



Research Article

Therapeutic Efficacy of Melatonin Against Polycystic Ovary Syndrome (PCOS) Induced by Letrozole in Wistar Rat

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Abstract

Background and Objective: Polycystic ovary syndrome (PCOS) is a common heterogeneous endocrinological and metabolic disorder in women of reproductive age which leads to infertility/subfertility. The present study was commenced to elucidate the therapeutic efficacy of melatonin in the pathogenesis of letrozole induced polycystic ovary syndrome (PCOS) in Wistar rat. **Materials and Methods:** Letrozole was administered orally (1 mg kg^{-1}) to induce PCOS condition in Wistar female rats for a period of 2-3 weeks followed by a dose of melatonin ($200 \mu\text{g}/100 \text{ g b.wt.}$) to PCOS induced rats. On the completion of experimental period the level of cytokines and expression level of different receptors was assessed. **Results:** The PCOs rats showed down regulation in melatonin (MT1 and MT2), estrogen (ER- α) and cytokine (IL-2R and IL-6R) receptors expression and high circulatory level of IL-6 and TNF- α during PCO condition. These anomalies in expression pattern and circulatory level of cytokines were restored following the treatment. **Conclusion:** Finding of present study showed the role of melatonin supplementation at receptor level and also suggesting a crosstalk between MT1R / MT2R via cytokine IL-2R and IL-6R resulting in modulation of ER- α receptors.

Key words: Melatonin, letrozole, melatonin receptors, metabolic disorder, estrogen receptor, polycystic ovary syndrome, endocrinological

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Polycystic ovary syndrome (PCOS) is one of the most common reproductive and endocrine disorders affecting ovarian follicular development and consequently ovulation which occurs due to imbalance in steroidal hormones^{1,2}. The diversity in its phenotypes and involvement of multiple organs contributes to complexity and heterogeneity of this syndrome of unknown etiology affecting 5-10% of women of reproductive age³. The main features of PCOS are chronic persistent anovulation, hyperandrogenism, bilateral cystic enlargement of ovaries⁴.

A direct action of neurohormone melatonin has already been reported on ovarian functions, steroidogenesis modulation in rodents and women^{5,6}. Further, there is evidence of presence of high concentration of melatonin in preovulatory follicular fluid⁷ and this high concentration of melatonin was noted to be associated with MT1 and MT2 receptor on ovarian tissue of rat and human⁸.

Reproductive failure during PCO condition indicates the inflammatory ovarian response and hence be immunologically determined. Earlier findings published provides a clear cut proof regarding melatonin's potent free radical scavenger with strong antioxidative action as well as immune enhancer in mammals including human⁹⁻¹² and hence, actively involved in immune regulation/immunoproliferation^{13,14}. Melatonin receptors also play a very important role in the physiology of an animal including inflammatory responses. Inflammatory events are particularly initiated and/or orchestrated by the sequential release of different anti/proinflammatory circulatory cytokine (s)/cytokine like molecules from local resident and infiltrating cells. Cytokines are critical to the success of reproductive process. They may act on germ cells, the embryo, non-hematopoietic cells in the gonads and uterus and hence can directly act on reproductive processes/functions. Further they also act indirectly by promoting immunologically receptive environment for the production of gametes as well as implantation and further gestational physiology till parturition. Therefore, it becomes important to understand the involvement of cytokines in the reproductive function which may help the researcher to design some therapeutic molecule aiming to reverse the reproductive pathologies like PCOS which may be a consequence of pathologies of ovarian immune origin. Therefore, the aim of present study was to examine the effect of melatonin in pathogenesis of PCOS induced by letrozole.

MATERIALS AND METHODS

Animals and maintenance: Female albino rats of Wistar strain (170 ± 10 g b.wt.) of same age were used in this study. Animals were housed under standard husbandry conditions ($25 \pm 2^\circ\text{C}$ temp, 60-70% relative humidity and 12 h photoperiod) and had access to standard rat feed and drinking water *ad libitum*. All the animals were treated and cared in accordance with the guidelines recommended by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forests, Government of India. The study was approved by Institutional Animal Ethics Committee of Guru Ghasidas Vishwavidyalaya, Bilaspur (994/GO/ERe/S/06/CPCSEA).

