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Protective effects of nobiletin against metabolic and reproductive disturbance in letrozole induced polycystic ovarian syndrome in rats

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ABSTRACT

Polycystic ovarian syndrome (PCOS) is the most prevalent metabolic and endocrine disorder that affects women globally. This research was planned to evaluate the curative effects of nobiletin (NOB) on biochemical, metabolic, androgenic as well as histological parameters of PCOS induced rats. 24 female albino rats were divided into 4 equal groups: Group-I served as control. Group-II was treated with letrozole (1 mg/kg) (dissolved in 0.5 % CMC) for 21 days, for the induction of PCOs. Group III received letrozole (1 mg/kg) and metformin (2 mg/100 g) for 21 days. Group-IV received letrozole 1 mg/kg and NOB 10 mg/kg for 21 days. Testosterone, estradiol, progesterone, triglycerides, antioxidants (CAT, SOD, GSR, GPx, GST, GSH,), reactive oxygen species, thiobarbituric acid reactive substances, glucose, cholesterol level (HDL-C, LDL-C, VLDL-C, Non-HDL-C) as well as histopathological analysis of ovaries were performed. However, nobiletin treatment demonstrated beneficial effects by lowering testosterone levels, glucose levels, cysts, and improving estradiol and progesterone level as well as number of follicles and corpus luteum in PCOS rats. Furthermore, dysregulated lipid as well as antioxidant profiles in PCOS rats were reverted to the normal level. The current investigation demonstrated that NOB shows the ability to mitigate the irregularities associated with PCOS.

1. Introduction

Polycystic ovarian syndrome (PCOS) is a prevalent gynecological disorder in the world. According to estimate, PCOS affects 5.6 % to 6.1 % of fecund women worldwide (Ding et al., 2017). It is delineated by the disturbance in endocrine hormones which is manifested by fluid filled cysts in one or both ovaries (Azziz et al., 2016). However, in PCOS, the neuroendocrine system is dysregulated, that reduces LH and FSH levels. This prevents the hormones from turning into estradiol which in turn increases androgens production (Coutinho and Kauffman, 2019). It has also been reported that there is a strong connection between androgens and insulin level (Tsilchorozidou and Conway, 2004). The hyperinsulinemia in PCOS is primarily brought about by increased basal insulin production and reduced hepatic insulin clearance (Dunaif, 1997). Thecal cells are stimulated by insulin to synthesize androgens, and increased levels of androgens are linked to a variety of disorders, including PCOS. Research has confirmed that insulin sensitivity increases as the level of androgen decreases (Moran et al., 2010).

Drugs including metformin, clomiphene, and hormone tablets (Estradiol and Progesterone) are used to treat PCOS. However, using these drugs for an extended period of time may have side effects (Badawy and Elnashar, 2011). Metformin regulates glucose concentration by reducing the release of glucose from hepatocytes and boosting peripheral glucose absorption (DeFronzo et al., 1991). Despite metformin's success in decreasing insulin resistance, concerns have been raised about long-term usage, because of its side effects, such as gastrointestinal discomfort, pharmacodynamic interactions (Huang et al., 2015), renal failure and lactic acidosis (Lashen, 2010). As a result, it may not be the best drug to use for long-term PCOS treatment.

Plant-based compounds have demonstrated antioxidative potential

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owing to their free radical scavenging ability (Moussaoui et al., 2021). Citrus peels contain nobiletin (NOB), a bioactive polymethoxylated flavonoid. NOB has been shown in numerous studies to possess anti-inflammatory, anti-diabetic, antioxidant, and hepatoprotective activities (Nguyen-Ngo et al., 2020). Because NOB protects the cells against apoptosis, which slows the progression of Type 2 diabetes mellitus (Kaneko et al., 2020). NOB has potential to maintain blood glucose homeostasis both in humans and animals. NOB shields the cells of pancreatic islets from oxidative damage (Keshtkar et al., 2019) and stimulates secretion of insulin in individuals with diabetes (Petrenko et al., 2020). Therefore, keeping the above-mentioned strong factor in mind, a current investigation was designed to explore the potential effect of nobiletin in metabolic and reproductive dysregulation in letrozole prompted PCOS rats.

