrawal of inhibitory effects of IR on high-density lipoprotein-cholesterol synthesis and therefore reverted back the atherogenic lipid profile to normal one.

Letrozole had been used for the development of PCOS and has proven hyperandrogenic effects in many experimental rat models (Mannerås et. al., 2007); hence the usage of the drug for the amelioration of the metabolic and ovulatory disturbances in experimentally induced hyperandrogenic milieu may be a conflicting methodology. However,

aromatase inhibitors are widely recommended for inducing ovulation in anovulatory women with PCOS after CC (Nader et al., 2007). Incidentally an increased rate of bone and cardiac anomalies in fetuses born to women after letrozole treatment was reported. Nevertheless, extensive examination of this issue has failed to substantiate these findings (Pritts et al., 2010). On the other hand, the safety of metformin therapy sounds theoretically appealing due to the lack of serious side effects like raised androgen levels observed due to hypoestrogenism in cases with letrozole. Recently, a metaanalysis of randomized clinical trials also revealed that metformin on its own or with CC was significantly superior to placebo or CC alone for ovulation induction (Xiao et al., 2012).

Metformin is well known to reduce hyperinsulinaemia and hyperandrogenaemia facilitating normal menses and pregnancy (Moghetti et al., 2000). However, the molecular mechanism of action of metformin remains uncertain. The action of metformin in muscle cells is via its easing of glucose transport and activation of tyrosine kinase activity mediated by IGF-1. Androgens increase the expression of IGF-1, and IGF-1 receptor in primate follicles and oocytes (Vendola et al., 1999). Hence, we evaluated the expression of StAR and IGF-1. Hyperinsulinemia, observed in our model could inhibit IGF-1-binding protein production by the liver. As a result, unbound IGF-1 in conjunction with LH could stimulate ovarian thecal cell androgen production (Stadtmauer et al., 2002). In the adult ovary, expression of P450scc and StAR geneis under the regulation of pituitary gonadotropins, via the hypothalamic-pituitary-gonadal axis feedback system (Chen et al., 2010) Steroidogenesis in the ovarian theca cell is primarily regulated by LH, which upon binding to the LH receptor promotes increased steroid production; gonadotropins activated cAMP-dependent PKA signalling and up-regulated multiple key steps in steroidogenesis, including StAR gene expression (Manna et. al., 2009). LH and insulin or insulin-like growth factor (IGF-I) act in synergy in stimulating StAR messenger RNA accumulation (Sekar et al., 2000). Thecal over-expression of StAR (Jakimiuk et al., 2001) improved androgen productivity. Metformin directly inhibits theca production of androgens (Rice et. al., 2009) through reduction of IGF-1 and StAR as reflected in our results. Hence, the attenuating effect of metformin on StAR expression may be a direct one (reducing androgens) ora change in the insulin status. However, letrozole do not have any impact on either of the expression profile.

In humans, administration of CC is done during the early follicular phase. Since rats have a 4-5 day cycle length, phase-dependent precise administration of CC is practically difficult, and hence was not attempted.

In summary, our data convincingly demonstrate that regulatory proteins and growth factor mRNAs that are over-expressed and causally associated with development of polycystic ovaries are mostly down regulated following treatment with metformin followed by the reversal of the metabolic arena of the enigma. The use of real time PCR analysis, instead of RT-PCR, however would have provided the array of molecular changes more precisely.

There is little agreement onthe ideal treatment of PCOS, which is largely symptom-based. IR plays a vital pathophysiological role in PCOS patients as manifested by causal relationship between IR and the reproductive and metabolic changes of PCOS. Our findings, although limited to androgen-induced PCOS model, demonstrate that letrozole improved the ovarian picture by reducing Cyp19 levels while metformin proves itself as a superior mode of treatment to recover the metabolic disturbances often observed in PCOS patients. In addition, metformin improves the ovarian response to gonadotropins. Based on these results metformin usagemay be useful in infertile patients with PCOS not only for the hyperinsulinemic-PCO subgroup but also for the purpose of induction of ovulation.