Preparation of letrozole and melatonin doses: Letrozole was dissolved in 1% CMC (Carboxymethyl cellulose) as a vehicle solvent and then the desired volume was added to obtain the desired concentration of 1 mg kg^{-1} . Melatonin solution was prepared by dissolving it in a few drops of ethanol and then diluted with normal saline upto the desired concentration.

Experimental design: After an acclimatization period of 2-3 weeks, rats were randomly divided into following experimental groups:

- Group I :** Normal control (1% CMC, p.o.)
- Group II :** PCOS control (letrozole -1 mg kg^{-1} p.o.)
- Group III :** Mel (*per se*) ($200 \mu\text{g}/100 \text{ g i.p}$)
- Group IV :** PCOS (letrozole -1 mg kg^{-1} p.o.)+Mel ($200 \mu\text{g}/100 \text{ g i.p}$)

Vaginal smear observation: The PCOS condition was confirmed by preparation of vaginal smear to observe reproductive cycle irregularities. Vaginal secretions were collected daily with a plastic pipette by inserting the tip in the vagina of female rats, filled with $15 \mu\text{L}$ of normal saline. One drop of collected vaginal fluid was placed on glass slides. Vaginal fluid was fixed by placing the slides on a slide warming table and stained with methylene blue staining solution. After staining, slides were washed to remove the excess stain, dried and observed under light microscope to assess the changes in the estrus cycle. Three types of cells were recognized: Round and nucleated cells were epithelial cells; irregular cells without a nucleus were the cornified cells and the small round cells were the leucocytes, their mutual proportion was used for the determination of different phases of the estrus cycle¹⁵.

Sample collection: After completion of experimental period, the rats of all the groups were sacrificed by euthanized in desiccator by using diethyl ether as an anesthesia. The blood samples were collected directly through cardiac puncture in heparinized tubes and centrifuged at 3000 g for 15 min to collect the plasma. The blood plasma was stored at -20°C for serum cytokine assay. The ovaries were collected and lysed for the assessment of receptor expression of various receptors.

Serum cytokine analysis

Circulatory level of IL-6: Sandwich ELISA was performed to quantify the level of IL-6 in serum collected from the rats according to the manufacturer's instruction (Koma Biotech, Seoul, Korea; Cat. No. K0331230). Lower and upper limits of analytic sensitivities were 16 and 1000 pg mL⁻¹. All the assays were carried out in triplicate.

Circulatory level of TNF-α: Sandwich ELISA was performed to quantify the level of TNF-α in serum collected from the rats according to the manufacturer's instruction (Koma Biotech; Cat. No. K0331186). Lower and upper limits of analytic sensitivities were 16 and 2000 pg mL⁻¹. All the assays were carried out in triplicate.

Receptor assay

qRT-PCR: The qRT-PCR was done for the expression assay of cytokine (IL-2R and IL-6R) by first extracting the total mRNA, followed by cDNA synthesis. The primers were purchased from Imperial life sciences (P) limited (Table 1).

RNA Isolation: Total RNA was isolated from the frozen tissue samples. Tissues were slowly crushed with the help of mortar and pestle. About 1 mL of RIBOZOL (Trizole) was added into it and homogenized for 5 min. The tissue samples homogenized in RIBOZOL was transferred to a fresh microtube and left for 5 min at RT. After incubation, 200 µL of chloroform was added, vortexed and left it for 10 min on RT, then centrifuged at 13000 rpm for 10 min at 4°C. Supernatant was collected in a fresh microtube and 0.5 mL isopropanol was added, vortexed and kept it for 10 min on RT. The sample was centrifuged at 13000 RPM on 4°C. The supernatant was discarded and the

pellet was washed with 80% ethanol by again centrifuging it on 7500 rpm for 5 min at 4°C. Washed pellet was air dried and dissolved in 50 µL of DEPC water and aliquoted it into 5 tubes and kept in -80°C for further processing.

cDNA synthesis: The cDNA synthesis was done by using Thermo kit K1632. About 1 µL of total RNA isolated from the tissues were used as template where 1 µL of primer oligo (dT) 18 and 10 µL of nuclease-free water was added. It was mixed gently, centrifuged briefly and incubated at 65°C for 5 min. Then the sample was transferred on ice, spins down and places the vial back on ice.