2. Materials and methods

2.1. Animals

24 sexually matured female albino rats were used to perform this experiment. Animals were kept in cages made up of stainless steel in the Animal House of the University of Agriculture Faisalabad (UAF) at 25 ± 3 °C for 21 days. All the rats were housed in a 12/12-hour dark/light cycle, & they were provided normal laboratory food pellets, and given unlimited access to tap water. The experimental animals were monitored and handled in compliance with the ethical guidelines of UAF.

2.2. Experimental design

24 Female rats were distributed into 4 groups (n = 6); the first group was considered as control (0.5 % CMC). The second group was treated with letrozole (Femara@, Novartis, Pakistan) (1 $mgkg^{-1}$ dissolved in 0.5 % CMC) for the induction of PCOS. The third group was treated with letrozole (1 $mgkg^{-1}$ dissolved in 0.5 % CMC) and metformin (2 mg/100 g body weight). The fourth group was treated with letrozole (1 $mgkg^{-1}$ dissolved in 0.5 % CMC) and NOB (10 mg/kg). On the 22^{nd} day, all the animals were anaesthetized by using ketamine (60 mg/kg) + xylazine (6 mg/kg) and decapitated.

2.3. Blood and tissue sampling

At the time of decapitation, blood from the trunk was taken & centrifuged for 15 min at 3000 rpm. Before conducting the biochemical and hormonal examination, plasma was isolated & kept at −20 °C. Ovaries were rendered fat-free & washed in saline. The right ovaries were fixed in 10 % formalin, whereas the left ovary was kept at − 80 °C to determine biochemical profile.

2.4. Estimation of antioxidant enzymes

The activity of CAT was estimated by a technique presented by Chance and Maehly, (1955). Activity of SOD was assessed by the procedure purposed by Kakkar et al. (1984). GSR activity was measured by adopting the technique of Carlberg and Mannervik, (1975). GPx activity was analyzed according to a process explained by (Rotruck et al., 1973). Activity of GST was examined by the protocol of Habig et al. (1974). GSH activity was estimated by protocol of Jollow et al. (1974). TBARS content was evaluated by a technique described by Iqbal et al. (1996). ROS content was estimated by a protocol of Tyan et al. (2014).

2.5. Glucose and Lipid profile analysis

The level of blood glucose was checked by using Accu Chek glucometer. The levels of triglycerides (TG), total cholesterol (TC), and high-density lipoprotein (HDL-C), were measured using commercially available kits (AMP diagnostics AMEDA Labordiagnostik GmbH, Austria). All the analyses were carried out according to the manufacturer's instructions. Low density lipoprotein cholesterol (LDL-C) and very low-density lipoprotein cholesterol (VLDL-C) were calculated by using Friedewald's formula. Non-HDL cholesterol (non-HDL-C) was calculated by determining the difference between TC and HDL-C.

2.6. Hormonal analysis

The levels of estradiol, testosterone and progesterone were evaluated by using the commercial ELISA kits manufactured by Los Angeles, CA USA (serial number-H090). The process was carried out in accordance with the catalogue provided with kits. The standard curve was used to assess the quantity of estrogen, progesterone, and testosterone.

2.7. Ovarian histology

The removed ovaries were fixed in 10 % formalin for further processing as a part of a standard histological technique. The ovaries were dehydrated and sectioned (5 μm) with the help of a microtome. Hematoxylin and eosin were used to stain the slides in accordance with standard procedure. Each section's follicula were counted and categorized.

2.8. Statistical analysis

Data were presented as mean ± SEM. Using Minitab software, one-way ANOVA followed by Tukey's test was performed. The normal distribution of the data was tested by Shapiro-wilk test. Differences with $P < 0.05$ were considered as significant.

3. Results

3.1. Biochemical profile status

CAT, GSR, SOD, GST, GPx & GSH activities in ovarian tissue of letrozole exposed animals were markedly reduced ($p < 0.05$) than those in the control group. Whereas these levels were reverted to the normal level after treatment with nobiletin and metformin. TBARS and ROS levels were notably increased in PCOS treated animals in comparison to control. However, metformin & NOB treatment significantly ($p < 0.05$) reduced the levels of TBARS and ROS (Table 1).

Table 1
Mean ± SEM of CAT, SOD, POD, GPx, GSR, GST, GSH, activities and TBARS and ROS concentrations.