Competing interests: The author(s) declare that they have no competing interests

Author's contributions: Conceived and designed the experiments: PC, SC. Performed the experiments: PC, IC, SC, DP. Analyzed the data: SNK, BNC. Contributed Reagents/materials/analysis tools: BNC, SNK, PC. Wrote the paper: PC, BNC.

References

1. Amalfi S, Velez LM, Heber MF, Vighi S, Ferreira SR, Orozco AV, Pignataro O, Motta AB. Prenatal Hyper androgenization Induces Metabolic and Endocrine Alterations Which Depend on the Levels of Testosterone Exposure. PLoS ONE 2012;7:e37658.
2. Casper RF, Mitwally M. Review: aromatase inhibitors for ovulation induction. J ClinEndocrinolMetab 2006;91:760–71.
3. Chen M-C, Wang S-W, Kan S-F, Tsai S-C, Wu Y-C, Wang PS. Stimulatory effects of propylthiouracil on pregnenolone production through up regulation of steroidogenic acute regulatory protein expression in rat granulosa cells. ToxicolSci. 2010;118:667-74.

4. Du Vigneaud V, Karr WG. Carbohydrate utilization, rat of disappearance of D-glucose from the blood.J. Bio Chem. 1925; 66:281-300.
5. Elter K, Imir G. and Durmusoglu F. Clinical, endocrine and metabolic effects of metformin added to ethinyl estradiol–cyproterone acetate in non-obese women with polycystic ovarian syndrome: a randomized controlled study Hum. Reprod. 2002; 17:1729-1737
6. Escobar-Morreale HF, Luque-Ramírez M, Millán JLS. The Molecular-Genetic Basis of Functional Hyperandrogenism and the Polycystic Ovary Syndrome. Endocr Rev. 2005; 2:251-282.
7. Franks S. Polycystic ovary syndrome. N. Engl. J. Med. 1995; 333:853–861.
8. Genazzani AD, Lanzoni C, Ricchieri F, Baraldi E, Casarosa E, Jasonni VM. Metformin administration is more effective when non-obese patients with polycystic ovary syndrome show both hyperandrogenism and hyperinsulinemia. Gynecol Endocrinol. 2007; 23:146-152.
9. Homburg R. Androgen circle of polycystic ovary syndrome. Hum Reprod. 2009; 24: 1548-1555.
10. Jakimiuk AJ, Weitsman SR, Navab A, Magoffin DA. Luteinizing hormone receptor, steroidogenesis acute regulatory protein, and steroidogenic enzyme messenger ribonucleic acids are overexpressed in thecal and granulosa cells from polycystic ovaries. J EndocrinolMetab 2001; 86:1318-1323.
11. King AJ, Olivier NB, Mohankumar PS, Lee JS, Padmanavan V, Fink GD. Hypertension caused by prenatal testosterone excess in female sheep.Am J Physiol Endocrinol Metab 2007; 292:E1837-E1841.
12. Legro, RS. Prevalence and predictors of dyslipidemia in women with polycystic ovary syndrome.Am J Med 2001; 111:607–613.
13. Lehtovirta M, Forsén B, Gullström M, Häggblom M, Eriksson JG, Taskinen MR, Groop L. Metabolic effects of metformin in patients with impaired glucose tolerance. DiabetMed. 2001; 18:578-583.
14. Manna PR, Dyson MT, Stocco DM. Regulation of the steroidogenic acute regulatory protein gene expression: present and future perspectives. Mol Hum Reprod 2009; 15:321-333.
15. Mannerås L, Stefan C, Agneta H, Zamira S, Theodore L, Malin L, Stener-Victorin E. A New Rat Model Exhibiting Both Ovarian and Metabolic Characteristics of Polycystic Ovary Syndrome. Endocrinol 2007; 148:3781-3791.
16. Mansfield R, Galea R, Brincat M, Hole D, Mason H. Metformin has direct effects on human ovarian steroidogenesis. Fertil & Steril. 2003; 79:956-962.