Then, 4 µL of 5X Reaction Buffer, 1 µL of RiboLock RNase Inhibitor (20 U µL⁻¹), 2 µL of 10 mM dNTP Mix and 1 µL of Revert Aid H Minus M-MuLV Reverse Transcriptase (200 U µL⁻¹) were added. Total volume of the reaction mixture was 20 µL. It was mixed gently and spin down. Finally for cDNA synthesis, the reaction mixture was incubated at 42°C for 60 min.

Western blot analysis: The western blot analysis was performed according to the method of Treek *et al.*¹⁶. Ovarian tissues were dissected in chilled PBS, homogenized and lysed in lysate buffer. The protein content of the lysates was quantified using the Bradford method¹⁷. The membranes were immunostained with β-actin antibodies in 1:1000 dilutions (A-2228; Sigma-Aldrich) as internal loading control. Immune detection of β-actin was performed with donkey anti-mouse IgG-HRP (1:1000). Bands were quantified by the measurement of optical density using Scion Image Analysis Software (Scion Corporation, MD, USA). Values were expressed as the ratio of the density of the specific signal to the β-actin signal. The ratio of density was calculated with respect to β-actin (house-keeping gene) and expressed as percentage relative integrated density values of MT1, MT2 and ER-α.

Statistical analysis: The experimental data were expressed as Mean ± SE. Statistical analysis was performed using one-way ANOVA followed by Student's t-test with the SPSS 16.0 statistical software (SPSS, Chicago, IL, USA). A probability value p<0.05 (*) or p<0.01 (**) was considered to be statistically significant.

Table 1: List of primers

Gene product	Forward	Reverse
IL-2	5-CCTCGAGTCCTGCCACAATG-3	5-GCGGCCGCTAGTGATCAACTC-3
IL-6	5-GGTACATCCTCGACGGCATCT-3	5-GT GCCTCTTTGCTGCTTTCAC-3
β-actin	5-GGAAATAGGGGTTAGCAC-3	5-CTCATGTGCGCCTACTTA-3

RESULTS

Expression of melatonin receptors MT1 and MT2: The MT1 receptor expression was noted significantly down regulated during PCO condition. Whereas, administration of exogenous melatonin resulted in the upregulation of expression level of MT1 significantly. A significantly decreased expression was observed in MT2 receptor, but the administration of melatonin showed recovery pattern for the expression towards the normal significantly (Fig. 1, 2).

Expression of estrogen receptor-ER- α : A significant down regulation in expression of ER- α receptors on ovarian tissue of PCO rats was noted. Administration of exogenous melatonin resulted in restoration of the disruption of expression level. Treatment of melatonin to PCO rats resulted with significant reversal of receptor of ER- α (Fig. 3).

Expression of cytokines-IL-2R and IL-6R: The IL-2R expression pattern was noted down regulated in letrozole induced PCO rats. Treatment of melatonin showed a significant increase (Fig. 4). Expression of IL-6R was found significantly reduced in PCO induced rats which was restored towards the control group on administration of exogenous melatonin (Fig. 5).

Circulatory level of cytokines: Circulatory level of cytokine IL-6 increased significantly in PCO rats when compared to the control group of rats (Fig. 6). The circulatory level of TNF- α was noted significantly increased in PCOS rats compared to the control group of rats. However, after the administration of melatonin restoration in the level was found significant towards the controlled group (Fig. 7). Treatment of melatonin resulted in the restoration of the circulatory cytokines (TNF- α and IL-6) level significantly and comparable to the controlled group.