Parameters	Groups			
	Control	PCOS	PCOS + MET	PCOS + NOB
CAT (U/mg protein)	11.56 ± 1.09[a]	5.74 ± 0.39[b]	12.27 ± 1.01[c]	11.42 ± 1.53[a]
SOD (U/mg protein)	7.48 ± 0.47[a]	2.81 ± 0.27[b]	7.66 ± 0.58[a]	7.34 ± 0.50[a]
GPx (U/mg protein)	5.64 ± 0.49[a]	1.74 ± 0.40[b]	5.16 ± 0.49[c]	5.92 ± 0.47[d]
GSR (nM NADPH oxidized/ min/mg tissue)	5.33 ± 0.38[a]	0.98 ± 0.23[b]	5.12 ± 0.33[a]	5.69 ± 0.46[a]
GST (nM/min/mg protein)	9.84 ± 0.79[a]	4.50 ± 0.38[b]	9.73 ± 0.68[a]	11.11 ± 0.61[a]
GSH (μM/g tissue)	16.10 ± 1.28[a]	7.40 ± 0.42[b]	16.47 ± 1.45[a]	16.36 ± 1.49[a]
TBARS (μmol/mg)	9.32 ± 1.05[a]	25.59 ± 2.26[b]	12.22 ± 1.66[c]	9.22 ± 1.03[a]
ROS (U/mg tissue)	0.33 ± 0.08[a]	1.67 ± 0.09[b]	0.66 ± 0.04[c]	0.31 ± 0.08[a]

The values with dissimilar letters are significantly distinct from other groups.

3.2. Lipid profile

There was a considerable increase in glucose, cholesterol & triglyceride levels in PCOS induced animals relative to control animals. But unlike the PCOS induced animals, metformin and NOB supplementation considerably lowered these levels. In contrast to the control, there was remarkable decrease in HDL-C whereas, considerable increase in LDL-C, non-HDL-C & VLDL-C levels in PCOS animals. Metformin & nobiletin supplementation lessened these levels as matched with PCOS group whereas, a profound increase in HDL-C was seen in metformin & nobiletin group (Table 2).

3.3. Hormonal assay

In PCOS rats, progesterone & estradiol levels were significantly decreased while testosterone and androgen levels were significantly increased relative to to control group. But the treatment with metformin and NOB significantly improved these values (Table 3).

3.4. Number of ovarian follicles

In contrast to control animals, the number of primordial, primary, secondary, tertiary & graffian follicles was significantly lowered ($p < 0.05$) in PCOS rats, however, metformin & nobiletin co-administration significantly enhanced the number of these follicles. Similarly, significant increase of cystic as well as atretic follicles were found in PCOS animals relative to metformin and nobiletin supplemented animals. Moreover, in PCOS animals, there was a reduction in corpus luteum that was reversed by metformin and NOB treatment (Table 4).

4. Discussion

The goal of the current investigation was to attenuate metabolic and reproductive disturbance in letrozole-prompted PCOS rats in order to examine any beneficial impact of nobiletin in reducing PCOS. In women during reproductive age, PCOS is a common endocrinopathy. The signs of hyperandrogenism, include hirsutism, acne & androgenic alopecia, irregular menstrual cycles, insulin resistance, chronic anovulation, disturbances in hormones, and decreased fertility (Goodarzi et al., 2011). The metabolic disorder, PCOS, is associated with multiple factors, i.e., type 2 diabetes mellitus, which is caused by high blood sugar levels and insulin resistance (Diamanti-Kandarakis and Dunaif, 2012). The rats were given letrozole, an aromatase inhibitor to cause PCOS. Letrozole inhibits the production of ovarian hormones, paracrine signaling, folliculogenesis, and the whole-body aromatization process (Maliqueo et al., 2013).

Table 2
Mean ± SEM of Total cholesterol, Triglycerides, HDL-C, LDL-C, VLDL-C, non-HDL-C, Glucose and Total protein concentrations.