17. Moghetti P, Castello R, Negri C, Tosi F, Perrone F, Caputo M, Zanolin 619 E, Muggeo M. Metformin effects on clinical features, endocrine and metabolic profiles, and insulin sensitivity in polycystic ovary syndrome: A randomized, double-blind, placebo-controlled 6-month trial, followed by open, long-term clinical evaluation. J ClinEndocrinolMetab 2000; 85:139-146.
18. Nader S. Ovulation induction in women with polycystic ovary syndrome. Nat Rev Endocrinol 2007; 3:438-439.
19. Neveu N, Granger L, St-Michel P, Lavoie HB. Comparison of clomiphene citrate, metformin, or the combination of both for first-line ovulation induction and achievement of pregnancy in 154 women with polycystic ovary syndrome. FertilSteril 2007; 87:113-120.
20. Ota H, Wakizaka A, Fukushima M, Maki M. Enhanced ovarian gonadotropin receptors in the testosterone-induced polycystic ovary in rats. Tohoku J Exp Med. 1986; 148:313-325.
21. Pasquali R, Gambineri A. Insulin-sensitizing agents in polycystic ovary syndrome. Eur J Endocrinol. 2006; 154:763–775.
22. Pritts EA. Letrozole for ovulation induction and controlled ovarian hyperstimulation. CurrOpinObstetGynecol 2010; 22:GCO200505.
23. Rice S, Pellatt L, Ramanathan K, Whitehead SA, Mason HD. Metformin inhibits aromatase via an extracellular signal-regulated kinase-mediated pathway. Endocrinol. 2009; 150:4794-4801.
24. Rincon J, Holma¨ng A, Wahlstrom EO, Lo¨nnroth P, Bjo¨rntorp P, Zierath JR, Wallberg-Henriksson H. Mechanisms behind insulin resistance in ratskeletal muscle after oophorectomy and additional testosterone treatment. Diabetes 1996; 45:615–621.
25. Ruel IL, Gaudet D, Perron P, Bergeron J, Julien P, Lamarche B. Effect of obesity on HDL and LDL particle sizes in carriers of the null P207L or defective D9N mutation in the lipoprotein lipase gene: the Québec LipD Study Int J Obesity 2003; 27:631–637.
26. Sattar N. PCOS, insulin resistance and long-term risks for diabetes and vascular disease. Br. J Diab & Vas Disease 2009; 9:15-18.
27. Sekar N, Lavoie HA, Veldhuis JD. Concerted regulation of steroidogenic acute regulatory gene expression by luteinizing hormone and insulin (or insulin-like growth factor I) inprimary cultures of porcine granulosa-luteal cells. Endocrinol. 2000; 141:3983–3992.
28. Stadtmauer LA, Wong BC Oehninger S. Should patients with polycystic ovary syndrome be treated with metformin? Benefits of insulin sensitizing drugs in polycystic ovary syndrome—beyond ovulation induction.Hum Reprod 2002; 17:3016-3026.
29. Tang T, Lord JM, Norman RJ,Yasmin E, Balen AH. Insulin-sensitising drugs (metformin, rosiglitazone, pioglitazone, Dchiro-inositol) for women with polycystic ovary syndrome, oligo amenorrhoea and subfertility. Cochrane Database Syst Rev. 2012; 5:CD003053.

30. Vendola K, Zhou J, Wang J, Bondy CA. Androgens promote insulin-like growth factor-I and insulin-like growth factor-I receptor gene expression in the primate ovary. Hum Reprod 1999;14:2328-32.

31. Wang Erica T, Calderon-Margalit Ronit, Bibbins-Domingo K. Polycystic ovary syndrome and risk for long-term diabetes and dyslipidemia. Obstet Gynecol 2011;117:6-13.

32. Xiao J, Chen S, Zhang C, Chang S. The effectiveness of metformin ovulation induction treatment in patients with PCOS: a systematic review and meta-analysis. Gynecol Endocrinol. 2012;28:956-60.

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