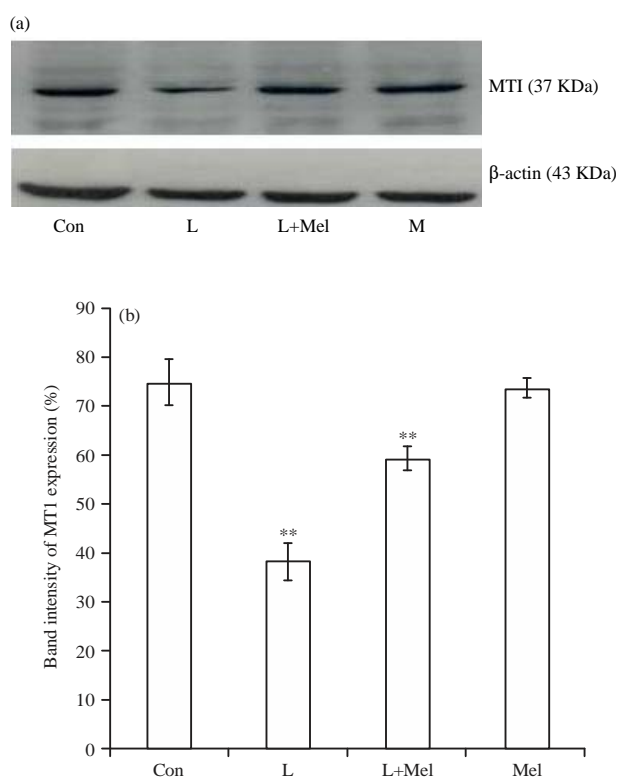


Fig.1(a-b): Western blot analysis for variation in the expression of melatonin receptor MT1 in ovary of female rats. The data are expressed as percentage band intensity of receptor expression in ovary. β -actin expression was used as control. Data are expressed as Mean \pm SE, n = 6 females per group Con: Control, L: Letrozole, Mel: Melatonin, **p<0.01, Con vs. L, L vs. L+Mel

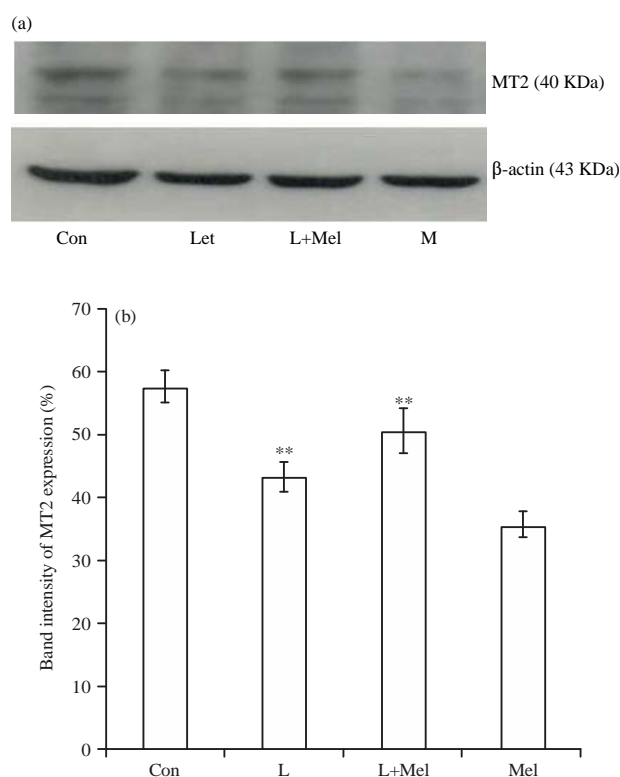


Fig.2(a-b): Western blot analysis for variation in the expression of melatonin receptor MT2 in ovary of female rats. The data are expressed as percentage band intensity of receptor expression in ovary. β -actin expression was used as control. Data are expressed as Mean \pm SE, n = 6 females per group Con: Control, L: Letrozole, Mel: Melatonin, **p<0.01, Con vs. L, L vs. L+Mel