Parameters	Groups			
	Control	PCOS	PCOS + MET	PCOS + NOB
Total cholesterol (mg/dL)	226.33 ± 6.76^{a}	308.87 ± 9.25^{b}	234.13 ± 4.92^{a}	241.10 ± 7.68^{c}
Triglycerides (mg/dL)	66.58 ± 5.17^{a}	93.14 ± 4.15^{b}	78.81 ± 2.98^{c}	72. 45 ± 1.27^{d}
HDL-C (mg/dL)	182.98 ± 4.74^{a}	114.02 ± 5.94^{b}	179.74 ± 6.15^{a}	166.31 ± 4.07^{c}
LDL-C (mg/dL)	13.60 ± 2.23^{b}	65.51 ± 2.18^{a}	14.09 ± 1.59^{b}	23.71 ± 1.98^{c}
VLDL-C (mg/dL)	33.41 ± 1.60^{a}	67.47 ± 2.11^{b}	38.61 ± 1.08^{c}	51.57 ± 2.16^{d}
non-HDL-C (mg/dL)	43.81 ± 2.46^{b}	84.12 ± 3.09^{a}	43.48 ± 2.23^{b}	46.09 ± 2.57^{b}
Glucose (mg/dL)	105.48 ± 7.27^{a}	170.23 ± 6.50^{b}	107.18 ± 7.05^{a}	119.78 ± 6.16^{c}

The values with dissimilar letters are significantly distinct from other groups.

Table 3
Mean ± SEM of Testosterone, Estradiol & Progesterone concentrations.

Parameters	Groups			
	Control	PCOS	PCOS + MET	PCOS + NOB
Testosterone (ng/ml)	0.53 ± 0.14^{a}	2.19 ± 0.13^{b}	0.60 ± 0.168^{a}	0.85 ± 0.14^{a}
Progesterone (pg/ml)	34.63 ± 1.09^{a}	9.59 ± 1.62^{b}	34.27 ± 1.212^{c}	29.39 ± 1.84^{a}
Estradiol (ng/ml)	7.37 ± 1.26^{a}	1.77 ± 0.25^{b}	7.06 ± 1.326^{c}	7.33 ± 1.14^{a}

The values with dissimilar letters are significantly distinct from other groups.

Table 4
Mean ± SEM of number of primordial, primary, secondary, tertiary, graffian, cystic, atretic follicle & corpus luteum.

Parameters	Groups			
	Control	PCOS	PCOS + MET	PCOS + NOB
Primordial follicle	9.17 ± 0.18^{a}	6.45 ± 0.31^{b}	9.44 ± 0.26^{a}	9.08 ± 0.25^{a}
Primary follicle	7.23 ± 0.21^{a}	3.95 ± 0.49^{b}	7.58 ± 0.28^{c}	7.16 ± 0.27^{a}
Secondary follicle	4.04 ± 0.42^{a}	1.36 ± 0.17^{b}	3.94 ± 0.39^{a}	3.73 ± 0.45^{c}
Tertiary follicle	3.37 ± 0.26^{a}	1.13 ± 0.10^{b}	3.18 ± 0.15^{a}	3.41 ± 0.34^{a}
Graafian follicle	2.54 ± 0.23^{a}	0.76 ± 0.22^{b}	2.75 ± 0.20^{c}	2.51 ± 0.27^{a}
Cystic follicle	0.00 ± 0.00^{a}	13.45 ± 1.43^{b}	3.71 ± 0.14^{c}	4.19 ± 0.39^{c}
Atretic follicle	1.84 ± 0.25^{a}	7.61 ± 0.34^{b}	3.57 ± 0.26^{c}	4.01 ± 0.24^{d}
Corpus luteum	6.83 ± 0.25^{a}	1.43 ± 0.41^{b}	6.87 ± 0.14^{a}	6.74 ± 0.27^{a}

The values with dissimilar letters are significantly distinct from other groups.

Oxidative stress (OS) has a pivotal role in PCOS (Lee et al., 2010). Regulated levels of CAT, SOD, GPx, GSR, GST & GSH are necessary for neutralizing elevated ROS level. Letrozole administration reduced CAT, SOD, GPx, GSR, GST & GSH activities, whereas elevated ROS & TBARS contents. By converting superoxide anion (O_2^-) to H_2O_2, superoxide dismutase (SOD) aids in detoxifying the O_2^- anion. The resulting products are further scavenged by catalase and glutathione peroxidase (GPx) into water (H_2O) (Agarwal et al., 2012). GSH protects membranes from free radicals, which is necessary for shielding the tissues from OS (He et al., 2012). Glutathione reductase (GSR), a non-protein substance that contains thiols as well as maintains GSH levels (Williams and Ford, 2005). Our investigation revealed that, GSR activity was significantly reduced in PCOS induced animals. However, nobiletin & metformin showed similar results. In PCOS induced animals, it was observed that, ROS and TBARS level was significantly increased in contrast to control group. Whereas, NOB and metformin showed similar result as in the control group. NOB treatment increased the antioxidant activities and counter-balanced the ROS & TBARS levels. TBARS is a reliable & frequently used marker for evaluating the level of lipid peroxidation (González et al., 2006).