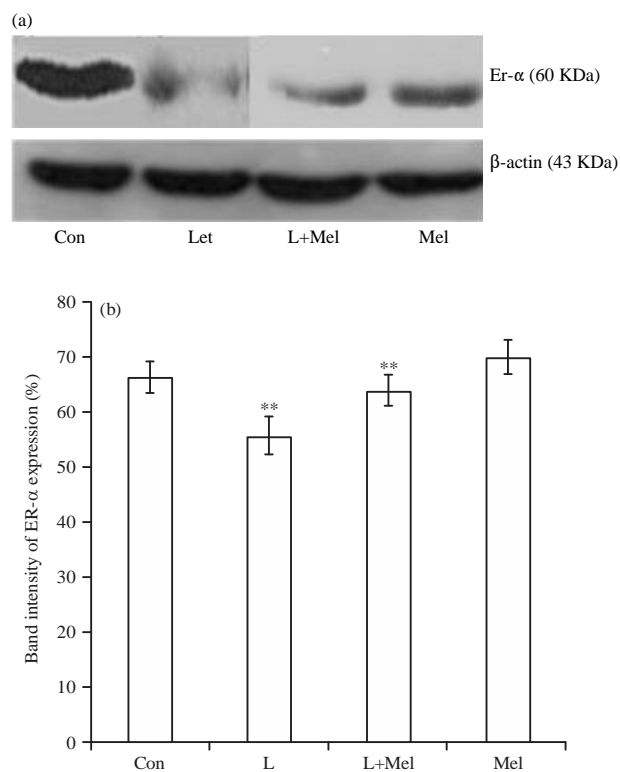


Fig. 3(a-b): Western blot analysis for variation in the expression of estrogen receptor ER- α in ovary of female rats. The data are expressed as percent band intensity of receptor expression in ovary. β -actin expression was used as control. Data are expressed as mean \pm SE, N= 6 females per group Con: Control, L: Letrozole, Mel: Melatonin, ** $p < 0.01$; Con vs. L, L vs. L+Mel

DISCUSSION

Present finding clearly showed the presence of MT1 and MT2 receptors in ovarian tissue of rat through which melatonin can exert its influence the reproductive system. Further ovarian tissue of PCO rats demonstrates down regulation of MT1 and MT2 receptors and melatonin treatment resulted in the restoration of the MT1 and MT2 receptors. Our observation confirms influence of exogenous melatonin and its receptor (MT1 and MT2) during such heterogeneous endocrine disorder of PCO which indicates the sensitivity of HPO axis for the treatment of melatonin. The expression of ER- α was significantly decreased in polycystic ovarian tissues.

Expression of ER- α receptor following administration of melatonin alone appeared static when compared to each other. The present finding can be correlated with the

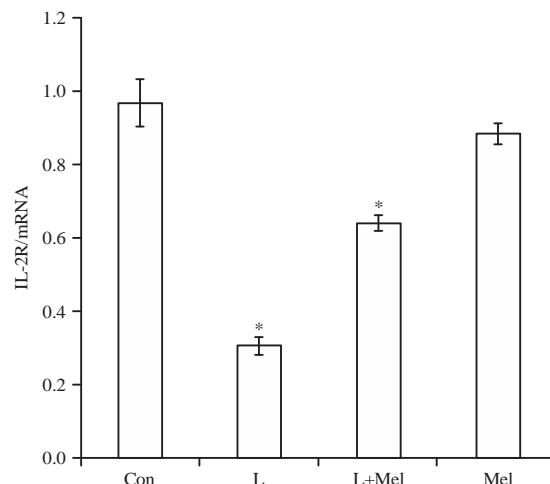


Fig. 4: Receptor expression analysis for variation in the expression of cytokine receptor IL-2R in ovary of female rats. The data are expressed as mRNA expression in ovary. Data are expressed as Mean \pm SE, n = 6 females per group Con: Control, L: Letrozole, Mel: Melatonin, * $p < 0.05$, Con vs. L, L vs. L+Mel

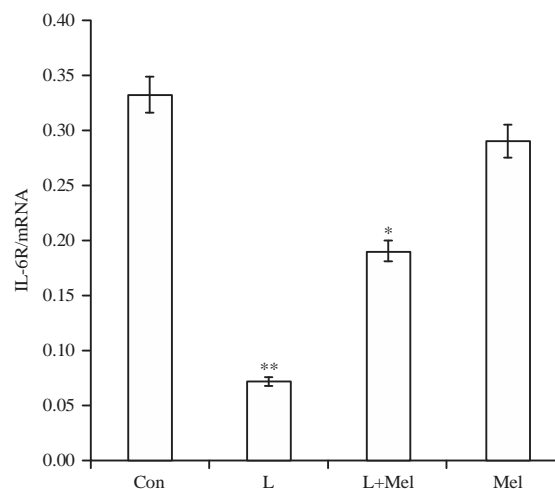


Fig. 5: Receptor expression analysis for variation in the expression of cytokine receptor IL-6R in ovary of female rats. The data are expressed as mRNA expression in ovary. Data are expressed as Mean \pm SE, m = 6 females per group Con: Control, L: Letrozole, Mel: Melatonin, * $p < 0.05$, ** $p < 0.01$, Con vs. L, L vs. L+Mel

fact that during PCO condition there is a failure to upregulation of aromatase enzyme in granulosa cell therefore, estradiol concentration of follicles fails to increase adequately and hence, led to the abnormal expression of ER- α ¹⁸.

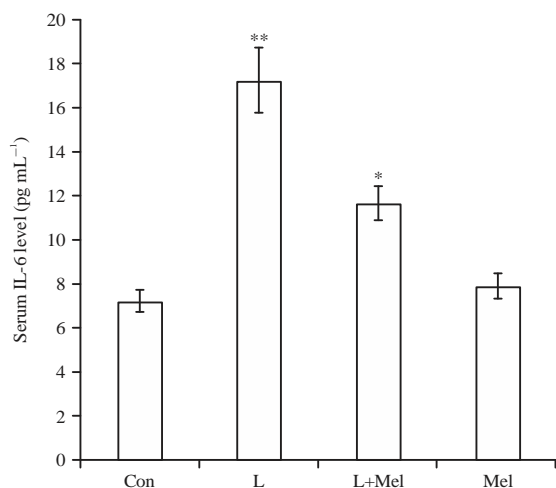


Fig. 6: Variations in the level of serum cytokine IL-6 in letrozole induced PCO rats and in recovery by melatonin. Data are expressed as Mean±SE, N = 6 females per group

Con: Control, L: Letrozole, Mel: Melatonin, *p<0.05, **p<0.01, Con vs. L, L vs. L+Mel

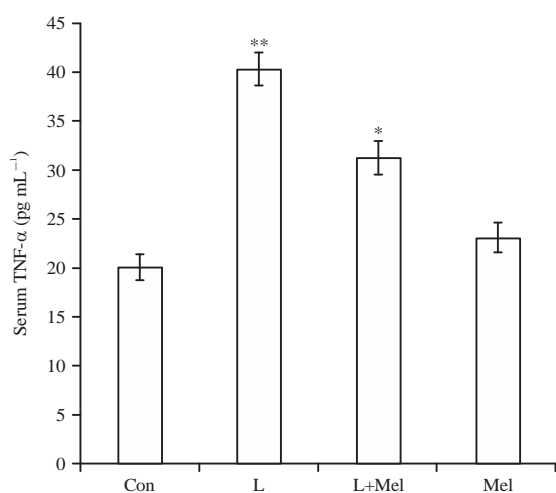


Fig. 7: Variations in the level of serum cytokine TNF-α in letrozole induced PCO rats and its recovery by melatonin. Data are expressed as Mean±SE, N = 6 females per group

Con: Control, L: Letrozole, Mel: Melatonin, *p<0.05, **p<0.01, Con vs. L, L vs. L+Mel

Further, present findings can be justified on the basis of the previous reports suggesting inhibitory action of melatonin during the binding of E2 ER complex to the estrogen responsive element on DNA^{19,20}.

Further, a differential expression of MT1 receptor noted in all experimental groups of rats where letrozole induced PCOs female showed down regulation in MT1 R expression in ovary. Upregulation of MT1 R in ovarian tissue following melatonin

injection to the control group provide a clear cut evidence of direct involvement of MT1 receptor in ovarian physiology specifically during the pathogenicity of PCOS.