During the course of this study, the glucose levels in the PCOS group was considerably increased when analyzed. Increased levels of androgen appear to be the primary factor to disrupt the animals' hormonal profiles. Higher levels of testosterone contribute in the insulin resistance and decreased glucose tolerance (Desai et al., 2012). Metformin lowers the release of glucose from hepatocytes and increases peripheral glucose absorption to control blood glucose level (DeFronzo et al., 1991). Although metformin has the ability to reduce insulin resistance, long-term use is limited due to its side effects, including abdominal pain

and pharmacological interaction (Huang et al., 2015). NOB is reported to display anti-diabetic & hepatoprotective properties (Nguyen-Ngo et al., 2020). Additionally, NOB is recognized for preventing apoptosis in cells, which delays the onset of Type 2 diabetes mellitus (Kaneko et al., 2020). It has also been revealed that NOB helps to regulate the glucose level in humans and other animals by shielding pancreatic islets from OS (Keshtkar et al., 2019). Furthermore, it stimulates the insulin secretion in diabetics (Petrenko et al., 2020). In the current study glucose concentration in PCOS group was significantly elevated that was significantly ameliorated in metformin and NOB treated group.

Lipid peroxidation is the oxidative depletion of lipids that leads to a polyunsaturated fatty acid membrane's polyunsaturated fatty acid chain reaction (Yildirim et al., 2007). Decreased level of HDL-C & elevated level of plasma triglyceride, total cholesterol as well as LDL-C was the outcome of PCOS that indicates dyslipidemia (Wild et al., 2011). Whereas, cholesterol, triglyceride, LDL-C, VLDL-C & non-HDL-C levels were increased in PCOS induced animals relative to control animals. Nevertheless, NOB supplementation significantly improves the lipid profile.

Aromatase inhibitors prevent the transformation of androgen into estrogens suggesting that elevated testosterone levels in PCOS are caused by an accumulation of androgens. Decreased testosterone levels in the metformin group are due to the reduction in ovarian androgen biosynthesis. (Attia et al., 2001). The excessive secretion of LH from hypothalamus and pituitary caused by the reduction in oestrogen production due to reduction in estrogen production followed by aromatase inhibition. Treatment with metformin and nobiletin dramatically decreased the elevated testosterone levels. It was also observed that, PCOS group showed significantly lowered progesterone level than control group, whereas values of progesterone in metformin and nobiletin treated group were significantly increased than PCOS induced animals. Elevation in LH & reduction in FSH levels stops the transformation of androgen into estradiol which leads to the accumulation of androgen (Coutinho and Kauffman, 2019). In the current research, level of estradiol was significantly reduced in PCOS induced animals. Whereas nobiletin and metformin supplemented animals showed increased level of estradiol.

The ovaries in PCOS induced group were bigger, reddish in color, and bulgier in morphology (Wang et al., 2017). PCOS induced rats had multiple larger cysts, paucity of oocytes and augmented follicular atresia. It has been observed that primordial, primary, secondary, tertiary & graffian follicle numbers were notably decreased in PCOS induced animals. In PCOS group, decreased numbers of secondary and tertiary follicles indicate that androgens are being produced in excess, which interferes with the normal maturation process of follicles (Rezvanfar et al., 2012). Antagonistic to PCOS group, cystic follicles numbers were remarkably lowered in metformin and nobiletin group; while there was not a single cystic follicle found in control group. It has also been found that the atretic follicles in PCOS group were significantly increased relative to control, whereas nobiletin & metformin supplemented animals showed decreased numbers of atretic follicles in comparison to PCOS. Moreover, PCOS group showed significantly decreased number of corpus luteum versus control group, whereas NOB and metformin group showed improved histological profile.

5. Conclusion

NOB is a potent flavonoid that displays the potential to reduce endocrine as well as metabolic irregularities in PCOS. NOB exhibited ameliorative role on glucose, hormonal levels as well as lipid profile. Additionally, it showed powerful antioxidant effects and restored normal follicles and ovarian cysts. An effective treatment for PCOS may be provided by NOB's ability to restore ovarian function. Furthermore, human based clinical trials are indispensable to evaluate the efficacy of NOB in PCOS patients.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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