Letrozole induced PCO female rats showed a significant increase in serum cytokine level of IL-6 as well as TNF-α in letrozole induced PCO rats indicating the internal inflammation which could be due to the tissue specific pathogenicity of local micro environment and stress induced. These finding is in concurrence with the previous report suggesting that free radical generation act via a variety of proinflammatory cytokines during various pathological condition of female reproduction including polycystic ovarian disease (PCOD)¹⁹. Since melatonin is reported a strong immunomodulatory which could be the main factor for the restoration in immune status of ovarian tissue. The present finding provides a clear cut evidence of involvement of exogenous melatonin resulted in the restoration of cytokine receptors as that of the control. Action of melatonin can be explained as this neurohormone can activate or deactivate the signaling pathway through its receptor MT1 and MT2 and also by upregulating few cytokines like IL-2 and others (IL-6, TNF-α) in seasonal breeder^{21,22}.

Previous studies provide the evidence that estrogen receptor are localized in the small antrum of granulosa cell and dominant follicles but not in the theca cells^{23,24}. Down regulation in ER-α receptor expression in ovarian tissue of PCO rats appears as one of the responsible component resulted into the arrhythmicity in the pulsatility of LH and FSH and could be because of the disturbances in the LH surge. Further, reports on ER knockout animals revealed that ER-α is a prerequisite for the proper functioning of hypothalamic pituitary ovarian (HPO) axis and therefore regulates the successful ovulation²⁵ and hence support partially the present finding. Knockout ER-α mice showed a similar phenotype to that of PCO with high circulating LH concentration which coincides our present finding. Such kind of hormonal balancing during the PCO may be considered as one of the major cause leading in the disarrangement of feedback mechanism and ultimately characterized by impaired ovulation such as PCOS with alteration in the expression of ER-α²⁶.

The ER-α receptors are present in the ovary differently in ovarian-theca and granulosa cells²⁷. ER-α, mRNA and protein which are expressed minimally in the ovary are located predominantly in the theca layer²⁷. The ER-β mRNA and protein which are highly expressed in the granulosa cells of the developing follicles. The disruption in the expression of ER-α in present findings can be explained because of elevated circulatory LH serum levels during PCO due to failure of the negative feedback action of estradiol on the hypothalamic-pituitary axis.

Nonetheless, since cytokine usually participate in ovarian physiology as paracrine/autocrine regulators of steroidogenesis, production of prostaglandins and LH, FSH receptor induction²⁸⁻³⁰. Literature suggested regarding origin of cytokines either leucocytes produced during ovulation or from resident ovarian cells³¹. Since cytokines are signaling molecule therefore may be involved in upregulating/downregulating particular cytokines like IL-2 and IL-6 in serum and additionally by associating its own receptor with cytokine receptor. Presence and participation of IL-6 is reported in normal ovary where it may participate in follicular development by reducing the hormone binding follicle stimulating hormone capacity of granulosa cell³². Therefore, exogenous melatonin given to PCO rats showed synchronization in the cytokine circulatory serum (IL-6, TNF- α) and receptor expression (IL-2R, IL-6R) via LH/FSH and hence modulating the ER- α expression with a crosstalk among MT1 and MT2 receptor. TNF α again is a pro inflammatory cytokine associated with the regulation of the tumor growth by increasing production of IL-6. TNF- α along with IL-1 inhibits the secretion of progesterone. The TNF α may also be involved in regression of corpora lutea³². Atresia is a hormonally controlled form of apoptosis of ovarian follicles and cytokines play a role in its regulation.

CONCLUSION

The present observation clearly explained that melatonin may have modulatory effect on steroidogenesis via its receptors (MT1/MT2) in association with ER- α in modulation of ovarian function during PCO condition. Present study further showed that during PCO disorder the molecular alterations might have brought the changes in steroidogenesis associated gene expression which following the treatment of melatonin was reversed via the crosstalk of the MT1/MT2, ER- α and the cytokines IL-2R and IL-6R.

SIGNIFICANCE STATEMENT

The findings of this study evidenced a novel therapeutic approach using melatonin exogenously. Alterations at molecular level which are observed during the PCOS pathogenicity might have changed the steroidogenic pathway related expression of genes. However, these alterations were reversed following melatonin supplementation. This study will help the researchers to uncover certain areas that many researchers have not been able to explore.

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